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Evolution and Flower Shape from the Hawaiian Lobelioids to the Campanulaceae

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EVOLUTION AND FLOWER SHAPE FROM THE HAWAIIAN LOBELIOIDS TO THE CAMPANULACEAE

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ABSTRACT

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Jingjing Tong

Campanulaceae, with roughly 2,400 species, is divided into five subfamilies, in part, by differences in their floral orientation and symmetry, with broad transitions between radial and bilateral floral symmetry. The Hawaiian lobelioids in the Campanulaceae is the largest endemic clade of Hawaiian angiosperms, and they have long been viewed as one of the most spectacular examples of an adaptive radiation in plants. In the first part of our study, we focus on *Clermontia*, which is one of the largest genera of Hawaiian lobelioids, employing variable low-copy nuclear gene sequences to determine their phylogenetic relationships. We use intron regions from four separate copies of *DIVRICATA* (*DIV*), as well as variable regions of two Pentatricopeptide Repeat (PPR) genes, to construct a phylogenetic history of Hawaiian *Clermontia* and highlight patterns of frequent hybridization across the group. In the second part of our study, we aim to clarify the *CYCLOIDEA*-like gene duplication and expression patterns across Campanulaceae. We identified all three *CamCYC* paralogs from across Campanulaceae. We show that gene duplication is most common in *CamCYC2*, specifically in the bilaterally symmetrical Lobelioideae, which is in line with hypotheses of *CYC2*-like gene function in the core eudicots. However, in bilaterally symmetrical Cyphioideae, we detected *CamCYC3* genes, but no *CamCYC*2 genes in this clade, suggesting that *CamCYC*3 genes might be involved in the development of bilaterally symmetrical flowers in this clade, instead of *CamCYC2*. To examine how tightly correlated the expression patterns of the *CYC*-like genes is with changes in flower morphology, we utilized *q*RT-PCR to determine *CamCYC2* gene expression patterns in four

species of bilaterally symmetrical Lobelioideae. The expression patterns of *CamCYC2* genes was like other core eudicots species, with restriction of expression to the dorsal zone of the flower in

bilateral symmetrical flowers. In Lobelioideae, with resupinate flowers, both *CamCYC2A* and *CamCYC2B* are highly expressed in the ventral petals. These ventral petals correspond to the dorsal side of the flower, suggesting conservation of dorsal identity in these upside down flowers. Additionally, individual copies of *CamCYC2* genes show slightly different expression levels in different petals, suggesting possible subfunctionalization between these copies.

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INTRODUCTION

1. Campanulaceae diversity

The Campanulaceae, or bellflower family, is a large core eudicot group, which encompasses 2,319 species in 84 genera. It is found on six continents and many oceanic archipelagoes, and is distributed from the tropics to the frigid zones. There are at least three putative synapomorphic characters shared in Campanulaceae: laticifers, stamens attached to the disc of the ovary, and epigynous flowers (Lammers, 2007). The Campanulaceae are divided into five monophyletic subfamilies, which had previously been treated as separate families (Lammers, 2007). The family includes the following five subfamilies: Campanuloideae, Cyphioideae, Lobelioideae, Cyphocarpoideae, and Nemacladoideae (Lammers, 2007) . Campanuloideae have radially symmetrical flowers, while the other four subfamilies have bilaterally symmetrical flowers (Fig. 1) (Crowl, et al., 2016; Lammers, 2007) .

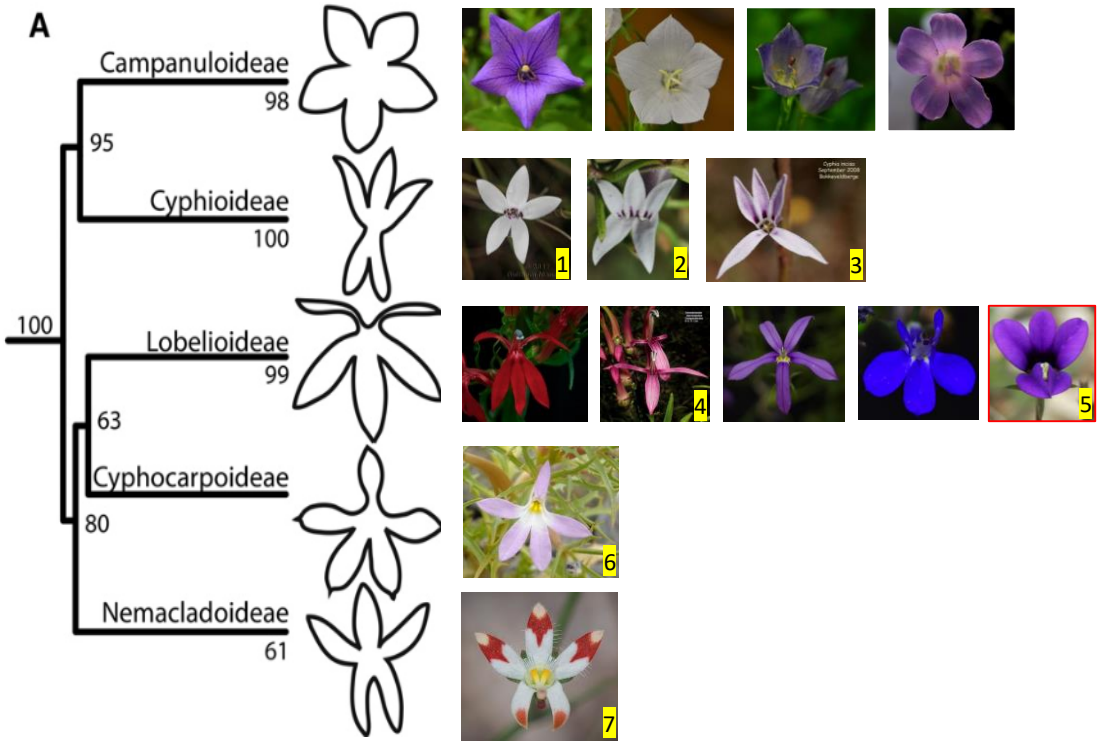

Figure 1. Different flower morphologies in the different Campanulaceae subfamilies. Campanuloideae is the only clade with radially symmetrical flowers. Cyphioideae and Nemacladoideae have un-resupinated flowers with a 3:2 corolla form. Cyphocarpoideae also have un-resupinated flowers, but with a 1:4 petal arrangement. Lobelioideae have resupinated flowers, with a 2:3 form in most genera, except *Monopsis* (red box) with un-resupinated and 3:2 form flowers. Phylogeny image from, Crowl et al.(2016).

Pictures sources:

1. https://www.guilhemmansion.com/en/album/botanical-families/campanulaceae/cyphia/cyphia-volubilis-20170905-007.html
2. http://angio.bergianska.se/Bilder/asterids/Campanulales/Campanulaceae/Cyphia/
3. https://ngoosen.fotki.com/campanulaceae-/cyphia/cyphiaincisa2.html
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6. https://www.patagoniawildflowers.org/search?&PlantName=Cyphocarpus+rigescens
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Subfamily Campanuloideae includes approximately 1050 species in 50 genera. They are dispersed worldwide, especially in temperate areas of the Old World, with the major centers of diversity in the Mediterranean Basin and the Middle East (Crowl, et al., 2014; Crowl, et al., 2016; Lammers, 2007). This is the only subfamily with radially symmetrical flowers (Fig. 1). Previous studies have shown that the common ancestor of Campanulaceae had bilaterally symmetrical flowers, and that there was a reversal back to radial floral symmetry in the Campanuloideae clade, early in their evolutionary history (Crowl, et al., 2016). This reversal is associated with a change in the pollinators, and was also accompanied by a change in the pollen presentation mechanism (Crowl, et al., 2016). The Lobelioideae encompasses about 1,200 species in 29 genera (Lammers, 2007). They are also dispersed nearly worldwide, with an origin in southern Africa, and the center of diversity in the New World tropics (Lammers, 2007). Most of the species of Lobelioideae have resupinate (turned 180 degrees upside down), bilaterally symmetrical flowers, connate (fused) stamens that form an anther tube, and styles with brush hairs (Fig. 1). The Lobelioideae species have a large diversification in growth-form, from small, herbaceous plants, to shrubs, to woody-rosette giant lobelias (Antonelli, 2008; Antonelli, 2009; Givnish, 2010). The Cyphioideae includes 64 species which are endemic in tropical and southern Africa (Fig. 1). In previous studies, Cyphioideae and Campanuloideae commonly grouped as sister clades, and the other three subfamilies, Lobelioideae, Cyphocarpoideae, and Nemacladoideae grouped together as a separate clade (Antonelli, 2008; Cellinese, et al., 2009; Crowl, et al., 2016; Knox, 2014; Linder, 2014). The Cyphocarpoideae, is a very small subfamily, with 3 species in 1 genus, narrowly endemic

in Chile. The Nemacladoideae, also a relatively small clade, contains 25 species in 3 genera is endemic in California and Mexico (Lammers, 2007).

2. Hawaiian Lobelioid diversity, including *Clermontia*

There are numerous endemic Lobelioideae on the small but highly isolated oceanic Hawaiian Islands. The Hawaiian Archipelago is a classic system for the study of speciation and adaptive radiation given its density of vastly different habitats in close proximity and the opportunity to increase variation rapidly (Sakai, et al., 1995; Sakai, et al., 2002). Because of this, the Hawaiian flora has an extremely high proportion of endemism, with the angiosperm flora being 89% endemic and the fern flora being 70% endemic, one of the highest percentages of any floristic region in the world (Givnish, et al., 2009; Givnish, et al., 2004; Keeley and Funk, 2011). The endemic Hawaiian Lobelioids are the most speciose plant group from a single colonization into the Hawaiian Islands, and it is one of the most spectacular examples of an adaptive radiation in plants. Hawaiian Lobelioids contains 8 genera (6 endemic, one indigenous, and one naturalized), and at least 127 species (Givnish, et al., 2009; Givnish, et al., 2004; Keeley and Funk, 2011; Montgomery and Givnish, 2008), and the flowers of Hawaiian Lobelioids are strongly bilaterally symmetrical with an adaxial (upper) corolla slit (Fig. 2). Based on previous research, the native species diversified from a common ancestor approximately 13 million years ago (Givnish, et al., 2013; Givnish, et al., 2004; Wagner, et al., 1999). *Cyanea* (ca. 88 spp.) and *Clermontia* (22 spp., listed in Table 1) are the largest two flowering plant genera in Hawaiian Lobelioids, and they diverged from each other only in the last 5 mya (Givnish, et al., 2004; Wagner, et al., 1999). Due to rapid diversification, Hawaiian *Clermontia* species have not accumulated genomic molecular variation, and

consequently, it is difficult to find variable markers to resolve their relationships at the interspecific level. Furthermore, on the Hawaiian Archipelago, due to the relatively close habitats and ecological niches, hybridization has played an important role in Hawaiian flowering plant evolutionary history (Givnish, et al., 2013; Howarth and Baum, 2005; Wagner, et al., 1999). Hybrid species have been shown to invade different habitats from those occupied by either parent (Howarth and Baum, 2005). Previous studies have suggested that there has been frequent hybridization between multiple species of Hawaiian Lobelioids (Wagner, et al., 1999), which complicates phylogenetic analyses.

In our study, we focused on Hawaiian *Clermontia*, and aim to reconstruct a robust interspecific phylogenetic tree. We utilized multiple low-copy nuclear genes and genomic intron sequences as phylogenetic tools instead of chloroplast markers, inter-simple sequence repeat polymorphisms (ISSRs), or non-transcribed spacer of 5S ribosomal DNA (Givnish, et al., 2013; Pillon, et al., 2013). Unlinked nuclear datasets can provide multiple and independent information for greater phylogenetic resolution in these recently diverged taxa. They can contain relatively higher sequence variation than chloroplast data, and are bi-parentally inherited, leaving more of a signature of evolutionary history. In our study, we also utilized intron sequences as a phylogenetic tool, as intron sequences can provide higher molecular variation compared with most exon regions (Howarth and Baum, 2005). We designed primers from the conserved flanking exon ends to isolate the intron sequences. In the first part of study, we choose the intron sequences from two *DIVARICATA*-like genes and four Pentatricopeptide repeat (PPR) gene loci and utilized

these nuclear loci to study the phylogenetic and hybridization history of Hawaiian *Clermontia*.

Figure 2. Hawaiian *Clermontia* flowers

Many species of Hawaiian *Clermontia* flowers have a double whorl of petals due to the petaloid sepals. This is seen in (A) *Clermontia micrantha*, (B) *Clermontia kohalae*, (C) *Clermontia kakeana*, and a few species have normal sepal and petal whorls, shown in (D) *Clermontia arborescens.* Photos from: http://www.botany.hawaii.edu/faculty/carr/clermontia.htm

3. *DIVARICATA*-like genes and PPR genes

The *DIV*-like genes function in ventral (lower) zones of flowers (Corley, et al., 2005; Jabbour, et al., 2009; Preston, et al., 2009). Three core eudicot clades of *DIV*-like genes have been identified: *DIV1*, *DIV2* and *DIV3* (Howarth and Donoghue, 2009), resulting from duplications of *DIV* occurred around the diversification of the core eudicots. The *DIV*-like genes contain an intron flanked by conserved MYB* domains, which makes it feasible to design universal primers to isolate *DIV*-like copies in other groups. Previous research in Dipsacales indicate that the exon sequences are highly conserved in Hawaiian lobelioids, and the intron sequences have relatively high variation (Howarth and Donoghue, 2009), which can be useful for species level phylogenetic reconstruction. *DIV*-like genes contain an intron which is roughly 400-500 bps in

*MYB: a large transcription factor family in plants with conserved MYB DNA-binding domain (Feller, et al., 2011; Stracke, et al., 2001).

Campanulaceae. We utilized the introns in *DIV1* and *DIV3* paralogs as phylogenetic markers to study Hawaiian *Clermontia*.

The Pentatricopeptide repeat (PPR) gene family is one the largest gene families found in land plants. The PPRs are characterized by 2-26 tandem repeats of a highly degenerate 35 amino acid motif (Yuan, et al., 2009). The PPR proteins are targeted to organelles and involved in many post-transcriptional processes in organellar transcripts, including splicing, editing, processing, and translation (Delannoy, et al., 2007). At present, studies have shown that the PPR gene family contains c. 450 members in *Arabidopsis thaliana* and 477 in *Oryza sativa*, with 80% of these PPRs being intronless (Yuan, et al., 2009). Most of the PPR genes have a single orthologue in both *A. thaliana* and rice genomes, and that means it should be straightforward to evaluate the orthology of most PPR genes across species. In addition, PPR proteins are characterized as RNA-binding molecules, binding in a sequence-specific manner (Hayes, et al., 2012). PPRs may have rapid rate of sequence evolution to adjust to the changes in the targeted RNA sequences. PPRs can be utilized as phylogenetic tools due to the enormous gene member, easily assessable orthology, and rapid rate of evolution (Yuan, et al., 2009), and PPR loci were utilized as phylogenetic tools in Crowl's study of Campanloideae clades (Crowl, et al., 2014).

Here we employed two *DIV*-like gene intron regions and four PPR loci to create a bi-parental phylogeny for *Clermontia*, and aim to clarify evolutionary relationships, speciation patterns, and hybridization history. We compare these data with a chloroplast dataset (Fig. 3) previously published of Hawaiian *Clermonita* (Givnish, et al., 2013).

