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Diversification of *CYCLOIDEA* expression in the evolution of bilateral flower symmetry in Caprifoliaceae and *Lonicera* (Dipsacales)

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• *Background and Aims* The expression of floral symmetry genes is examined in the *CYCLOIDEA* lineage following duplication, and these are linked to changes in flower morphology. The study focuses on Dipsacales, comparing *DipsCYC2* gene expression in *Viburnum* (radially symmetrical Adoxaceae) to members of early-diverging lineages of the bilaterally symmetrical Caprifoliaceae (*Diervilla* and *Lonicera*).
• *Methods* Floral tissue from six species, which included dorsal, lateral and ventral regions of the corolla, was dissected. RNA was extracted from these tissues and each copy of *DipsCYC2* was amplified with reverse transcriptase PCR.
• *Key Results* Members of *DipsCYC2* were expressed across the corolla in the radially symmetrical *Viburnum plicatum*. A shift to bilaterally symmetrical flowers at the base of the Caprifoliaceae was accompanied by a duplication of the *DipsCYC2* gene, resulting in *DipsCYC2A* and *DipsCYC2B*, and by loss of expression of both of these copies in the ventral petal. In *Lonicera* (Caprifolieae), there is a shift from flowers with two dorsally and three ventrally oriented corolla lobes to a clear differentiation of dorsal, lateral and ventral lobes. This shift entailed a decoupling of expression of *DipsCYC2A* and *DipsCYC2B*; *DipsCYC2B* continues to be expressed in the dorsal and lateral lobes, while *DipsCYC2A* expression is restricted to just the two dorsal lobes. A reversion to more radially symmetrical flowers within *Lonicera* was accompanied by a re-expansion of expression of both *DipsCYC2A* and *DipsCYC2B*.
• *Conclusions* The transition to bilateral symmetry in Caprifoliaceae involved: (*a*) duplication of an ancestral *DipsCYC2* gene; (*b*) the loss of expression of both of these copies in the ventral petal; and (*c*) changes in the zone of expression, with one copy continuing to be expressed across the dorsal and lateral petals, and the other copy becoming restricted in expression to the dorsal corolla lobes.

Key words: Flower symmetry, *CYCLOIDEA*, Dipsacales, Caprifoliaceae, *Lonicera*, RNA expression.

INTRODUCTION

Shifts between radially symmetrical (polysymmetrical, actino-morphic) and bilaterally symmetrical (monosymmetrical, zygomorphic) flowers have been common within angiosperms (Weberling, 1989; Endress, 1996, 1999). Such morphological shifts are of special interest in relation to shifts in pollination (e.g. see Neal, 1998) and perhaps to shifts in rates of diversification (Sargent, 2004). A shift from radial to bilateral symmetry entails a transition of a single petal or corolla lobe form to the individualization, ultimately, of three types: a medially positioned (abaxial or ventral) petal, two lateral petals and two adaxial or dorsal petals (Donoghue *et al.*, 1998). To tease apart the mechanisms underpinning the evolutionary transition from radial to bilateral symmetry it is helpful to compare representatives of early-diverging bilaterally symmetrical lineages within a clade to their closest relatives with radial symmetry, with the aim of identifying any changes in the number of genes and/or shifts in the expression of genes that may underlie the morphological differences.

Most studies of floral symmetry genes have focused on species with highly derived morphologies (e.g. Luo *et al.*, 1996; Citerne *et al.*, 2006; Feng *et al.*, 2006; Busch and Zachgo, 2007; Broholm *et al.*, 2008; Chapman *et al.*, 2008; Kim *et al.*, 2008; Wang *et al.*, 2008). A few studies have correlated gene duplications in *CYCLOIDEA* (*CYC*) with morphological shifts in floral symmetry (e.g. Howarth and Donoghue, 2005; Chapman *et al.*, 2008; Kim *et al.*, 2008; Zhang *et al.*, 2010). Here, gene expression patterns following duplication are examined, and shifts in expression linked to changes in flower morphology. Additionally, different forms of symmetry are examined, including radially symmetrical flowers in *Viburnum*, and several forms of bilateral symmetry in *Diervilla* and *Lonicera*. It is shown how changes in the zones of expression of duplicated copies of *CYC* within the corolla appear to be related to morphological changes and how a series of changes in the deployment of duplicated genes serves to developmentally individuate different regions of the corolla along the dorsoventral axis.