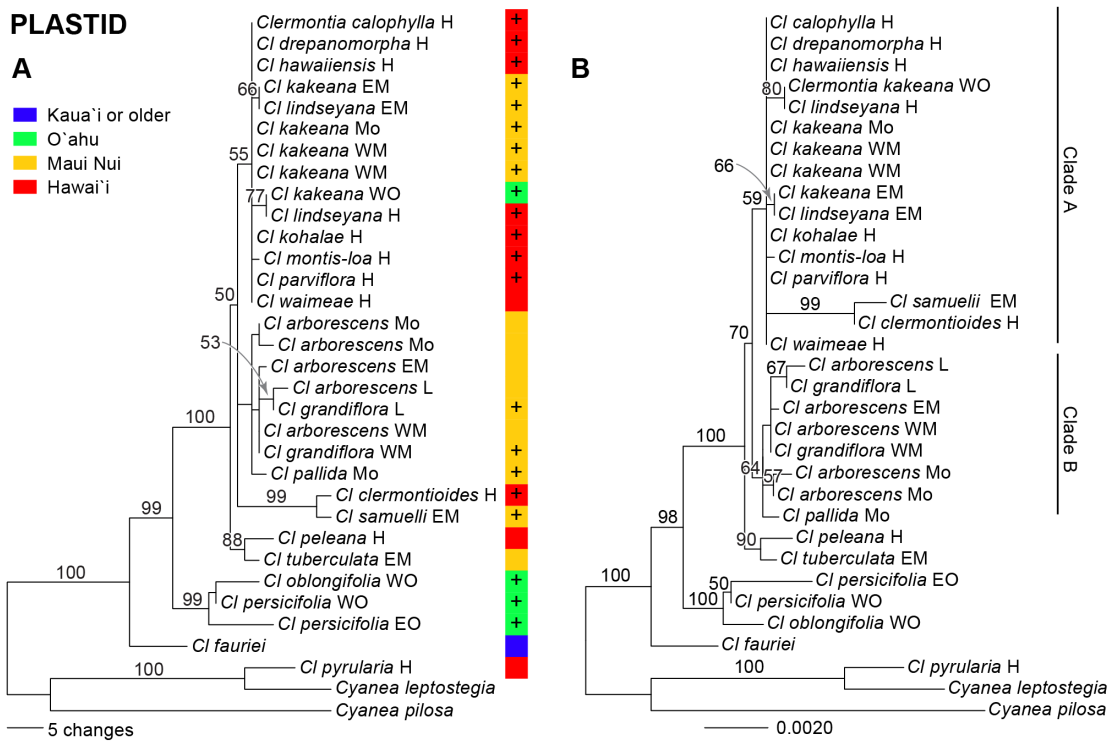

Figure 3. Phylogenetic tree of Hawaiian *Clermontia* based on chloroplast makers (Givnish, et al., 2013).

4. Flower architecture and morphology

The evolutionary development of flowers is associated with their pollination, adaptive radiation, speciation, and diversification (Coen and Nugent, 1994; Endress, 1999; Zhang, et al., 2010). Floral symmetry can be classified into two main types: radially symmetrical (actinomorphic; polysymmetric), in which the flower has two or more central axes; and bilaterally symmetrical (zygomorphic, monosymmetric), which have a flower with only one central axis (Donoghue, et al., 1998; Endress, 1999). Generally, bilaterally symmetrical flowers have floral organs of three different sizes or shapes (especially in the petal whorl): dorsal (abaxial), lateral, and ventral (adaxial). In core eudicots, bilateral symmetrical flowers can vary in multiple morphologies with the most frequent being 2 dorsal petals, 2 lateral petals, and 1 ventral petal (2+3 form). Other common forms include (4+1) and (0+5), with all of these types including a central ventral petal pointed downward, while the other four petals shift in location (Donoghue, et al., 1998) .

Previous studies in flowering plants have found four transcription factors from two gene families that directly affect floral symmetry: *CYCLOIDEA (CYC)* and *DICHOTOMA* (*DICH*) from the TCP* family and *DIVARICATA* (*DIV*), *RADIALIS* (*RAD*), and DRIF (*DIV*-and-*RAD*-Interacting-Factor) from the MYB family (Corley, et al., 2005; Cubas, 2004; Feller, et al., 2011; Preston, et al., 2009; Specht and Howarth, 2015). The four genes play important roles in dorsoventral asymmetrical development in

*TCP: a plant transcription factor gene family first described in 1999 (Endress, 1999; Martín-Trillo and Cubas, 2010). TCP genes encode proteins that share a conserved 59-amino acid basic helix-loop-helix TCP domain. The name "TCP" is from: *teosinte branched1* (T) first found in maize, *CYCLOIDEA* (C) from snapdragon, and the *PROLIFERATING CELL FACTORS* (*PCF*) *1* and *2* (P) from rice.

the corolla and androecium of bilateral flowers (Corley, et al., 2005; Cubas, 2004; Jabbour, et al., 2009; Preston, et al., 2009)). *CYC* was the first characterized member and it has the strongest dorsal phenotypic effect (Schwarz-Sommer, et al., 1990). In *Antirrhinum majus* (snapdragon), *CYC*-like genes are necessary to establish the dorsoventral axis in bilaterally symmetrical flowers (Luo, et al., 1996). *CYC*, together with its paralog *DICH*, co-express in the dorsal domain of the floral meristem from initiation and cause retarded growth of the petals and stamen. *RADIALIS* (*RAD*), is activated by *CYC* and also expresses in the dorsal domain of the floral meristem. The molecular structure of *RAD* and *DIV* proteins are closely related, with previous research suggesting that *RAD* acts antagonistically with *DIV* in the dorsal domain of flower, and this causes the *DIV* genes to only function in the ventral petals of the floral meristem. In *cyc-dich* double mutants in *Antirrhinum, DIV* expresses all over the meristem, and these

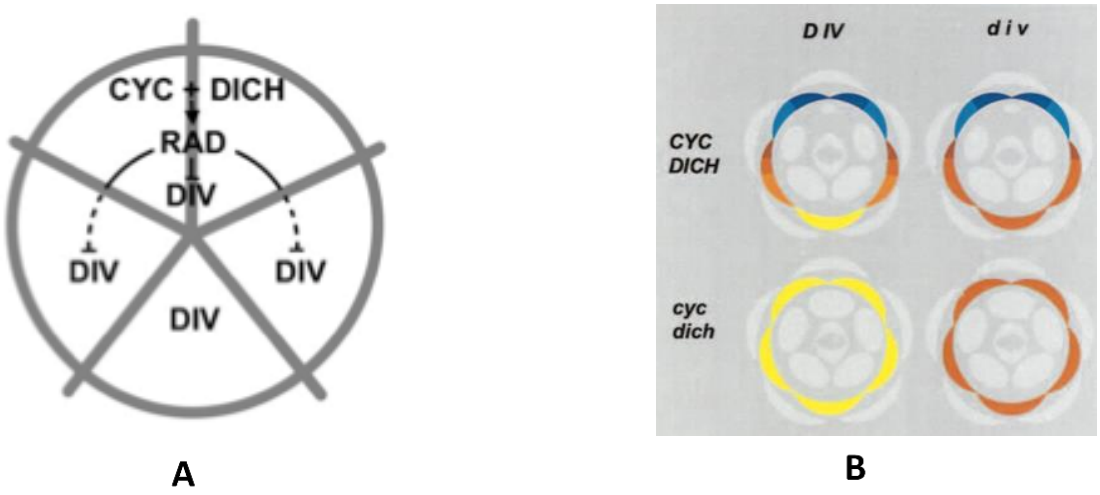

Figure 4. The relationship of transcription factors, *CYC*, *DICH*, *RAD*, and *DIV*, in flower morphological development. *CYC* and *DICH* are co-expressed in the abaxial region during the formation of the early flower primordium, and then activate *RAD*, which can negatively regulate *DIV* in the dorsal region, resulting in *DIV* being functional only in ventral regions (Corley, et al., 2005; Knox, et al., 1993). In the *cyc-dich* mutant flowers, all petals are ventralized, and flowers are radially symmetrical (Galego and Almeida, 2002; Knox and Li, 2017).

plants form radially symmetrical, ventralized flowers (Fig. 4) (Corley, et al., 2005; Gubitz, et al., 2003).

Evidence from numerous comparative studies across flowering plants has shown that the duplication of *CYC*-like genes is highly correlative with the development of floral morphology in bilaterally symmetrical flowers (Bello, et al., 2017; Berger, et al., 2016; Carlson, et al., 2011; Chapman, et al., 2012; Chen, et al., 2018; Citerne, et al., 2003; Citerne, et al., 2000; Claßen-Bockhoff, et al., 2013; Damerval, et al., 2007; Hoshino, et al., 2014; Howarth, et al., 2011; Tähtiharju, et al., 2012; Xu, et al., 2013). Duplications of *CYC*-like genes or loss of function of *CYC*-like genes is highly associated with shifts between radially symmetrical and bilaterally symmetrical flowers. Previous research has shown that two duplication events around the diversification of the core eudicots resulted in three clades of *CYC*-like genes: *CYC1*, *CYC2* and *CYC3* (Howarth and Donoghue, 2006). The *CYC1* genes have not been shown to be directly involved in floral morphological development, but *CYC1* genes may be responsible for branching architecture or/and inflorescence development (Aguilar-Martínez, et al., 2007; Luo, et al., 1999; Preston, et al., 2009). The function of *CYC3* genes are still unclear in floral morphological development (Aguilar-Martínez, et al., 2007); however, based on preliminary data from Dipsacales and Asterales, *CYC3* genes may play a role in ventral specialization (Berger, et al., 2016; Chapman, et al., 2008) (Han *et al*., unpublished). Members of the *CYC2* clade are widely involved in controlling bilateral symmetry across the core eudicots (Howarth and Donoghue, 2006). There are at least two or more paralogs of *CYC2* genes in nearly all characterized bilaterally symmetrical clades of core eudicots. In Asteraceae or Dipsacaceae, some clades have capitate inflorescences, which contain

both radially and bilaterally symmetrical flowers. In these groups there are multiple copies of *CYC2* genes, and they appear to be differentially expressed across the flowers of the inflorescence (Berger, et al., 2016; Carlson, et al., 2011; Chapman, et al., 2008; Chapman, et al., 2012; Chen, et al., 2018; Tähtiharju, et al., 2012). In Asteraceae, the over expression of *CYC2* genes can result in radially symmetrical disc flowers shifting to be more bilaterally symmetric (Tähtiharju, et al., 2012).

5. Flower symmetry across Campanulaceae

The common ancestor of extant Campanulaceae is hypothesized to have had bilaterally symmetrical flowers, with a reversal to radially symmetrical flowers in the Campanuloideae (Fig. 1, Fig. 5 A—D), (Crowl, et al., 2016). We therefore hypothesize a change in expression or function of *CamCYC*-like genes in Campanuloideae, which resulted in the rearrangement of floral organs. In the other major clade, Lobelioideae, almost all the taxa have resupinate flowers with (2+3) or (0+5) forms (Fig. 1, Fig. 5 E—J. Fig. 6), except in *Monopsis* in which flowers are not resupinate. The rest of the three subfamilies have non-resupinated flowers, with Cyphioideae and Nemacladoideae having a (3+2) form, and Cyphocarpoideae having a (1+4) form (Fig. 1) (Crowl, et al., 2016; Lammers, 2007). Cyphocarpoideae and Nemacladoideae (Fig. 1) are narrowly endemic and very difficult to obtain seeds from. Therefore, in our study, we have not included any species from these two subfamilies. The shift in this family not just in symmetry but also in resupination, provides novel variation to examine how these genes effect plant orientation and twisting of structures (Fig. 6).

In this study, we sampled species from Campanuloideae, Lobelioideae, and Cyphioideae, and are using a well-resolved species tree for Campanulaceae (Table 2)

(Antonelli, 2008; Crowl, et al., 2014; Knox and Li, 2017) and thorough sampling of *CamCYC*-like genes. The main aims in this study were to pinpoint the duplication events of *CamCYC*-like genes across Campanulaceae and use *qRT*-PCR to investigate the expression of *CamCYC2* genes in Lobelioideae species with resupinate, bilaterally symmetrical flowers. We examined expression in four species with different forms of bilateral symmetry and relative petal lengths. *CamCYC2A* and *CamCYC2B* are highly expressed in the ventral petals, which correspond to the dorsal side of the flower, suggesting conservation of dorsal identity in upside down flowers. Additionally, individual copies of *CamCYC2* genes show slightly different expression levels in different petals, suggesting possible subfunctionalization between these copies.

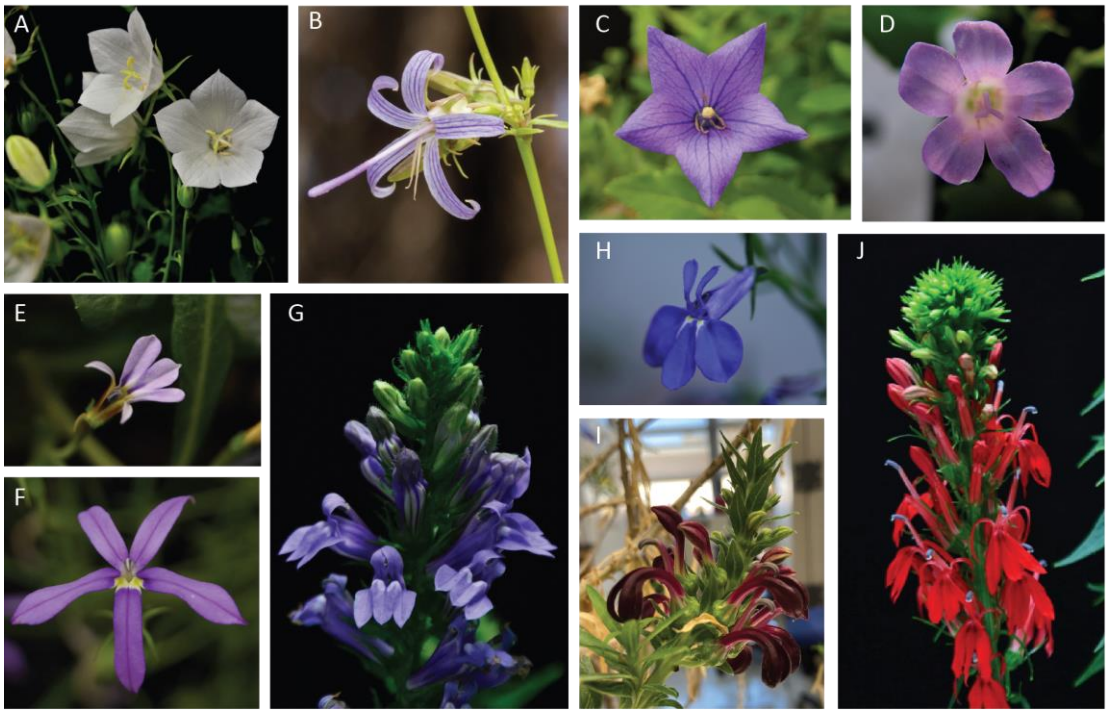

Figure 5. Campanulaceae species. A-D. Campanuloideae species, have radially symmetrical flowers, and E– J. Lobelioideae species, have different forms of bilaterally symmetrical flowers. A. *Campanula carpatica*, B. *Asyneuma prenanthoides**, C. *Platycodon grandiflorus*, D. *Campanula portenschlagiana*, E. *Lobelia anceps*, F. *Lithotoma axillaris*, G. *Lobelia siphilitica*, H. *Lobelia erinus*, I. *Lobelia polyphylla*, J. *Lobelia cardinalis.* *Picture right reserved to Marlin Harm. Other pictures taken by Jingjing Tong.

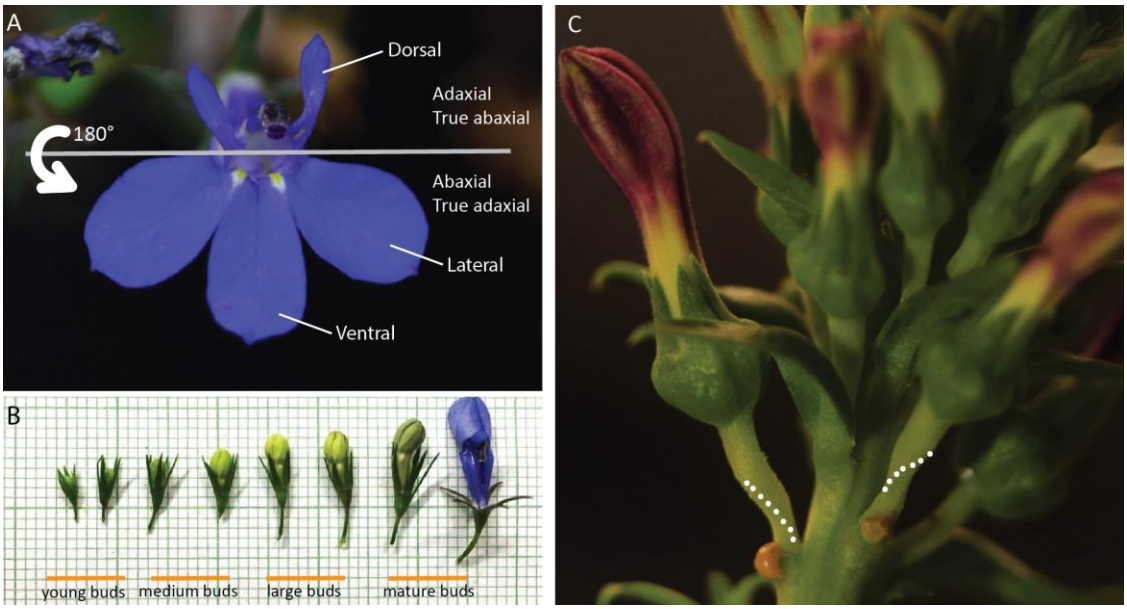

Figure 6. Lobelioideae flowers. A. *Lobelia erinus* flower, B. *Lobelia erinus* floral buds in different stages. Lobelioideae have resupinated flowers with the entire bud turning180-degrees during development, resulting in a reversal of the adaxial domain and abaxial domains. C. *Lobelia polyphylla* large flower bud. During floral bud growth, pedicels turn around at a relatively early stage of bud development and are completely turned 180-degrees upside-down by later stages of bud development. White dots show the twist in the pedicels. Pictures taken by Jingjing Tong.

MATERIAL AND METHODS

1. Sampling and Plant material

In the phylogenetic study of Hawaiian *Clermontia*, we have a total of 48 individuals, from 22 *Clermontia* species, and 4 *Cyanea* species (Table 1). DNA was extracted from fresh material, and prepared by our collaborators, Dr. Richard Pender and Dr. Clifford W. Morden, University of Hawai'i (Table 1). All the *Clermontia* species are included as well as all the sub-species in *Clermontia arborescens*, *Clermontia clermontioides*, *Clermontia grandiflora, Clermontia oblongifolia*, and *Clermontia samuelii*. DNA was extracted by DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany), and then stored in -20ºC.

In the study of duplication and expression patterns of *CYCLOIDEA*-like genes in Campanulaceae, we sampled a total of 132 DNA samples, from 128 species, including 9 Cyphioideae species, 9 Campanuloideae species, and 110 Lobelioidea species (Table 2). All Cyphioideae species and 93 Lobelioideae samples were shared from Dr. Eric Knox, Indiana University (Table 2). Twelve of the Hawaiian lobelioid samples were shared from Dr. Richard Pender and Dr. Clifford W. Morden, University of Hawaii, 1 Campanuloideae species was shared from Dr. Cellinese, University of Florida, and 8 Campanuloideae and 8 Lobeloideae species were from live plants growing in the greenhouse at St. John's University. All DNA was extracted using a DNeasy Plant Mini Kit (QIAGEN) and stored in -20ºC.

Table 1. The Hawaiian *Clermontia* and *Cyanea* samples used in the phylogenetic study of Hawaiian *Clermontia.* Included 22 Hawaiian *Clermontia* species and their subspecies, and 4 *Cyanea* species as the outgroup.

	Species	Location Area
1	*Clermontia arborescens arborescens*	West Maui
2	*Clermontia arborescens waihiae*	West Maui, East Maui
3	*Clermontia arborescens waikoluensis*	Molokai, Lanai
4	*Clermontia calophylla*	Hawaii
5	*Clermontia clermontioides rockiana*	Puu Waa Waa, Hawaii
6	*Clermontia clermontioides clermontioides*	Hawaii
7	*Clermontia drepanomorpha*	Kohala Mt, Hawaii
8	*Clermontia faurei*	Kauai
9	*Clermontia grandiflora grandiflora*	West Maui
10	*Clermontia grandiflora munroi*	West Maui, East Maui, Molokai, Lanai
11	*Clermontia hawaiiensis*	Hawaii
12	*Clermontia kakeana*	Puu Waa Waa, Oahu, Maui, Hawaii
13	*Clermontia kohalae*	Hawaii
14	*Clermontia lindseyana*	East Maui-Nakula NAR, Hawaii
15	*Clermontia micrantha*	West Maui
16	*Clermontia montis-loa*	Hawaii
17	*Clermontia oblongifolia brevipes*	Molokai
18	*Clermontia oblongifolia oblongifolia*	Oahu, West Oahu
19	*Clermontia pallida*	Molokai
21	*Clermontia peleana peleana*	Volcano Rare Plant facility
22	*Clermontia persicilolia*	Oahu
23	*Clermontia pyrularia*	Hawaii
24	*Clermontia samuelii samuelii*	East Maui
25	*Clermontia tuberculata*	East Maui
26	*Clermontia waimeae*	Kohala Mt, Hawaii
27	*Clermontia samuelii hanaensis*	East Maui
28	*Clermontia sp. "x"*	Hawaii
29	*Cyanea calycina*	Hawaii
30	*Cyanea grimesiana*	Hawaii
31	*Cyanea leptostegia*	Hawaii
32	*Cyanea superba*	Hawaii

Table 2. Campanulaceae species in the *CamCYC*-like genes study. Included a total of 132 DNA samples, from 128 species, including 9 Cyphioideae species, 9 Campanuloideae species, and 110 Lobelioideae species.

Clade	Genus	Species	CYC1	CYC2B	CYC2A	CYC3
Camp	*Campanula*	*persicifolia*	2			
Camp	*Campanula*	*carpatica*	1			
Camp	*Campanula*	*glomerata*	1			
Camp	*Campanula*	*portenschlagiana*	2			
Camp	*Campanula*	*cochleariifolia*	2			
Camp	*Jasione*	*montana*	1			
Camp	*Platycodon*	*grandiflorus*	1			
Camp	*Phyteuma*	*scheuchzeri*	2			
Camp	*Campanula*	*drabifolia*				
Cyphia D	*Cyphia*	*lasiandra*	1			1
Cyphia D	*Cyphia*	*comptonii*	1			1
Cyphia D	*Cyphia*	*digitata*	1			2
Cyphia D	*Cyphia*	*longipetala*	1			1
Cyphia D	*Cyphia*	*zeyheriana*	1			1
Cyphia D	*Cyphia*	*eckloniana*	1			1
Cyphia D	*Cyphia*	*volubilis*	1			1
Cyphia D	*Cyphia*	*longipetala*	1			1
Cyphia D	*Cyphia*	*smutsii*				1
Cyphia D	*Cyphia*	*rogersii*	1			1
Genistoid E	*Lobelia*	*baumannii*	1	1	1	
Genistoid E	*Lobelia*	*hartlaubii*	1	1	1	
Genistoid E	*Lobelia*	*goetzei*	1	1	1	
Genistoid E	*Lobelia*	*tomentosa*	1	1		
Genistoid E	*Lobelia*	*comptonii*	1		1	
Genistoid E	*Lobelia*	*malowensis*	1	1	1	
Genistoid E	*Lobelia*	*pteropoda*	1		1	
Genistoid E	*Lobelia*	*dasyphylla*	1		1	
Genistoid E	*Lobelia*	*patula*	1	1	1	
Genistoid E	*Lobelia*	*vanreenensis*	1		1	
Impares F	*Lobelia*	*heterophylla*	1			2
Impares F	*Lobelia*	*cleistogamoides*	2			1
Impares F	*Lobelia*	*tenuior*	1			1
Impares F	*Lobelia*	*winifrediae*		1	1	
Impares F	*Lobelia*	*rhytidosperma*	1		1	
Impares F	*Lobelia*	*simplicicaulis*	2	1	1	
Impares F	*Lobelia*	*rhombifolia*	2		1	
Impares F	*Lobelia*	*rarifolia*	2		1	
Impares F	*Lobelia*	*trigonocaulis*	1		1	1

Impares F	*Colensoa*	*physaloides*	2			1
Monopsis G	*Monopsis*	*stellarioides*	1	1		
Monopsis G	*Monopsis*	*decipiens*	1		1	
Monopsis G	*Monopsis*	*unidentata*	1		2	
Monopsis G	*Monopsis*	*alba*	1	1	1	
Monopsis G	*Monopsis*	*debilis*	1			
Monopsis G	*Monopsis*	*flava*	1			
Broom H	*Lobelia*	*lasiantha*	1			
Broom H	*Lobelia*	*capillifolia*				
Broom H	*Lobelia*	*linearis*	1			
Grammatotheca I	*Lobelia*	*thermalis*			1	
Grammatotheca I	*Grammatotheca*	*bergiana*				
Mezlerioid1 J	*Lobelia*	*quadrisepala*				
Mezlerioid2 K	*Lobelia*	*galpinii*				
Erinoid L	*Lobelia*	*inconspicua*			1	
Erinoid L	*Lobelia*	*wilmsiana*	1		1	
Erinoid L	*Lobelia*	*cymbalarioides*	1		1	
Erinoid L	*Lobelia*	*minutula*	1		1	
Erinoid L	*Lobelia*	*boivinii*			1	
Erinoid L	*Lobelia*	*laxa*				
Erinoid L	*Lobelia*	*erinus*	1	1	1	
Wimmerella M	*Wimmerella*	*bifida*	1	1		
Wimmerella M	*Wimmerella*	*pygmaea*	1	1	1	
Wimmerella M	*Wimmerella*	*hederacea*	1	1	1	
Wimmerella M	*Wimmerella*	*secunda*		1	1	
Wimmerella M	*Lobelia*	*anceps*		1		
Mezlerioid3 N	*Lobelia*	*laurentioides*	1	1		
Mezlerioid3 N	*Lobelia*	*jasionoides*	1	1		
Mezlerioid3 N	*Lobelia*	*muscoides*	1	1		
Solenopsis O	*Lobelia*	*urens*		1	1	
W North America P	*Downingia*	*bicornuta*	1			
W North America P	*Downingia*	*cuspidata*				
W North America P	*Porterella*	*carnosula*	1	1	1	
W North America P	*Palmerella*	*debilis*	1		1	
Diastatea Q	*Diastatea*	*micrantha*		2	1	
E North America R	*Lobelia*	*dortmanna*		1		
E North America R	*Lobelia*	*puberula*		1		
E North America R	*Lobelia*	*fenestralis*	1	1		
E North America R	*Lobelia*	*cardinalis*		1	1	
E North America R	*Lobelia*	*siphilitica*		1	1	
South America S	*Centropogon*	*comosus*	2	1	1	
South America S	*Burmeistera*	*crispiloba*	2		1	
South America S	*Lobelia*	*tupa*	2	2		

South America S	*Lobelia*	*bridgesii*	2	1	1	
South America S	*Lobelia*	*polyphylla*		2	1	
Australasia T	*Isotoma*	*gulliveri*	2	1		
Australasia T	*Hypsela*	*reniformis*	1	1		
Australasia T	*Lobelia*	*roughii*	1	1	1	
Australasia T	*Lobelia*	*macrodon*	1	1	1	
Australasia T	*Pratia*	*arenaria*	2	1		
Australasia T	*Pratia*	*gelida*	1	1		
Australasia T	*Lobelia*	*pratioides*	1	1	1	
Australasia T	*Pratia*	*pedunculata*	1	1	1	
Australasia T	*Lithotoma*	*axillaris*		1	1	
Australasia T	*Lithotoma*	*petraea*	1	1	1	
Australasia T	*Isotoma*	*hypocrateriformis*	1	1	1	
Giants U	*Lobelia*	*sessilifolia*		2		
Giants U	*Lobelia*	*doniana*	1	2	1	
Giants U	*Lobelia*	*yuccoides*	2	2		
Giants U	*Lobelia*	*niihauensis*	1	1		
Giants U	*Lobelia*	*kauaensis*	2	1		
Giants U	*Cyanea*	*leptostegia*	2			
Giants U	*Apetahia*	*longistigmata*	2	2		
Giants U	*Delissea*	*rhytidosperma*				
Giants U	*Lobelia*	*sancta*	1	2	1	
Giants U	*Lobelia*	*lukwangulensis*	3	1	1	
Giants U	*Lobelia*	*longisepala*	1		2	
Giants U	*Lobelia*	*morogoroensis*	1		1	
Giants U	*Lobelia*	*thuliniana*	2	2		
Giants U	*Lobelia*	*stuhlmannii*	2	1		
Giants U	*Lobelia*	*wollastonii*	1	2		
Giants U	*Lobelia*	*bequaertii*	2	2	1	
Giants U	*Lobelia*	*giberroa*	2	1		
Giants U	*Lobelia*	*mildbraedii*		1		
Giants U	*Lobelia*	*acrochilus*				
Giants U	*Lobelia*	*aberdarica*	2	2	1	
Giants U	*Lobelia*	*bambuseti*		2	1	
Giants U	*Lobelia*	*telekii*	2			
Giants U	*Lobelia*	*gregoriana*	1			
Giants U	*Lobelia*	*burttii*		2	1	
Giants U	*Lobelia*	*columnaris*	2	2		
Giants U	*Clermontia*	*micrantha*		1	1	
Giants U	*Clermontia*	*persicifolia*	1	1	1	
Giants U	*Cyanea*	*acuminata*	2	1	1	
Giants U	*Cyanea*	*superba*	1	2		
Giants U	*Brighamia*	*insignis*	2	1	2	

Giants U	*Delissea*	*rhytidosperm*	4	2	2	
Giants U	*Delissea*	*subcordata*	2	2	1	
Giants U	*Lobelia*	*villosa*	3	2	1	
Giants U	*Lobelia*	*yuccoides*				
Giants U	*Trematolobelia*	*kaulensis*	2			
Giants U	*Trematolobelia*	*macrostachys*	2	2	1	
Giants U	*Lobelia*	*oahuensis*		1		

2. Dissecting and Collection Floral tissues

Four Lobelioideae species were grown in the greenhouse at St. John's University, Queens, NY, USA, *Lobelia erinus* (Fig. 5 H), *Lobelia siphilitica* (Fig. 5 G), *Lobelia polyphylla* (Fig. 5 I), and *Lithotoma axillaris* (Fig. 5-F). All the plant seeds were ordered from online plant nurseries (Botanical interests ®, Hazzard's Seeds, Plant World Seeds). Flower buds were collected at three different stages: small buds, medium buds, and large buds. The small buds of *Lobelia erinus* were about 2.5~4mm, medium buds were about 5~6mm, and large buds were about 7~8 mm. For *Lobelia siphilitica*, small buds were about 5~6 mm, medium buds were about 8~12 mm, and the large buds were about 14~18 mm. For *Lobelia polyphylla*, small buds were about 7~10 mm, medium buds were about 15~20 mm, and the large buds were about 25~30 mm. For *Lithotoma axillaris*, small buds were about 10~13 mm, medium buds were about 15~25 mm, and the large buds were about 25~35 mm. Medium flower buds were dissected into separate dorsal, lateral, and ventral petals. Leaf tissue was separately collected as a control. All tissues were immediately frozen with liquid nitrogen and stored in a -80 freezer until extraction. Roughly 20~30mg of tissue was collected for each RNA extraction. The exception was tissue from *L. erinus* flower buds, which are extremely small, with 3~4 mm medium size buds, so therefore, only roughly 15~20 mg was collected for RNA extraction in this species. Three biological replicates were collected for each type of tissue.