Background on CYCLOIDEA *and related genes*

Understanding the evolution of floral symmetry has been greatly advanced by the study of three transcription factors from two gene families: the TCP family, including *CYCLOIDEA* (Luo *et al.*, 1996) and the MYB family,

including *DIVARICATA* (Almeida *et al.*, 1997; Galego and Almeida, 2002) and *RADIALIS* (Corley *et al.*, 2005; Costa *et al.*, 2005). *CYCLOIDEA* (*CYC*) is the most thoroughly studied to date and has been shown to be involved in specifying dorsal flower identity (Luo *et al.*, 1996, 1999; Almeida *et al.*, 1997). A duplication in Antirrhineae (which includes the snapdragon, *Antirrhinum majus*; Hileman and Baum, 2003), resulted in two closely related copies, *CYC* and *DICH* (*DICHOTOMA*), which have overlapping expression patterns in floral meristems (Luo *et al.*, 1996, 1999; Hileman *et al.*, 2003). In *Antirrhinum*, a fully radial and ventralized flower (a peloric form) is produced only in *CYC*/*DICH* double mutants (Luo *et al.*, 1996; Almeida *et al.*, 1997). Additionally, *CYC* and *DICH* both inhibit stamen growth in *A. majus*, with expression in stamen primordia resulting in abortion (Luo *et al.*, 1996, 1999).

Similar CYC function has been corroborated in the closely related *Linaria* (Cubas *et al.*, 1999), as well as in two legumes, *Lotus japonica* and *Pisum sativum* (Feng *et al.*, 2006; Wang *et al.*, 2008). Additionally, CYC orthologues in Asteraceae play a role in specifying disk versus ray florets (Broholm *et al.*, 2008; Kim *et al.*, 2008). *CYC* has also been shown to be dorsally expressed in an array of other core eudicots: *Arabidopsis*, *Bournea*, *Iberis*, *Lonicera*, *Lupinus* and Malpighiaceae (Cubas *et al.*, 2001; Citerne *et al.*, 2006; Howarth and Donoghue, 2006; Busch and Zachgo, 2007; Zhou *et al.*, 2008; Zhang *et al.*, 2010). An exception is the radially symmetrical *Cadia*, where one *CYC*-like copy (*LegCYC1A*) has an apparently derived, expanded expression across the corolla (Citerne *et al.*, 2006). These data suggest that *CYC* expression and function may be similar across rosid and asterid angiosperms.

Previously it has been shown that *CYC*-like genes duplicated twice around the divergence of the core eudicots, resulting in three paralogues (Howarth and Donoghue, 2006). These three paralogues fall into a clade of TCP genes that contain an ECE motif (Howarth and Donoghue, 2006). All of the studies of the role of *CYC*-like genes in floral symmetry have focused on a single core eudicot copy, members of the CYC2 clade (Howarth and Donoghue, 2006). This gene clade also contains the greatest number of secondary gene duplications, with a pattern emerging of extra independent duplications in bilaterally symmetrical groups. Duplications in *CYC*-like genes have been common in the Asteridae (e.g. Reeves and Olmstead, 2003). In *Antirrhinum* and its relatives there is one clear duplication within the CYC2 clade at the level of the Antirrhineae (resulting in both *CYC* and *DICH* (Gübitz *et al.*, 2003; Hileman and Baum, 2003). *Digitalis* (also in Plantaginaceae) lacks this duplication, but has three additional copies (Hileman and Baum, 2003). In Gesneriaceae, three broad duplications have been hypothesized across Cyrtandroideae (Citerne *et al.*, 2000; Gao *et al.*, 2008; Song *et al.*, 2009; Pang *et al.*, 2010), and one more within Gesnerioideae (Smith *et al.*, 2004). Chapman *et al.* (2008) documented ten separate copies in *Helianthus* (Asteraceae), with one copy exclusive to bilaterally symmetrical ray florets. Previously extra duplications have also been noted in Dipsacaceae, Morinaceae and Valerianceae (Dipsacales), as well as in *Scaevola* (Goodeniaceae) (Howarth and Donoghue, 2005, 2006; S. Carlson and M. J. Donoghue,

University of Neuchâtel, Switzerland, unpubl. res.). Outside the Asteridae, the same pattern has also been documented in legumes, with three or four copies present in many of the groups that have been examined (Citerne *et al.*, 2003; Fukuda *et al.*, 2003; Ree *et al.*, 2004; Feng *et al.*, 2006; Wang *et al.*, 2008). Additionally, in Malpighiaceae, a duplication appears to have occurred near the origin of bilaterally symmetrical flowers (Zhang *et al.*, 2010).