3. Amplification

All PCR reactions were performed using *Taq* DNA Polymerase (Go*Taq*® Flexi DNA polymerase). All DNA were amplified in 25 µl PCR reactions containing: 1 µl DNA, 5 µl 5X buffer, 2.5 µl 25mM $MgCl_2$, 0.5 µl 10 mM dNTP, 1 µl of 10mM primers, 1 µl *Taq* polymerase, and distilled water was added to bring up to total volume. Amplifications utilized the following cycling program: (1) initial denaturation was carried out at 94ºC for 2 min; (2) 39 cycles of 94ºC for 45 seconds, 51ºC (vary by different pairs of primers) for 1 min, and 72ºC for 1 min 30 seconds; (3) a final elongation step at 72ºC for 20 min. We used previously published degenerate *DIV* primers based on the conserved regions of the two MYB domains in *DIV*-like genes (Howarth and Donoghue, 2009). Primers used and their sequences are provided in Table 3.

To amplify *CYC*-like genes in Campanulaceae, previously designed degenerate primers were used from Howarth and Donoghue (Howarth and Donoghue, 2005). Primers for *CamCYC1* were designed based on *CYC*-like sequences from other lineages in asterids available from NCBI. All primer sequences are provided in Table 3. Amplifications utilized the following cycling program: (1) initial denaturation was carried out at 94ºC for 2 min; (2) 39 cycles of 94ºC for 45 seconds, 53ºC (*CamCYC1* 53 ºC, *CamCYC2* and *CamCYC3* 52 ºC) for 1 min, and 72ºC for 1 min 30 seconds; (3) a final elongation step at 72ºC for 20 min.

Table 3. Primers for different loci in Campanulaceae. Two *DIV* loci and three PPR loci were examined as nuclear markers in the Hawaiian *Clermontia* phylogenetic study. Three *CYC* loci were examined as floral symmetry genes in *CYC*-like study in Campanulaceae.

Locus	Primer	Primer Sequences (5'-3')
DIVARICATA1	CampDIV1Fb	CYCSTTTTACTYTASAATGGGG
	Clerm DIV1Fa	GCTTTGAKGGATTCAAGCCGCCAAAT
	CampDIV1R	GARATRTTTYTCCAGTYYCCTTTTCCA
DIVARICATA3	DIVFa	GTGGGGGAYGTGATCAAACAGTAYAG
	CampDIV3F	GGATTCGATGGGWTRAAACAAYTC
	Clerm DIV3 Fa1	GGYAGGCTTTATACCGGTACCTGGC
	Clerm DIV3 Fa2	GCTTTATACCGGTACCTGGCTRCAG
	DIVRa	CCATACTTRTTWAGCCCSAGCAAAAATTGCCTG
AT1G09680/PPR11	PPR11 F	TTTGTTATGTTGATKTGGGTTTT
	PPR11 R	GCCAGAAATAATAGCCGTGTAAG
AT5G39980/PPR123	PPR123-1362 F	AARGCYAAYAATCTTATTCARGARATGCAG
	PPR123-1957 R	TAHAGASTHAGCATYTGAAAGTGAACTTC
CYCLOIDEA1	Astl CYC1Fa	CGRAGRATGAGRYTRTCNCTTGATG
	Astl CYC1Ra	GCCCTTKCYCTTGCYCTTTCCCTTG
CYCLOIDEA2	CYC 73b	GCNCGNARRTTYTTYGATCTDCAAG
	CYC Ra	CTTGCTCTTTCYCTYGCYTTYGCCC
CYCLOIDEA3	CYC 73b	GCNCGNARRTTYTTYGATCTDCAAG
	CYC Ra	CTTGCTCTTTCYCTYGCYTTYGCCC
	Astl CYC3 Fa	GGGAAGAMAGAYMGGCAYAGC
	Astl CYC1Ra	GCCCTTKCYCTTGCYCTTTCCCTTG

Cloning used the StrataClone PCR Cloning Kit (Agilent, Santa Clara, CA), following the manufacturer's instructions. We picked four to eight colonies per plate and amplified them by using construct primers, M13F and M13R. DNA cleaning utilized the P.E.G method (Rosenthal, et al., 1993), and DNA was sent to Yale University DNA

Analysis Facility. Sanger sequencing was run on a 3730xl DNA Analyzer (Applied Biosystems, Thermo Fisher Scientific, Inc.).

4. Quantitative Real-Time PCR and statistical analysis

Total RNA was extracted from plant tissues used for qPCR using the RNeasy Plant Mini Kit and RNase-free DNase kit (QIAGEN), and then stored in -80 ºC. The concentrations and purities of all RNA samples were determined by a Thermo Scientific NanoDrop 2000 (Thermo Scientific, Waltham, MA). The *q*RT-PCR primers were designed in Geneious Pro v.7.1.2 (http://www.geneious.com) based on *CamCYC2* gene sequences and ACTIN sequences collected in our study. Specific primer sets were designed for each species (Table 4). The One-Step RT-PCR kit (QuantaBio) was used to investigate the expression patterns of *CamCYC2A* and *CamCYC2B* gene expression in the collected tissues from *Lobelia erinus*, *Lobelia siphilitica, Lithotoma axillaris,* and *Lobelia polyphylla*. In the *q*RT-PCR experiments, each type of tissue included three biological and two technical replicates. Samples were run on a Bio-Rad MyIQ Single Color Real-Time RCR Detection System (Bio-Rad, Hercules, CA). The melting curve and threshold cycle (Ct) values were analyzed by the $2^{-\Delta C_T}$ method (Livak and Schmittgen, 2001). Because all of the tissues used were from natural or wildtype plants, there was no "untreated control" to normalize the second delta that is standard in these methods. ANOVA and post hoc Tukey HSD were performed on the web site: https://astatsa.com/OneWay_Anova_with_TukeyHSD/.

Table 4. The *q*RT-PCR primers for each species examining *CamCYC2A* and *CamCYC2B* gene expression patterns. The annealing temperature for all primers is 60℃.

	Lobelia erinus
CamCYC2A	CYC2 37F 5'-GCTAGTAAAACCCTTGATTGGCT-3' CYC2A 314R 5'-GCCCTGGACTCTTTTGCAAAGT-3'
CamCYC2B	CYC2 37F 5'-GCTAGTAAAACCCTTGATTGGCT-3' CYC2B 291R 5'-GCGATGAGATGCAGGTTTATAACTG-3'
CamActin	Le-act1046F 5'-ATCCACGARACSACCTACAACT-3' Le-act1216R 5'- MACCACCTTAATCTTCATGCTGCT-3'
	Lobelia siphilitica
CamCYC2A	ls CYC2A-37F 5'-TTCGACAAAGCTAGTAAAACTCTTGATTGG-3' Ls CYC2A 264R 5'-TTTCTCTTTGGCTCTCGTTGTAGC-3'
CamCYC2B	ls CYC2B-47F 5'-CTAGTAAAACCCTTGATTGGCTTTTCAC-3' Ls CYC2B 298R 5'-CTAGGCGATGAGATGCAGGTTTATAAC-3'
CamActin	Le-act477F 5'-AGATYTGGCATCAYACTTTCTACA-3' Le-act729R 5'- CCTTCGTARATTGGAACCGTGTG-3'
	Lithotoma axillaris
CamCYC2A	Ls CYC2A F41 5'-ACAAAGCTAGTAAAACTCTTGATTGGCT-3' ISCYC2A260R 5'-TTCTCTTTGGCGCTCGATGTAGCTG-3'
CamCYC2B	ISCYC2B38F 5'-TTGACAAAGCTAGTAAAACCCTTGATTGG-3' ISCYC2B203R 5'-GCTCCTTCATTTTGTTCAGCTGC-3'
CamActin	Le-act1046F 5'-ATCCACGARACSACCTACAACT-3' Le-act1216R 5'-MACCACCTTAATCTTCATGCTGCT-3'
	Lobelia polyphylla
CamCYC2A	LP CYC2AF1a 5'-TCGACAAAGCTAGTAAAACTCTTGATTGG-3' LP CYC2A R4 5'-TTTGCAAGATAAAGTGCAGGTTTATACG-3'
CamCYC2B	Ls CYC2B F43 5'-AAAGCTAGTAAAACCCTTGATTGGCT-3' LP CYC2B R1 5'-TTTGTGCTCTCATCGTTTTCGCTTCAC-3'
CamActin	Le-act477F 5'-AGATYTGGCATCAYACTTTCTACA-3' Le-act729R 5'-CCTTCGTARATTGGAACCGTGTG-3'

5. Alignment and Phylogenetic Analyses

All individual colony sequences were edited in Geneious® (Pro v. 7.1.2), including removing the plasmid and primer sequences. Consensus sequences were generated from similar clones from the same DNA sample. In order to determine orthology, we used BLAST in NCBI. *DIV*-like gene sequences were determined by the presence of the conserved two MYB domains flanking the two ends of the intron. PPR loci also contain conversed sequences. *CYC*-like genes were determined by the TCP domain and R domain. The species level sequences were aligned in the Geneious®, using the MUSCLE Alignment tool (default parameters) based on nucleotide sequences, and then manually adjusted according the amino acid sequences or nucleotide sequences in Mesquite (Wayne P. Maddison, David R. Maddison) or Geneious®. The phylogenetic trees were generated with CIPRES science gateway (https://www.phylo.org), using the Maximum Likehood analysis by the RAxML-HPC BlackBox, default parameters, except with added option to estimate the proportion of invariable sites (GTRGAMMA + I).

Chapter 1

Phylogeny of *Clermontia* (Hawaiian Lobelioids) based on *DIVARICATA (DIV)*-like genes and Pentatricopeptide Repeat (PPR) genes

1.1 RESULTS

1.1.1 *ClermDIV*-like genes and PPR loci isolated from Hawaiian *Clermontia*

In our phylogenetic study of Hawaiian *Clermontia*, we sampled a total of 4 *Cyanea* and 22 *Clermontia* species including all subspecies (Table 1). We identified Clem*DIV*1 and Clem*DIV*3 genes from Hawaiian *Clermontia*. Because *Clermontia* is tetraploid, each gene had duplicated, resulting in two separate clades of each gene. This resulted in four genes, *ClermDIV1A*, *ClermDIV1B*, *ClermDIV3A*, and *ClermDIV3B*. We also isolated the four genomic loci, PPR11, PPR 123, PPR70, and PPR81, from Hawaiian Lobelioids by using specific primers for each locus (Table 3). Species level variation was found in all the regions except PPR70 and PPR81.

The matrix for *ClermDIV*1 has 74 DNA sequences from *Clermontia* and 8 DNA sequences from sister genus, *Cyanea*. The matrix for *ClermDIV*3 has 84 sequences from *Clermontia* and 5 DNA sequences from *Cyanea*. The average length of *ClermDIV1* sequences is 720 bps, the average length of *ClermDIV3* sequences is 560 bps. In general, sequence length is relatively conserved within the *Clermontia* genus, especially the *ClermDIV*1 sequences and PPR loci. There are no deletions or insertions in *ClermDIV1A*

intron sequences, except one 19 bp deletion in *Cyanea leptostegia*, and in *ClermDIV1B* intron sequences there are two 2 bp insertions found in a few sequences, and one 9 bp deletions found in *Cy. leptostegia*. The *ClermDIV*3 loci show higher variation than all of the other loci. Sequences of the *ClermDIV3* loci are divided into two groups, *ClermDIV3A* and *ClermDIV3B*, with both groups containing several big deletions/insertions. In the PPR11 locus, there are two 2 bp insertions found in *Cy. grimesiana*, *Cy. leptostegia* and *Cl. pyrularia* sequences. In the PPR123 locus, there are also two 2 bp deletions. There are very few DNA sequences isolated from PPR70 and PPR81 loci, and we did not include the PPR70 and PPR81 datasets in our phylogenetic analyses.

A combined matrix (Fig. 7) of *ClermDIV1A*, *ClermDIV1B*, *ClermDIV3A*, *ClermDIV3B*, PPR123, and PPR11 was analyzed and a Maximum Likelihood tree was generated with RAxML-HPC BlackBox on the CIPRES science gateway (https://www.phylo.org). The phylogeny was consistent with the chloroplast phylogeny (Givnish, et al., 2013) (Fig. 3) in many clades. However, there were clear differences in a few species. *Clermontia persicifolia* was most closely related to the species in clade A rather than clade D. Additionally, *Clermontia kakeana* and *Clermontia arborescens* fell out in multiple places in the phylogeny and had frequent conflict between markers (Fig. 8).

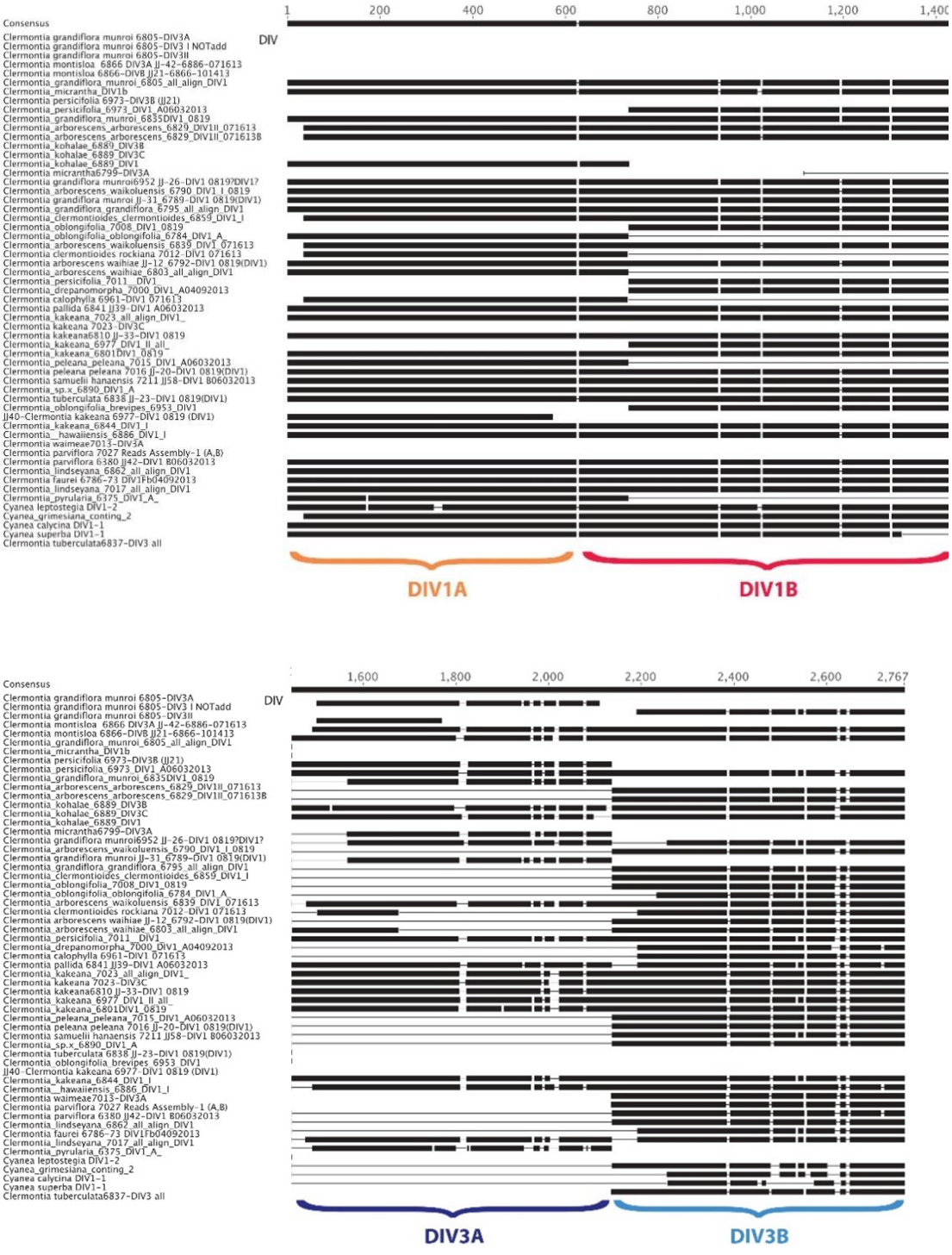

Figure 7. The alignment of *ClermDIV* intron sequences combining *ClermDIV1A*, *ClermDIV1B*, *DIV3A*, and *DIV3B*. The alignment containing a total of 2,767 nucleotide characters.

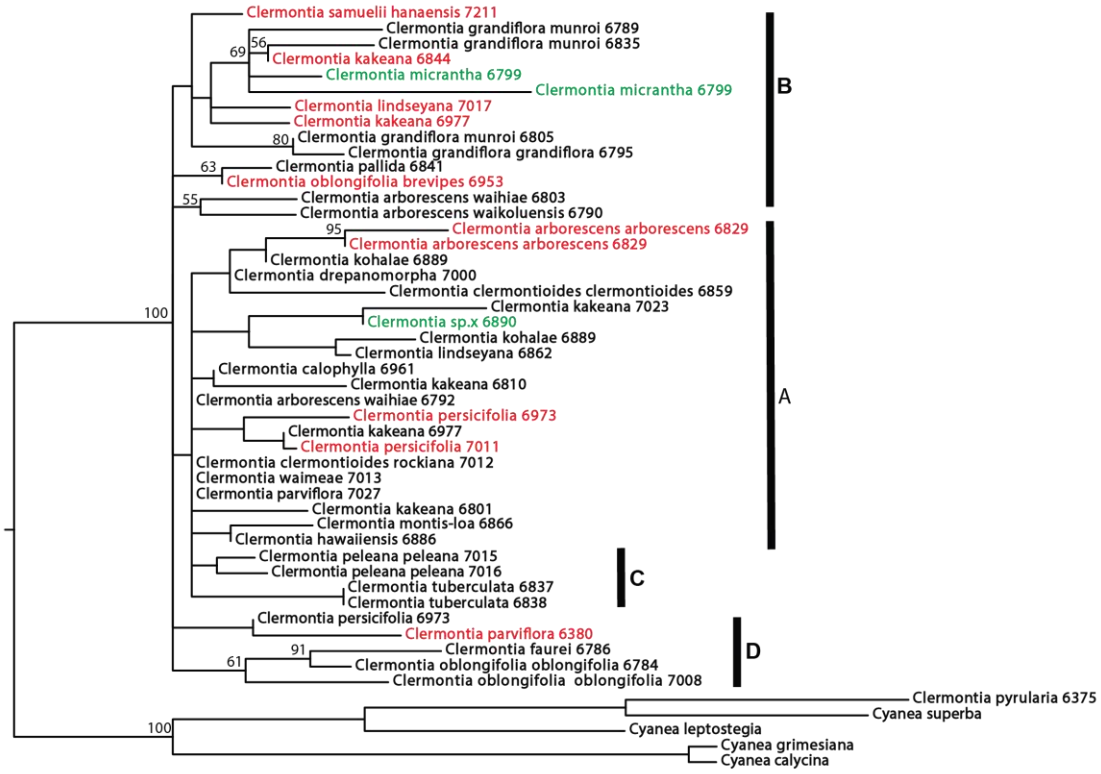

Figure 8. ML Phylogenetic tree of Hawaiian *Clermontia* using ML combining *ClermDIV1A*, *ClermDIV1B*, *ClermDIV3A*, *ClermDIV3B*, *ClermPPR123*, and *ClermPPR11*. Groups are numbered following the clades in Fig. 3. Species that disagree with labeled chloroplast clades are highlighted in red. Species not sampled in Givnish et al. (2013) are highlighted with green.

1.2 DISCUSSION

Species relationships and hybridization in Hawaiian *Clermontia*

Hawaiian Campanulaceae is the largest adaptive radiation of flowering plants in the Hawaiian Islands, with roughly 130 species in 6 genera. The group has been shown to be the result of a single colonization event to the Hawaiian Islands, roughly 13 Mya (Givnish, et al., 2009). *Clermontia*, the second largest genus with 22 species, has been estimated to have only diversified roughly 5 Mya and has therefore been difficult to examine phylogenetically, due to low sequence variability. This suggests that *Clermontia* began diversifying around the time that the oldest modern island, Kaua'i, emerged from the ocean (Givnish, et al., 2013; Givnish, et al., 2009). Given its young age, very little sequence variation has evolved among *Clermontia* species, despite distinctively different morphologies (Fig. 2). The most notable characteristic, occurring in 15 of the 22 species of *Clermontia* involves petaloidy of the sepals resulting in a "double whorl" or petals (Fig. 2), (Hofer, et al., 2012; Wagner, et al., 1999). This has been shown to be the result of a homeotic change in the MADS box gene *PISTILLATA* leading to the sepals looking like petals (Hofer, et al., 2012).

Previous studies have attempted to examine the evolutionary history of *Clermontia*; however, these studies have focused on plastid data, inter-simple sequence repeat polymorphisms (ISSRs), or 5S-NTS ribosomal DNA (Givnish, et al., 2013; Hofer, et al., 2012), (C. Morden, unpublished data). Each of these methods can provide variation to analyze, but only provide a fragment of the evolutionary history, especially in groups with frequent hybridization among species. Chloroplast data is only maternally inherited, and is subject to chloroplast capture, a process by which inter-species hybridization leads

the chloroplast not matching the nuclear genome (Rieseberg and Soltis, 1991). 5S-NTS is subject to similar processes, since concerted evolution, which maintains high sequence similarity between copies and alleles (Nei and Rooney, 2005), leads to sequences being uniform. ISSR markers can provide significant variation, however, they are not direct DNA sequences that can be properly assessed for homology, instead providing presence or absence data for polymorphisms in repeat sequences.

Comparing the sequences from multiple, targeted low-copy nuclear genes and introns can be the most effective way to uncover evolutionary history of closely related species (Howarth and Baum, 2005). This was successfully utilized in a separate Hawaiian genus, *Scaevola*, in which the genealogy clearly showed that two species in the small clade of seven species were the result of past hybridization events (Howarth and Baum, 2005). We employ a similar method here with *Clermontia*, utilizing the intron region of four *ClermDIV* genes and two PPR genes, as the phylogenetic tools. In addition, *Clermontia* species are tetraploid, due to genomic duplication before the group's dispersal to the Hawaiian Islands. These duplicated copies clearly form two separate clades of *Clermontia* in our phylogenetic trees.

Previous data from Givnish's study, provide a phylogenetic tree based on chloroplast sequence information. Their tree roughly follows a dispersal pattern from older islands to younger islands (Kaua'i to Hawai'i). In the chloroplast data set, the *Clermontia* species grouped into two major clades, Clade A and Clade B, and two basal *Clermontia* clades. One exception is *Clermontia pyrularia,* which falls in the *Cyanea* clade. The clade of species on the island of Hawai'i (Fig. 3, clade A) contains almost no resolution among species due to low sequence variation. However, there was minimal

resolution to fully resolve the phylogeny and several points of conflict among the trees generated from these different datasets.

Here we show data from multiple nuclear genes. After combining intron sequence from four *ClermDIV* genes with PPR123 and PPR11, we uncovered a tree which generally agrees with a phylogenetic tree based on chloroplast data (Fig. 3 and Fig. 8). In the nuclear phylogeny, *Clermonita* species are also grouped into two major clades, Clade A and Clade B, and the basal *Clermontia* clades, which we named as Clade C and Clade D. We were able to pinpoint specific species that likely have resulted from past hybridization based on comparisons among these datasets. *Clermontia pyrularia* falls into the *Cyanea* clade. Tt may be a intergeneric hybrid involving a cross between species from *Cyanea* and *Clermontia*, which was also supported by the plastid phylogeny in Givinish et al. (2013). *Clermontia kakeana* samples fall out in multiple places in the phylogeny and this species, which occurs across multiple islands, likely has hybridized in each of its locations. Despite the close relationship between *Clermontia persicifolia* and the *C. faurei* + *C. oblongifolia* clade based on chloroplast data, *C. persicifolia* does not share that relationship based on nuclear gene regions. Instead, it is related to members of clade A (Fig. 3). Finally*, Clermontia arborescens* falls out in multiple clades and we had to remove two sequences from the final analysis because of conflicting information among the datasets, suggesting that this species and its varieties have undergone frequent introgression. There are some conflicts between the chloroplast phylogeny and the nuclear phylogeny, which may be the result of different individual sampling in each study. Overall, phylogenetic relationships of *Clermontia* species show a strong signal of geographic location, suggesting high levels of introgression within each island.

Chapter 2

Duplication and Expression Patterns of *CYCLOIDEA*-like genes in Campanulaceae

2. 1 RESULTS

2. 1. 1 *CamCYC1*, *CamCYC2,* and *CamCYC3* from Campanulaceae

In this study, we sampled a total of 128 species, including 9 Cyphioideae species, 9 Campanuloideae species, and 110 Lobelioideae species. In *CamCYC*-like genes, the TCP and R domains are relatively conserved across Campanulaceae. We isolated *CamCYC1* from 83 Lobelioideae species, 8 Campanuloideae species, and 7 Cyphioideae species, *CamCYC2* from 90 Lobelioideae species and 4 Campanuloideae species, and *CamCYC3* from 5 Lobelioideae species, 3 Campanuloideae species, and 8 Cyphioideae species. There were no *CamCYC2* gene sequences isolated from Cyphioideae. The tree topologies across the *CamCYC1*, *CamCYC2*, and *CamCYC3* clades were congruent with known species phylogenies, especially in the best sampled Lobelioideae, in these groups. All three subfamilies were monophyletic and were consistent with a sister group relationship between Campanuloideae and Cyphioideae.

The *CamCYC1* matrix was 492 bps long with 140 sequences, which included 12 sequences from Campanuloideae and 9 sequences from Cyphoideae. Campanuloideae and Cyphoideae grouped in one clade (ML bootstrap = 97). The rest of 119 sequences, all isolated from 83 Lobelioideae species, formed the other clade (Fig. 9). Our data support a duplication in *CamCYC1* within the Campanuloideae that is not shared with the other

subfamilies (ML bootstrap = 100), with *Campanula cochlearifolia*, *C. persicifolia*, *C. portenschlagiana*, and *Phyteuma scheuchzeri* occurring in both clades. Cyphioideae *CamCYC*1 sequences form a single clade, although multiple *CamCYC1* gene sequences were isolated from most Cyphioideae samples, likely due to allelic diversity.

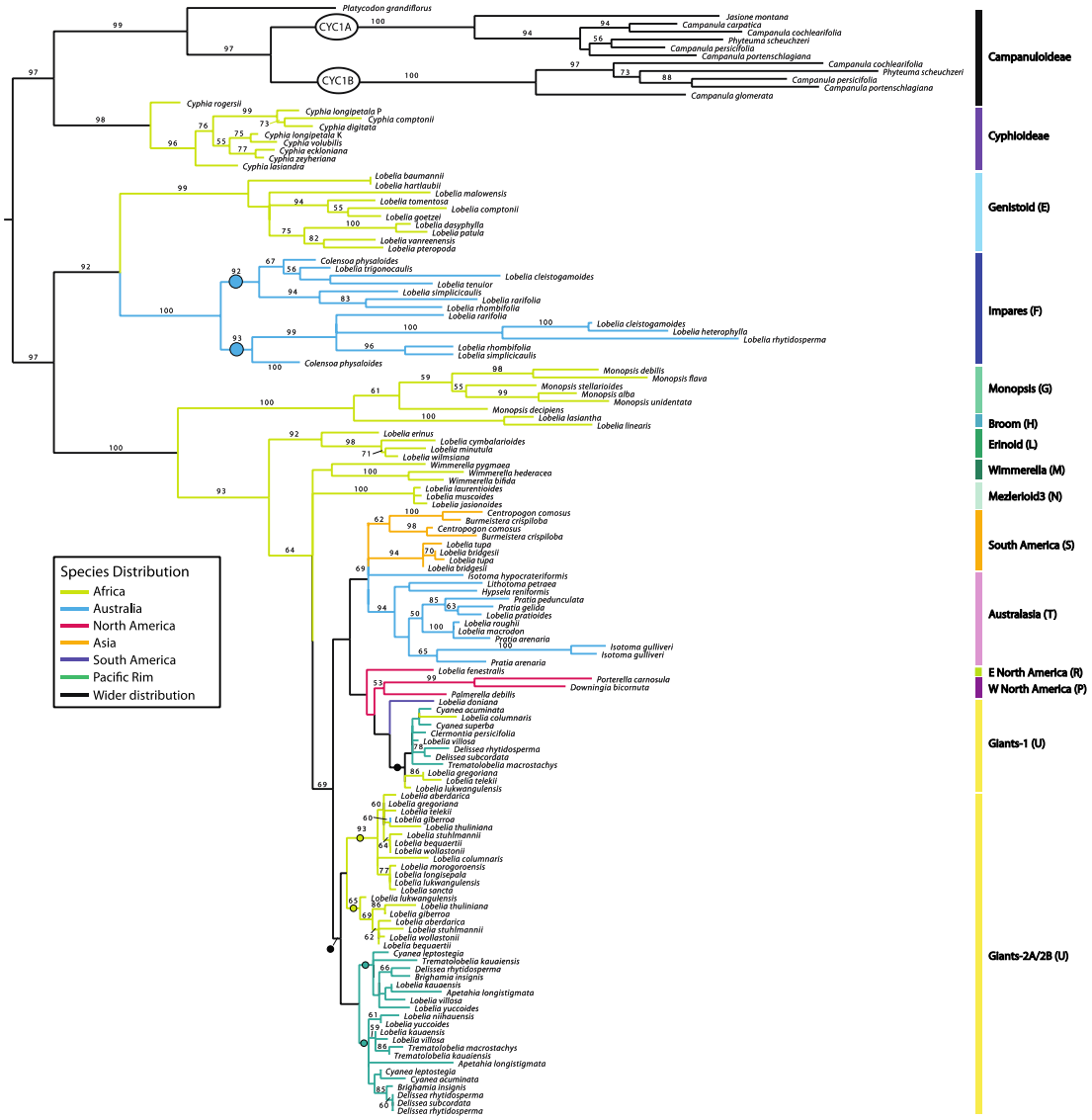

Figure 9. *CamCYC1* RAxML phylogenetic tree. Campanuloideae and Cyphoideae group into one clade, sister to Lobelioideae. The *CamCYC1* gene duplicated in Campanuloideae, which is not shared with the other two subfamilies. Lobelioideae *CamCYC1* sequences are congruent with previously published Lobelioideae phylogenies (Antonelli, 2008; Knox, et al., 1993; Knox and Li, 2017; Knox and Palmer, 1998), with letter designations provided by Knox (unpublished).

Lobelioideae *CamCYC1* formed a single clade with no clear broad duplication detected across Lobelioideae. Species distribution in Lobelioideae *CamCYC*1 subclades are congruent with previously published Lobelioideae phylogenetic relationships (Antonelli, 2008; Knox, et al., 1993; Knox and Li, 2017; Knox and Palmer, 1998). Clade names and letter designations are used from (Antonelli, 2008; Knox, et al., 1993; Knox and Li, 2017; Knox and Palmer, 1998) and Knox (unpublished data), with Genistoid (E) and Impares (F) subclades forming a grade subtending the rest of the subclades. The Impares (F) subclade appears to have duplicated in *CamCYC1* with 5 out of 10 sampled species detected in both subclades including *Lobelia cleistogamoides*, *L. simplicicollis*, *L. rhombifolia*, *L. rarifolia*, and *Colensoa physaloides*, (ML bootstrap = 92). The U subclade, often called the Giant Lobelias, occur in three duplicate clades in the phylogeny, likely due to tetra- and hexa-ploidy in this group. The U1 clade in embedded in a clade with members of the P, R, S, and T subclades, and the U2 clade, including duplicated U2-A and U2-B subclades, is sister to that group.