Using a well-resolved species tree for Dipsacales (see Donoghue *et al.*, 2003), and a thorough sample of *CYC*-like genes in this group (Howarth and Donoghue, 2005), it was possible to map clearly *CYC*-like duplications within this group. Previously it has been shown that the radially symmetrical clade of Dipsacales, the Adoxaceae, have only a single base copy of *DipsCYC2*, with a duplication within the *Viburnum* lineage (Howarth and Donoghue, 2005), and that a duplication occurred along the branch leading to the bilaterally symmetrical Caprifoliaceae. This duplication in Caprifoliaceae is concurrent with the shift to bilateral flower symmetry.

Background on Dipsacales, Caprifoliaceae and Lonicera

The Dipsacales phylogeny is now quite well resolved, based on a series of molecular and morphological phylogenetic analyses (Judd *et al.*, 1994; Backlund and Donoghue, 1996; Bell *et al.*, 2001; Donoghue *et al.*, 2001b, 2003; Pyck, 2001; Zhang *et al.*, 2003; Smith and Donoghue, 2008). This understanding provides a solid basis for inferring the location of evolutionary changes in flower characters (Donoghue *et al.*, 2003), as well as a secure framework within which to infer the evolution of *CYC* and related genes. A variety of floral forms are found within Dipsacales, including polysymmetric, monosymmetric and asymmetric flowers (Donoghue *et al.*, 2003). Recent phylogenetic analyses of campanulid angiosperms support the radially symmetrical Paracryphiaceae as sister to Dipsacales (Winkworth *et al.*, 2008b; Tank and Donoghue, 2010), which supports an ancestral condition of radial symmetry in the entire clade. The primary split within Dipsacales separates the Adoxaceae from the Caprifoliaceae (Fig. 1). Adoxaceae (including *Viburnum*, *Sambucus* and *Adoxa* and its relatives) have radially symmetrical flowers, with small calyx lobes, and rotate corollas, whereas Caprifoliaceae (including Diervilleae, Caprifolieae, Linnaeeae, Morinaceae, Dipsacaceae and Valerianaceae) typically have bilaterally symmetrical flowers, larger calyx lobes, and tubular corollas (Fukuoka, 1972; Donoghue *et al.*, 2003). Caprifoliaceae appear to represent an independent derivation of bilaterally symmetrical flowers within the Asteridae (Fig. 1).

There are several forms of bilateral symmetry present within the Caprifoliaceae. Two-lipped flowers, in which the two dorsal petals are differentiated from the two lateral petals and the medial ventral petal (the 2:3 form), are widespread within the clade and appear to be ancestral. Members of the sister group of the rest of the Caprifoliaceae, the Diervilleae, are only weakly bilaterally symmetrical with the 2:3 form. In *Diervilla*, for example, the corolla lobes are more or less indistinguishable except for a modified ventral petal. Another early-diverging lineage, the Caprifolieae, includes species with two-lipped flowers in which the two dorsal and the two lateral
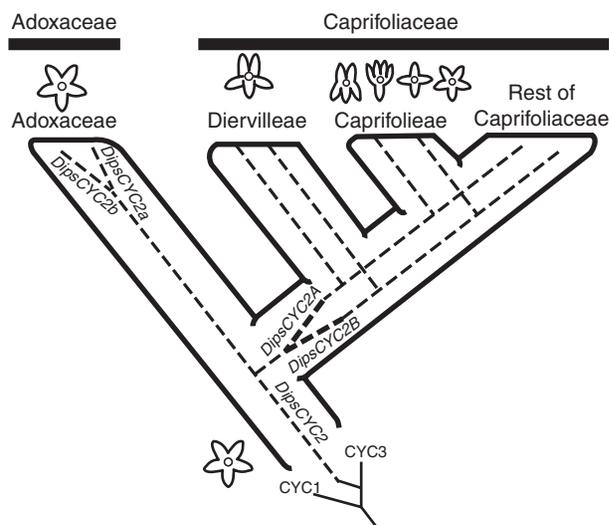
Fig. 1. Floral symmetry shifts and *DipsCYC2* duplication events. The ancestor of Dipsacales is hypothesized to have been radially symmetrical. This radial symmetry is maintained in Adoxaceae. Within *Viburnum* (Adoxaceae), a duplication event resulted in two copies, *DipsCYC2a* and *DipsCYC2b*. The shift to bilateral symmetry in the ancestor of Caprifoliaceae is correlated with a duplication of *DipsCYC2* into *DipsCYC2A* and *DipsCYC2B*.