The *CamCYC2* matrix was 348 bps long with 154 sequences, which included 151 sequences from Lobelioideae and only 4 sequences isolated from Campanuloideae (Fig. 10). There were no *CamCYC2* sequences uncovered from Cyphioideae. In the *CamCYC2* gene tree (Fig. 10), sequences from Lobioideae species formed two clades, which were broadly congruent with the hypothesized species relationships of Lobelioideae across two separate duplicate clades, *CamCYC2A* (ML bootstrap = 92) and *CamCYC2B* (ML bootstrap = 90). Species relationships in both Lobelioideae *CamCYC2* clades shared the similar pattern, and also corresponded with the *CamCYC1* tree. In the *CamCYC2* tree, forty-three Lobelioideae species occurred in both *CamCYC2A* and *CamCYC2B* clades.

We also detected two U subclades in both *CamCYC2* paralogs. Our data indicate that *CamCYC2* genes have duplicated in the Lobelioideae, a duplication that does not appear to be shared with the *CamCYC2*-like genes isolated from Campanuloideae.

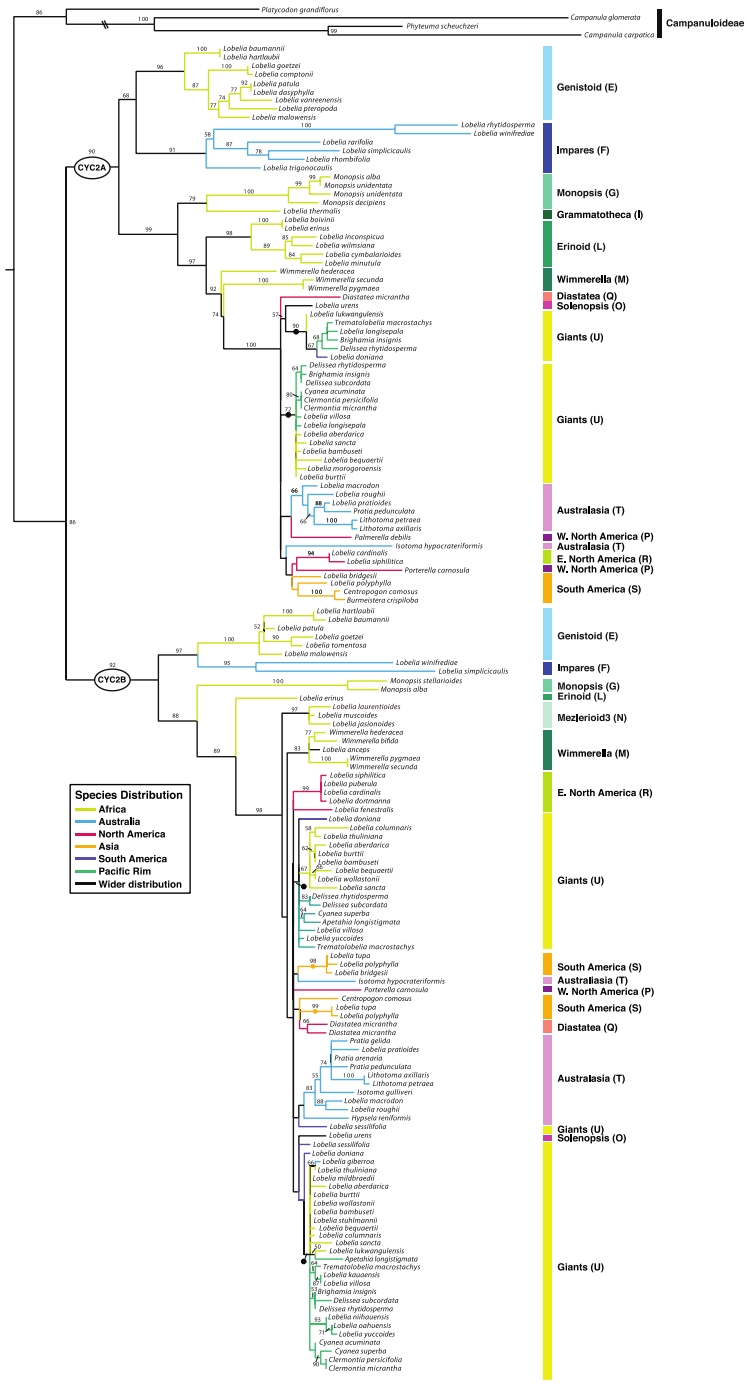

Figure 10. *CamCYC2* RAxML phylogenetic tree. The *CamCYC2* tree only includes sequences isolated from Lobelioideae and Campanuloideae, with no *CamCYC2* gene detected in Cyphioideae, likely lost in this lineage. In Lobelioideae there is a clear duplication across the entire clade, which is not shared with Campanuloideae. It is in line with known *CYC2*-like gene function in core eudicots. The species relationship patterns are congruent between the two Lobelioideae subclades, and also share similarities with Lobelioideae subclades in the *CamCYC1*tree. The U clade includes multiple duplicate lineages in both *CamCYC2* paralogs, likely due to polyploidy.

The *CamCYC3* gene tree included species from all three sampled subfamilies (Fig. 11). The *CamCYC3* matrix was 329 bps long with 23 sequences, including 6 sequences from Lobelioideae, 3 sequences from Campanuloideae, and 14 sequences from Cyphioideae. Fewer sequences were uncovered from *CamCYC3* compared to the other clades. Three sequences from Campanuloideae formed one clade. Cyphioideae sequences were grouped into two clades, and the occurence of two species, *Cyphia longipetala* and *Cyphia comptonii*, across both clades, suggests that *CamCYC3* duplicated in *Cyphia*. In Lobelioideae, these data suggest that *CamCYC3* has been lost in the group except for the "F" clade.

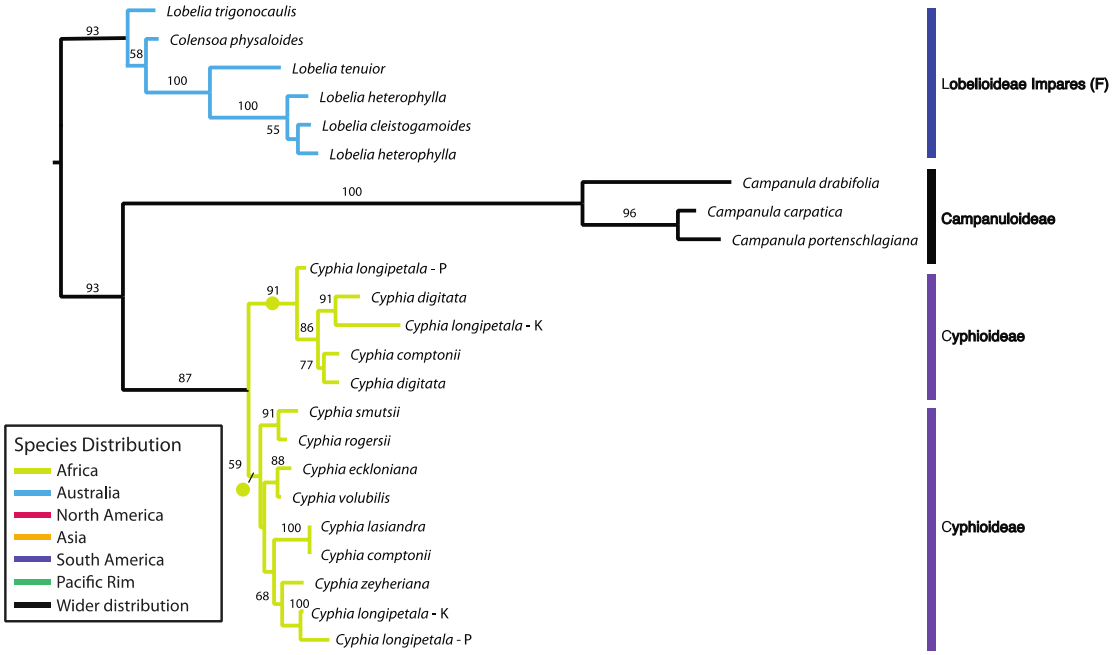

Figure 11. *CamCYC3* RAxML phylogenetic tree. Bootstrap values provided. Dots indicate hypothesized duplication. Campanuloideae and Cyphioideae form a clade sister to Lobelioideae. A duplication is suggested in Cyphoideae. In Lobelioideae, only the F clade was recovered and is potentially lost from other lineages. *CamCYC3* might be play a role in patterning flower symmetry in Cyphioideae instead of *CYC2*-like genes.

2. 1. 2 Expression of *CamCYC2* genes in Lobelioideae species

CamCYC2A and *CamCYC2B* expression levels were assayed with *q*RT-PCR across four Lobelioideae species with different floral morphologies, *Lobelia erinus*, *Lobelia siphilitica*, *Lithotoma axillaris,* and *Lobelia polyphylla* (Fig. 12). The overall expression patterns were broadly similar across all four species (Fig. 12 A-I, B-I, C-I, C-I), however, the expression level between the two paralogs varied. In all species, *CamCYC2A and CamCYC2B* are strongly expressed in flowers and not leaves, and in most cases, the expression levels were not significantly different in different flower bud stages. In the dorsal, lateral, and ventral petal lobe dissections, across all four species, both *CamCYC2A and CamCYC2B* were highly expressed on the abaxial region (the true adaxial region), and only minimally expressed in the adaxial region (the true abaxial region). *CamCYC2A* was expressed similarly in lateral and ventral lobes and significantly reduced in dorsal lobes. *CamCYC2B* was expressed in a gradient, with the highest expression in ventral lobes, medium expression in lateral lobes, and lowest expression in dorsal lobes. This pattern held among the different species, however, which paralog was dominant was markedly different across species (Fig. 12 A-II, B-II, C-II, D-II).

In *Lobelia erinus*, which has the flowers with smallest ratio of dorsal petals to lateral and ventral petals, the lateral and ventral petals are a similar size, and the dorsal petals are about 15~ 20% of the size of lateral and ventral petals (Fig. 5 A, or Fig 6 A). *CamCYC2* genes are highly expressed on the lateral and ventral petals (the true abaxial/top position) and have extremely low expression on the dorsal petals (the true adaxial/bottom position) (Fig. 12 A-II). *CamCYC2A* is highly expresses at lateral and ventral petals at similar levels ($p = 0.762$). By contrast, the dorsal petal lobe expression is

significantly less expressed (dorsal/lateral $p = 0.0017$, dorsal/ventral $p = 0.0002$). *CamCYC2B* shows a dorsoventral gradient of expression, being most highly expressed in the ventral petal, moderately expressed in the lateral petals, and only minimally expressed in the dorsal petals. The expression of *CamCYC2B* was significantly different in the three petal lobe types (dorsal/lateral $p = 0.006$, dorsal/ventral $p = 0.0008$, and lateral/ventral $p = 0.0031$). Dorsal petal expression in both *CamCYC2A* and *CamCYC2B* was similar to leaf expression. Temporally, *CamCYC2A* and *CamCYC2B* genes express in very early stages of flower development, and steadily express through bud growth stages, with no significant differences in expression levels in either *CamCYC2A* or *CamCYC2B* (Fig. 12 A-I). Comparing the two paralogs, *CamCYC2A* is much more highly expressed than *CamCYC2B* in floral tissue and flower buds in *L. erinus*. For instance, *CamCYC2A* is roughly 15 times more expressed than *CamCYC2B* in the ventral petal (Fig. 12 A-II).

Lobelia siphilitica flowers have relatively big dorsal petals compared with *L. erinus*, and dorsal petals are about 40% of the size of lateral and ventral petals (Fig. 5 G, or Fig. 12 B). The expression patterns of *CamCYC2A* and *CamCYC2B* are similar to that of *L. erinus*. In *L. siphilitica*, *CamCYC2A* is highly expressed in a similar level in lateral and ventral petals ($p = 0.6438$), and barely expressed in dorsal petals (dorsal/lateral $p = 0.0025$ and dorsal/ventral $p = 0.0032$ (Fig. 12 B-II). *CamCYC2B* is expressed the highest in the ventral petal, intermediate in the lateral petals, and extremely low in the dorsal petals (dorsal/lateral $p = 0.0009$, dorsal/ventral $p = 0.0004$, and lateral/ventral $p- = 0.0029$) (Fig. 12 B-II). Temporally, *CamCYC2A* and *CamCYC2B* genes express in very early stages of flower development, and steadily express through bud growth stages, with no significant differences in expression levels in either *CamCYC2A* or *CamCYC2B*

(Fig.12 B-I). Similar to in *L. erinus*, *CamCYC2A* is more highly expressed than *CamCYC2B* in floral tissue and flower buds in *L. siphilitica*, however, with less of a differential between the paralogs. For instance, *CamCYC2A* expression is only roughly 2 times greater in the ventral petal than *CamCYC2B* (Fig. 12 B-II).

Lithotoma axillaris flowers have dorsal, lateral, and ventral petals that all have a similar size and shape, but due to the petal arrangement and a deep dorsal slip, they form a bilaterally symmetrical flower (Fig. 5 F, Fig. 12 C). *CamCYC2A* and *CamCYC2B* have much higher expression in lateral and ventral petals and are barely expressed in dorsal petals, with similar expression patterns overall to *L. erinus* and *L. siphilitica* (Fig. 12 C-II). *CamCYC2A* is significantly less expressed in dorsal petals than lateral ($p = 0.0163$) and ventral ($p = 0.0015$) petals, with similar expression between dorsal and lateral petals ($p = 0.5634$). In *CamCYC2B* each petal type has significantly different expression along a gradient as in the other species, with ventral/lateral ($p = 0.0139$), ventral/dorsal ($p = 0.0012$), and lateral/dorsal ($p = 0.0016$). Temporally, both paralogs are expressed early and continued to be expressed through development but with a statistically significant decrease in expression in large buds (Fig. 12 C-I). In *CamCYC2A* large bud/medium bud $p = 0.0136$, large bud versus small bud *p*-value is 0.1508 and medium bud versus small bud *p*-value is 0.2016, which shown the *CamCYC2A* consistently expressed through the bud development, and only slightly up-regulation in the medium buds. *CamCYC2B* expression peak is in the medium buds, and then decreases in large buds ($p = 0.0021$). A major difference in expression between *Lithotoma axillaris* and previously discussed *Lobelia* species is that *CamCYC2B* is more highly expressed overall, compared to *CamCYC2A* in floral buds and petal lobes (more than 7 times greater in ventral petals).

Therefore, the overall expression patterns among petal types are the same across species, however, the gene copy that is the most highly expressed flips.

Lobelia polyphylla flowers have dorsal, lateral, and ventral petals that all have a similar size and shape, and all five petals bend to abaxial/bottom position (true adaxial/top position), and the dorsal petals are slightly longer than the lateral and ventral petals (Fig. 5 I, Fig. 12 D). *CamCYC2A* and *CamCYC2B* have significantly higher expression in lateral and ventral petals and are barely expressed in dorsal petals, with similar expression patterns as *L. axillaris* (Fig. 12 D-II). *CamCYC2A* is significantly less expressed in dorsal petals than lateral ($p = 0.0079$) and ventral ($p = 0.0002$) petals, with similar expression between dorsal and lateral petals ($p = 0.5783$). In *CamCYC2B* there is a dorsoventral gradient of expression, highest in ventral petal lobes, with each petal type having significantly different expression between dorsal/lateral ($p = 0.0161$), dorsal/lateral ($p = 0.0011$), and lateral/ventral ($p = 0.0099$) petal lobes. Temporally, both paralogs are expressed early and continued to be expressed through development but with a statistically significant decrease in expression in large buds in only *CamCYC2A* (Fig. 12 D-I). In *CamCYC2A*, expression in large buds is significantly less than that of small buds ($p = 0.0004$) or medium buds ($p = 0.0042$). In *L. polyphylla CamCYC2B* is more highly expressed than *CamCYC2A*, similar to the pattern in *L. axillaris*, with *CamCYC2B* roughly 3.5 times more expressed than *CamCYC2A* in the ventral petal.

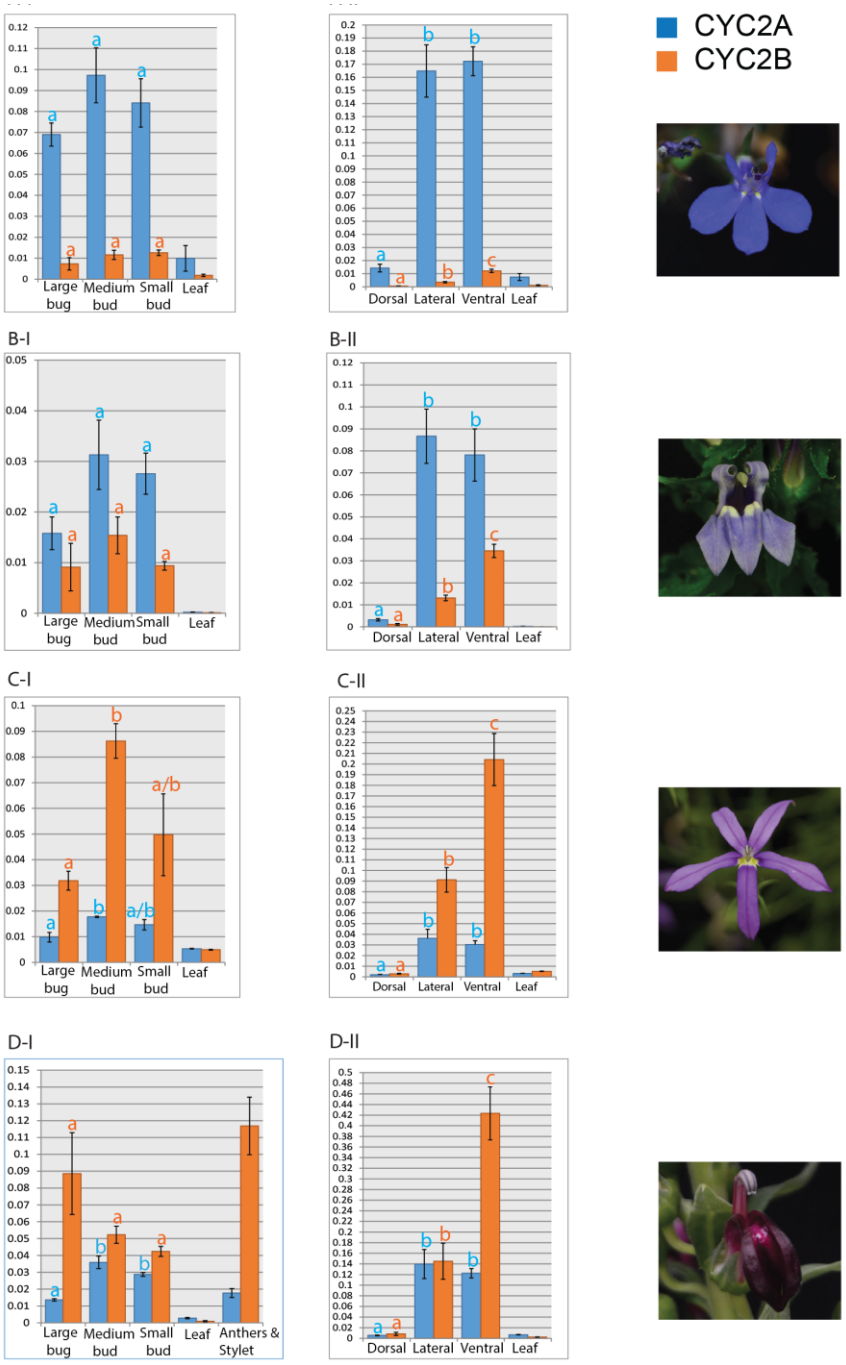

Figure 12. Relative expression levels of *CamCYC2A* and *CamCYC2B* genes in Lobelioideae species, A. *Lobelia erinus*, B. *Lobelia siphilitica*, C. *Lithotoma axillaris*, D. *Lobelia polyphylla*. A-I, B-I, C-I, D-I show *CamCYC2* genes in different floral bud stages, both *CamCYC2* genes are expressed through the whole floral growth stage; A-II, B-II, C-II, D-II show *CamCYC2* genes in different floral petals. *CamCYC2A* is highly expressed in the ventral and lateral petals, exhibiting lower expression in dorsal petals; *CamCYC2B* is highly expressed in the ventral petal, exhibiting intermediate expression in lateral petals and low expression in dorsal petals. Lines of the Y-axis are labeled with the same scale across all diagrams except D-II. Y-axis is the relative expression level, normalized to *CamActin* as the reference gene.

2. 3 DISCUSSION

The three subfamilies of Campanulaceae sampled in this study have distinctly different floral symmetry modifications with radially symmetrical flowers in Campanuloideae, non-resupinate bilaterally symmetric flowers in Cyphioideae, and largely 180 degree resupinated flowers in Lobelioideae (Antonelli, 2008; Crowl, et al., 2016; Lammers, 2007). In these three groups we uncovered broad gene duplications and losses that correlate with these morphological shifts. We detected all three core eudicot *CYC*-like genes from the *CYC1*, *CYC2*, and *CYC3* clades (Howarth and Donoghue, 2006). *CamCYC1* was thoroughly sampled from all three subfamilies, while *CamCYC2* was likely lost in Cyphioideae and *CamCYC3* was likely lost from all but the F clade of Lobelioideae that are sister to the rest of the subfamily. Additionally, we found evidence for subfamily duplications, *CamCYC1* duplicated in Campanuloideae, *CamCYC2* duplicated in Lobelioideae, and *CamCYC3* duplicated in Cyphioideae.

2. 3. 1 Radially symmetric Campanuloideae duplicated in *CamCYC1*

Despite limited sampling in Campanuloideae we isolated all three *CamCYC* genes. Campanuloideae plants have radially symmetrical flowers, which are hypothesized to be a reversion from the ancestral Campanulaceae plants with bilaterally symmetrical flowers (Crowl, et al., 2016). *CamCYC2*, which has been shown to be functionally conserved in patterning floral bilaterally symmetry (Donoghue, et al., 1998; Howarth and Donoghue, 2006; Luo, et al., 1999; Specht and Howarth, 2015), was present across the Campanuloideae, from four species that span the major clades of the group (Fig. 9). There was no evidence for duplications in *CamCYC2*, which is in line with that found in other radially symmetrical groups (Hileman, 2014; Howarth and Donoghue, 2006).

Additionally, Campanuloideae *CamCYC2* copies had high sequence diversity, being on very long branches, and difficult to align with Lobelioideae species (Fig. 10). In other linages with both radially symmetrical and bilaterally symmetrical flowers, such as Fabales, Malpighiales, and Dipsacales, species with radially symmetrical flowers have *CYC2*-like genes expressed uniformly across the whole corolla or have lost floral expression (Berger, et al., 2016; Busch and Zachgo, 2007; Carlson, et al., 2011; Citerne, et al., 2003; Citerne, et al., 2000; Damerval, et al., 2007; Feng, et al., 2006; Hoshino, et al., 2014; Howarth, et al., 2011; Preston, et al., 2009; Preston, et al., 2011; Tähtiharju, et al., 2012; Xu, et al., 2013; Zhang, et al., 2012; Zhang, et al., 2013). *CamCYC*3 was also uncovered in Campanuloideae, but only in the C2 clade (Crowl, et al., 2014) and also on a long branch, compared to Cyphioideae and Lobelioideae sequences (Fig. 11). *CYC3* has been shown to be involved in axillary bud outgrowth (Aguilar-Martínez, et al., 2007) and in floral symmetry (Berger, et al., 2016), but with variable function in different plant groups.

Campanuloideae shows the most diversification in *CamCYC1* genes, with a duplication shared either across the Campanuloideae clade (Fig. 9). Each of these duplicate clades is in agreement with the Campanuloideae species phylogeny (Crowl, et al., 2014; Crowl, et al., 2016). As the *CamCYC1* gene tree suggests (Fig. 9), *CamCYC1* appears to have duplicated across Campanuloideae, and only in the Impares and Giants clades in the lineages with bilaterally symmetrical flowers. Studies in plant groups across core eudicots suggest that *CYC1* genes are functionally conserved, regulating the number and position of axillary bud development (Aguilar-Martínez, et al., 2007; González-Grandío, et al., 2013; Hubbard, et al., 2002; Lewis, et al., 2008; Martín-Trillo and Cubas,

2010), as well as inflorescence architecture and development (Dixon, et al., 2018; Hubbard, et al., 2002). Loss-of-function mutants in *Arabidopsis* and *Populus* lead to a marked increase in bud outgrowth and plant branching (Aguilar-Martínez, et al., 2007; Finlayson, 2007; Muhr, et al., 2018). It is possible that the duplication of *CamCYC1* set the stage for the variation in plant and inflorescence architecture in Campanuloideae. Flowers vary from solitary to complex inflorescences such as capitulate heads (Lammers, 2007). Broad duplications in *CYC1* are less common than in the other *CYC* clades, although they are consistently duplicated in lineages known for capitulate heads such as Asteraceae (Chapman, et al., 2008; Chapman, et al., 2012), Dipsacaeae (Carlson, et al., 2011), and *Actinodium* (Claßen-Bockhoff, et al., 2013).

2. 3. 2 Cyphioideae have lost *CamCYC2* and duplicated *CamCYC3*

Cyphioideae typically have a bilaterally symmetrical flowers that are not resupinate with a 3+2 form, with one dorsal petal, two lateral petals, and two ventral petals (Fig. 1). In all other core eudicot bilateral symmetrical lineages studied to date, *CYC2* is differentially expressed across the dorsoventral axis and functions to pattern that bilateral symmetry (Hileman, 2014). Occasionally, *CYC2* genes appear to lose floral expression or be lost from the genome of certain species; however, these are always marked by shifts to radial symmetry (Zhang, et al., 2012) . Additionally, in almost all cases, *CYC2* genes are duplicated in bilaterally symmetrical lineages (Hileman, 2014; Howarth and Donoghue, 2006). Here we report the first case of an apparent loss of *CYC2* in a bilaterally symmetrical group, Cyphioideae. Sampling across 9 species across multiple primer sets, no *CamCYC2* sequences were found, despite easily recovering them from Campanuloideae and Lobelioideae. Cyphioideae is sister to Campanuloideae in the

latest phylogenetic analyses (Crowl, et al., 2016) and does not turn upside down like that of most species of Lobelioideae. However, unlike most bilaterally symmetrical core eudicots, *Cyphia* flowers have 3 dorsal petals lobes and 2 ventral petal lobes. Standard orientation of core eudicot bilaterally symmetrical flowers have a single ventral petal, pointed downward with two lateral and two dorsal petals each acting as pairs that can shift along the dorsoventral axis in tandem (Donoghue, et al., 1998). Therefore, a 3+2 petal lobe arrangement necessitates a shift in that axis, likely through rotation.

Along with a likely loss of *CYC2* in Cyphioideae, *CamCYC3*, on the other hand, appears to have duplicated in this lineage (Fig. 11). This is in stark contrast to Lobelioideae, which appears to have lost *CamCYC3* in all but the Impares (F) clade and is not duplicated. *CYC3* is the most understudied paralog across core eudicots and also appears to be the most variable in function. *CYC3* members duplicate in some lineages such as Dipsacales and Asteraceae (Carlson, et al., 2011; Chapman, et al., 2008; Howarth and Donoghue, 2005) but have been likely lost in others such as Leguminosae and Gesneriaceae (Song, et al., 2009). In *Arabidopsis* and *Populus*, *CYC1* (*Branched1*) and *CYC3* (*Branched2*) orthologs have redundant function in regulating bud outgrowth (Aguilar-Martínez, et al., 2007; Muhr, et al., 2016) with an increase in branching in loss-of-function mutants. Interestingly, *branched1* had the stronger phenotype in *Arabidopsis* (Aguilar-Martínez, et al., 2007) and *branched2* had the stronger phenotype in *Populus* (Muhr, et al., 2018). Although, *CYC3* gene function in floral symmetry has not previously been shown, studies in Dipsacales and Asteraceae report expression patterns suggestive of this role with dorsoventral expression of *KmCYC3B* in *Knautia macedonica* (Berger, et al., 2016) and *HaCYC3a* expression specific to ray florets in *Helianthus annuus*

(Tähtiharju, et al., 2012). All of this suggests that *CYC3* function is highly labile. Additionally, function specific to plant branching appears to be found in rosids while floral expression has been seen in campanulid asterids. All of these data suggest that *CYC3* could play a role in floral symmetry in campanulids such as *Cyphia*, and possibly replaced the role of *CYC2*.

2. 3. 3 In Lobelioideae, *CamCYC1* duplicated in two subclades while *CamCYC3* appears to be lost in all but Impares clade.

In Lobelioideae, *CamCYC1* is congruent with the hypothesized species phylogeny with no obvious subfamily wide duplications (Fig. 9)(Antonelli, 2008; Antonelli, 2009; Givnish, 2010; Knox, et al., 1993; Knox and Li, 2017; Knox and Palmer, 1998). There are multiple sequences in a few species; however, these are likely alleles or more recent isolated duplications. Species distribution in the Lobelioideae *CamCYC1* subclades are highly congruent with Lobelioideae biogeography, with the Genistoid (E) clade from Africa and the Impares (F) clade from Australia and New Zealand each being monophyletic and forming a clade sister to the rest of the Lobelioideae. *CamCYC1* has not been implicated in bilateral symmetry in any groups, instead being involved in plant and inflorescence branching in several lineages (Aguilar-Martínez, et al., 2007; Martín-Trillo and Cubas, 2010; Muhr, et al., 2018). Within the Lobelioideae there is no broad duplication correlating with a shift to bilateral symmetry, which is in line with what has been found in other groups (Howarth and Baum, 2005; Howarth and Donoghue, 2005). However, there are duplications found in the Impares (F) clade as well as the Giant Lobelioids (U), likely due to of independent ancient genome duplications.

The Impares clade, appearing to have duplicated *CamCYC1* early in its diversification (Fig. 9), is notable for having a diversity of chromosome numbers, varying among 8, 9, 10, and 11 (Stace and James, 1996), while most of Lobeliaceae have multiples of 7 chromosomes. This suggests that a genome duplication occurred around the diversification of the Impares clade, followed by subsequent frequent chromosome losses. The duplication in *CYC1* likely correlates with that genome duplication; however, we have no hypothesis for why these genes were maintained in this lineage. The Impares clade also appears to be the only group to have not lost *CamCYC3* genes. This means that this lineage maintains both an extra *CYC1* and an extra *CYC3* gene compared to most other Lobelioideae clades. The Impares corolla shape does differ from other groups in having large, broad ventral and lateral petal lobes and greatly reduced, nearly scale-like dorsal lobes (Walsh, et al., 2010). However, there are no data that tie this morphology with extra *CYC1* and *CYC3* gene copies.

The Giant Lobelias (U clade) grow in montane habitats in tropical regions around the globe and have synapomorphies of a tree-like habit, often with lignification, and are polyploid with a chromosome number of N=14 (Antonelli, 2008; Chen, et al., 2016; Knox, et al., 1993). In the U clade, there are 3 subclades of *CamCYC1*, with the U1 clade grouping with other Neotropical, Australia, and South American Lobelioideae species, and sister to a clade including U2A and U2B. The current topology suggests separate duplications in Pacific basin species (Fig. 9, green) and non-Pacific basin species (Fig. 9, yellow); however, there was no bootstrap support for the relationships of these clades, so these duplications could be shared across all the Giant Lobelias. These groups were difficult to tease apart because sequence divergence is minimal and they were amplified

and cloned together, which resulted in some mixing of sequences among copies. Nevertheless, *CYC1* duplicates are maintained in the Giant Lobelias and better sampling could shed light on the precise ancestor(s) of this clade. For instance, in the U1 clade, *L. doniana* is sister to the rest, supporting the East Asian origin hypothesis of Giant Lobelias (Knox and Li, 2017) , although they are nested withing a grade of North American species.