petals are differentiated from the medial ventral petal (the 4:1 form); such flowers are found in *Lonicera* and *Triosteum*, and radially symmetrical flowers appear to have re-evolved in *Symphoricarpos*. There have also been several shifts in the number of corolla lobes, most notably the reduction from five to four lobes in *Symphoricarpos* and in Dipsacaceae. Stamen number became reduced from five to four in the remainder of the Caprifoliaceae (excluding Diervilleae and Caprifolieae) through loss of the medial dorsal stamen. Further reductions in stamen number within this group occurred within Morinaceae, and multiple times within the Valerianaceae (Donoghue *et al.*, 2003).

This study is focused on two early-diverging lineages within the Caprifoliaceae, the Diervilleae and the Caprifolieae. Diervilleae is made up of two sublineages, *Diervilla* (three species) and *Weigela* (ten species). Caprifolieae is a much larger group, containing *Leycesteria* (six species, 2:3 floral form), *Lonicera* (approx. 180 species, 4:1 floral form), *Symphoricarpos* (approx. 15 species, radial flower) and *Triosteum* (six species, 4:1 floral form). Caprifolieae is well supported as a clade and *Heptacodium* (one species, 2:3 floral from) is typically reconstructed as sister to these four genera (Bell *et al.*, 2001; Donoghue *et al.*, 2001*a*, *b*; Zhang *et al.*, 2003; Theis *et al.*, 2008; Winkworth *et al.*, 2008*a*). However, relationships among these four genera have remained problematic, possibly owing to rapid radiation (Bell and Donoghue, 2005). The best-supported results to date suggest that *Triosteum* is sister to the other three genera, and that *Leycesteria* and *Symphoricarpos* form a clade that is sister to *Lonicera* (Theis *et al.*, 2008; Smith, 2009). This has implications for the evolution of bilateral symmetry; specifically, the 4:1 floral form may have arisen separately in *Lonicera* and in *Triosteum* within the Caprifolieae.

Lonicera is divided into two clades, traditionally recognized as the subgenera *Lonicera* and *Caprifolium* (Rehder, 1903;

Theis *et al.*, 2008; Smith, 2009). The two groups are morphologically distinctive; members of subgenus *Lonicera* have two-flowered cymes and free leaves, while species of *Caprifolium* have three-flowered cymes in whorls and perfoliate leaves subtending the inflorescences. Although *Lonicera* species all have the 4:1 flower form, they vary from being weakly bilaterally symmetrical (see *L. sempervirens*; Fig. 2J) to very strongly bilatiate, with the two dorsal and two lateral petals nearly entirely fused and pointed upward (see *L. periclymenum*; Fig. 2I). In contrast, in *L. morrowii* and *L. × bella* (studied here) the two dorsal lobes are distinct in orientation from the two lateral lobes, though all are similar in size and shape (Fig. 2G, H).

MATERIALS AND METHODS

Plant collections

Dissections were performed on the following six taxa: *Viburnum plicatum*, *Diervilla sessilifolia*, *Lonicera sempervirens*, *Lonicera periclymenum*, *Lonicera morrowii* and *Lonicera × bella*. *Viburnum plicatum* was collected on Raff Ave. in Floral Park, NY. *Diervilla sessilifolia* and *L. periclymenum* were collected from propagated plants in the greenhouses of the Marsh Botanical Gardens, Yale University, New Haven, CT. *Lonicera sempervirens* was collected near the Stewart Manor train station in Garden City, NY. *Lonicera morrowii* was collected in two locations. One was in Guilford, CT and the other was on The Grand Central Pkwy access road, where it crosses with the Midland Pkwy, in Jamaica, NY. *Lonicera × bella* is a horticultural hybrid between *Lonicera tatarica* and *L. morrowii*. It was also collected in Jamaica, NY in the same location as *L. morrowii*. Vouchers for these species have been deposited at Yale University Herbarium, New Haven, CT.