2. 3. 4 Duplication of *CamCYC2* in Campanulaceae are highly associated with bilateral symmetry in Lobelioideae

CamCYC2 genes are the orthologs of *CYCLOIDEA*, a gene which has been shown repeatedly to restrict dorsally in expression in bilaterally symmetrical groups (see Hileman 2014). Additionally, the evolution of bilateral symmetry has been correlated with duplications in *CYC2* genes (Hileman, 2014; Howarth and Donoghue, 2005) . These genes are of interest in bilaterally symmetrical species of Campanulaceae, where we expect that gene expression will be restricted to one side of the flower and that duplications will likely be frequent. *CamCYC2* in Lobelioideae was well sampled and as expected had a clear duplication across the entire clade (Fig. 10). The *CamCYC2* duplication very likely occurred in Lobelioideae after it diverged from Campanulaceae. Phylogenetic relationships in both Lobelioideae *CamCYC2* clades share a similar pattern and are congruent with previous research (Antonelli, 2009; Knox, et al., 1993; Knox and Li, 2017; Knox and Palmer, 1998), and with *CamCYC1* Lobelioideae clades. As in *CamCYC1*, we also detected duplications in the U subclade in both *CamCYC2* paralogs, likely due to polyploidy. Flowers of all Lobelioideae are resupinate, twisting their pedicle (Fig. 6 A, C). However, since mature flowers, after turning, end up having a flower that

looks right side up (i.e., a standard core eudicot 2+3 petal arrangement); this suggests there is a developmentally earlier change in orientation to create an initial 3 up, 2 down petal arrangement. Taxa such as species in *Monopsis* (G) clade do not twist their pedicle and end up with mature 3+2 flowers, retaining the hypothesized ancestral Lobelioideae flower orientation. That said, this group did not lose their *CamCYC2* copies like that of Cyphioideae, which similarly does not undergo resupination. There are currently no known genes involved in twisting of plant tissues, for instance, to present the flower upside down, allowing us to potentially uncover novel gene functions of *CYC-like* genes with further studies of these groups.

Within both of the *CamCYC2A* and *CamCYC2B* clades, the U subclade, or Giant Lobelias, occur in two duplicate locations in the phylogeny, likely due to their tetraploid (Antonelli, 2009; Knox and Li, 2017; Knox and Palmer, 1998) and even hexaploidy (Wagner, et al., 1999) ancestry. This means that there are four separate clades of *CYC2* in Giant Lobelias. One of the U clades in each of *CamCYC2A* and *CamCYC2B* have no clear sister group, however, the other clades in each are most closely related to *Lobelia urens*. This relationship to *L. urens* is not well supported in either clade; however, it does suggest that this species, with a Lusitanian distribution, should be explored with other phylogenetic markers. *Lobelia urens* has not been included in previous publications on the evolutionary history of Giant Lobelias (Antonelli, 2008; Antonelli, 2009; Chen, et al., 2016; Knox, et al., 1993; Knox and Li, 2017). The Tupa group of South America have evolved a giant growth habit independently from the Giant Lobelias (Knox, et al., 1993; Lagomarsino, et al., 2014). The Tupa group appears to have independently duplicated in *CamCYC2B*, similar to that of the Giant Lobelia (U) group.

2. 3. 5 Gene expression of *CamCYC2* in Lobelioideae species

In Lobelioideae species, we isolated two copies of *CamCYC2* genes and utilized *q*RT-PCR to examine the temporal and spatial expression patterns of *CamCYC2* genes. As previous researchers have shown, *CYC2*-like genes are dorsally restricted, limited to the adaxial region of the flower tissues. In most examined bilaterally symmetrical species, *CYC2*-like paralogs are diverged in their expression with one copy being more restricted dorsally than the other (Hileman, 2014; Howarth, et al., 2011). Lobelioideae have resupinate flowers and we hypothesized that the expression patterns would show an upside pattern compared with other un-resupinate bilaterally symmetrical flowers. Using four Lobelioideae species, *Lobelia erinus*, *L. siphilitica*, *L. polyphylla*, and *Liothotoma axillaris*, we found that 1) the paralogs varied in how restricted they were on the dorsoventral axis, 2) that resupinate flowers led to upside-down expression with the highest expression in ventral regions, and 3) the overall patterns of expression among petals was similar across species; however, the paralog with the greatest expression shifted.

The temporal expression of *CamCYC2* genes expression patterns were relatively uniform through development, which is similar to that observed in other groups (Luo, et al., 1996) . The spatial expression patterns of *CamCYC2A* and *CamCYC2B* are relatively concordant among the four species (Fig. 12 A-II, B-II, C-II, D-II, and Fig. 14). *CamCYC2A* is expressed similarly in lateral and ventral petals, or the whole abaxial region of the flower (the real adaxial region) and has very low expression in the dorsal petals (the real ventral petals, adaxial region of the flower). *CamCYC2B* is always highly expressed on the ventral petal (the real dorsal petal), and has an intermediate expression

level in lateral petals, and is barely expressed in dorsal petals (the real ventral petals). Both *CamCYC2A* and *CamCYC2B* have little to no expression in the dorsal petals, similar to leaf expression.

Previous work has shown in Dipsacales that even subtle differences in the dorsoventral gradient of *CYC2* expression can lead to significantly different growth patterns of the petals (Berger *et al.* 2016). In *Lobelia erinus*, flowers with small dorsal petals, *CamCYC2A* is significantly more expressed than *CamCYC2B* (Fig. 12 A-II). In *Lobelia siphilitica*, flowers with relatively bigger dorsal petals than *L. erinus*, the pattern is the same, but the distinction between the level of expression is not as great especially in the ventral petal, where the *CamCYC2B* gene expression level is almost 50% of the *CamCYC2A* gene expression level (Fig. 12 B-II). In *Lithtoma axillaris* and *L. polyphylla*, there is no distinct difference in shape or size between dorsal, lateral, and ventral petals. In an opposite pattern, the *CamCYC2B* gene is more highly expressed than the *CamCYC2A* gene (Fig. 12 C-II, and 12 D-II). This is effectively an increase in expression of the gene with the broader zone of expression, which has been shown to result in a more radialized flower (Howarth, et al., 2011). In flower primordia, *CYC* genes repress cell growth and control organ number, and in later stages, *CYC* and *DICH* also can upregulate cell division via enhancing *RAD* (Luo, et al., 1999; Preston, et al., 2009). In this case, Lobelioideae species have relatively bigger lateral and ventral petals (the true adaxial region), very likely due to the high *CamCYC2* gene expression. But it does not easily explain how *Liothotoma. axillaris* and *Lobelia polyphylla* flowers have petals with almost the same size and shape. Nonetheless, this change in the expression ratio among paralogs sets up an intriguing system to study not just *CYC* function and evolution, but

also how morphology can be substantially altered by shifts in expression dominance among gene paralogs.

2. 4 CONCLUSION

Campanulaceae is a large core eudicot family, which exhibits a variety of floral symmetries, including varying types of resupination and pollination syndromes. The family occurs nearly world-wide and has become a model for studying adaptive radiations in many locations. We sequenced all three core eudicot paralogs of *CamCYC* genes, *CamCYC1*, *CamCYC2*, and *CamCYC3* (Fig. 13). The *CamCYC1* genes duplicated in radially symmetrical Campanuloideae, but not in other bilaterally symmetrical flower subfamilies. As we expected, *CamCYC2* genes have duplicated in the Lobelioideae clade with bilaterally symmetrical flowers. However, we show for the first time a loss of CYC2-like genes in a bilaterally symmetrical group, with no sequences found in Cyphioideae. Instead of *CamCYC2*, we found a potential duplication of *CamCYC3* in this group. It is possible that in Cyphioideae, *CamCYC*3 genes may have replaced the role of *CYC2*-like genes. Future studies would include examining RNA floral expression in *Cyphia*.

In Lobelioideae, expression patterns of *CamCYC2* genes were similar to previous studies across core eudicots species, with *CamCYC2A* and *CamCYC2B* both highly expressed in the ventral petals (Fig. 14), which correspond to the dorsal side of the flower, suggesting conservation of dorsal identity in these upside down flowers. In addition, the *CamCYC2A* and *CamCYC2B* show a distinctly different expression pattern in different species with a different dorsal petal size ratio. *CamCYC2A* is the dominant *CamCYC2* gene in the species with smaller dorsal petals, like *Lobelia erinus*, and *Lobelia siphilitica*. *CamCYC2B* is the dominant *CamCYC2* gene in the species with bigger dorsal petals, in which the dorsal petals are almost the same size as the lateral and ventral petals,

like *Lithotoma axillaris* and *Lobelia polyphylla*. We illustrate here for the first time that *CYC* expression is conserved along the dorsoventral axis of the flower even as it turns upside down, suggesting that *CYC* expression is not regulated by extrinsic factors such as gravity. Additionally, the shift in expression dominance among paralogs provides intriguing data that differences in ratios of expression in *CYC* could lead to shifts in morphological growth ratios in the flower.

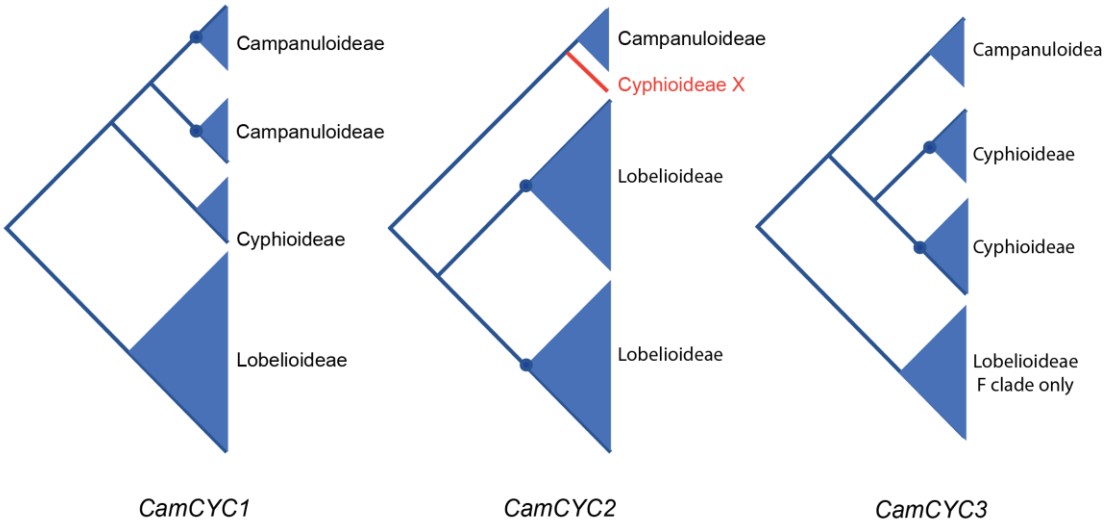

Figure 13. Summary *CYC*-like duplication events across Campanulaceae. *CamCYC1* duplicated in Campanuloideae and might have narrower duplications in the F and U clades in Lobelioideae. *CamCYC2* showed a clear duplication event specific to Lobelioideae and an apparent loss in Cyphoideae. *CamCYC3* duplicated in Cyphioideae, and is apparently lost in all by one clade of Lobelioideae. *CamCYC3* might be play a key role in bilateral symmetry instead of *CYC2*-like in Cyphoideae. Blue dots indicate hypothesized location of broadly duplicated clades.

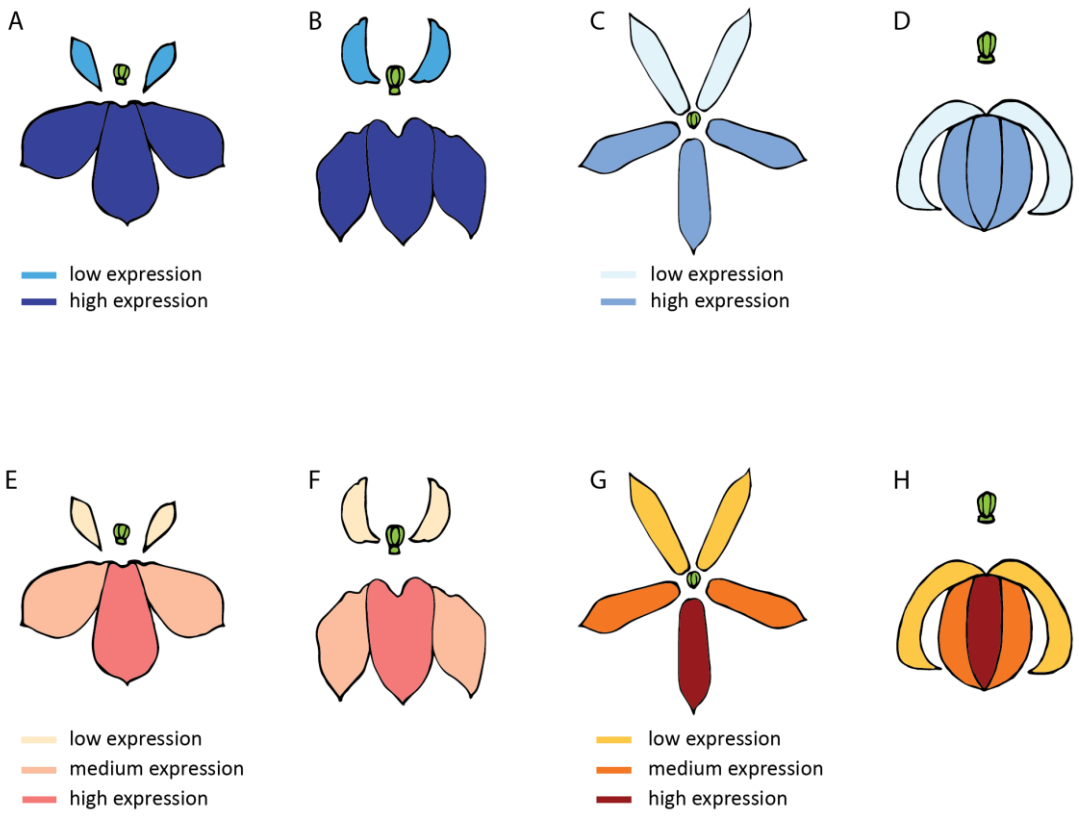

Figure 14. *CamCYC2A* and *CamCYC2B* expression pattern in Lobelioideae species A-D. *CamCYC2A* expression pattern, E-F. *CamCYC2B* expression pattern. A, E. *Lobelia erinus*, B, F. *Lobelia siphilitica*, C, G. *Lithotoma axillaris*, D, H. *Lobelia polyphylla*. Low saturation of color represents minor expression, high saturation of color represents high expression in flower buds. *CamCYC2A* is more highly expressed in species with relatively small dorsal corolla lobes, like in A. *Lobelia erinus* and B. *Lobelia siphilitica*. *CamCYC2A* has weak expression in the dorsal corolla lobes (the true ventral domain) and is highly expressed in the ventral domain (the true dorsal domain). *CamCYC2B* is the more highly expressed *CamCYC2* gene in species with relatively large dorsal corolla lobes, like in G. *Lithotoma axillaris* and H. *Lobelia polyphylla*. *CamCYC2B* has weak expression in the dorsal corolla lobes (the true ventral corolla lobes), medium expression in lateral corolla lobes, and high expression in the ventral corolla lobe (the true dorsal corolla lobe).

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