Plants were collected by cutting off large branches that contained flower buds at multiple stages. The branches were taken to the laboratory and immediately dissected. The dissected material was housed in a freezer at −80 °C until RNA extraction could be performed.

Dissections

Whenever possible, dissections were performed on multiple accessions of each species. Every accession contained three separate corolla lobe dissections (Fig. 2F, K) consisting of dorsal (two dorsal petals), lateral (two lateral petals) and ventral (single ventral petal). Corolla lobes were separated from the corolla tubes of multiple flower buds from early stage (first stage possible to dissect) to nearly open flower buds. The floral parts were already well developed and only elongating through all of these dissectible bud stages. The bud stages were mixed because the comparisons were across multiple taxa that do not have comparable stages, CYC2 clade members are expressed throughout corolla development, and, in several species, there was a limited supply of buds from plants that flower for only a short window of time. Late-stage buds alone (from buds about to open) were also used in accessions of three of the taxa. Dissections targeted only the lobes, without any of the fused corolla tube tissue. At least one
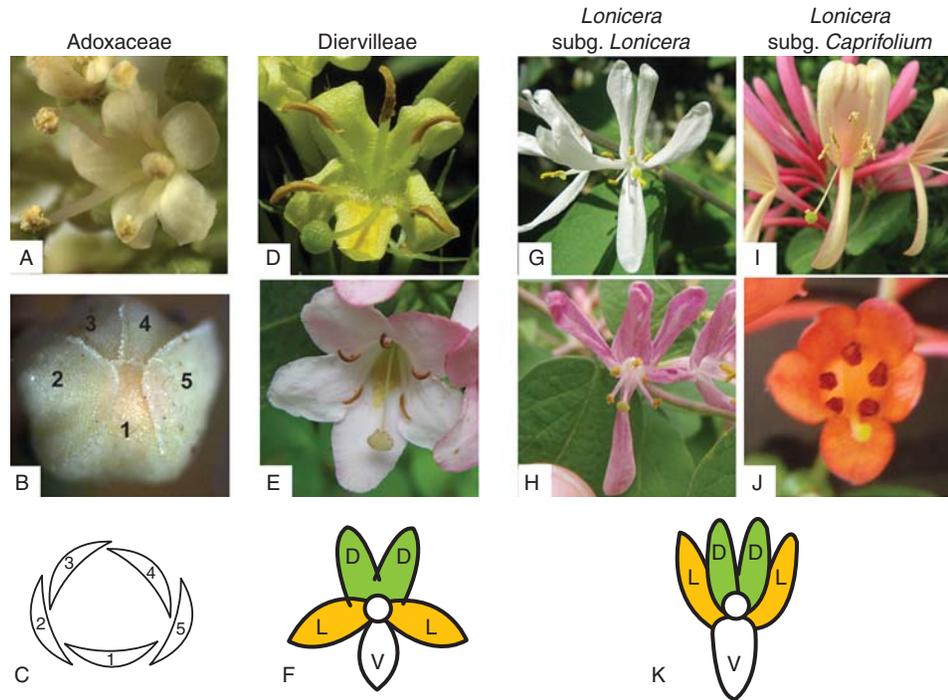
FIG. 2. Images of Dipsacales examined in this study: *V. plicatum* (A–C), *V. plicatum* aestivation in bud (B) and floral diagram (C), *D. sessilifolia* (D), *W. florida* (E), petal arrangement of Diervilleae (F), *L. morrowii* (G), *L. × bella* (H), *L. periclymenum* (I), *L. sempervirens* (J), and general petal arrangement in *Lonicera* (K). In (B) and (C), numbers indicate the order of dissected petals. In (F) and (K), D = dorsal, L = lateral, V = ventral. Photos taken by D. G. Howarth (A, B, G, H, J), M. J. Donoghue (E, I) and Alan Cressler (D).

dissection from each species also included whole bud, tube only (no petal lobes) and leaf for comparison.

In Adoxaceae, the petals of *V. plicatum* were dissected individually based on aestivation. Petal 1 was determined in each flower as the lone innermost petal of the bud (see Fig. 2B, C). Petals 2–5 were then dissected in a clockwise pattern. We hypothesize that petal 1 is the ventral petal; however, this is extremely difficult to determine in these small radially symmetrical flowers. Whole bud dissections from central reproductive flowers and sterile marginal flowers were also included, as well as leaf tissue. The extractions required significant tissue, requiring petal lobes from at least 40 buds.

Reverse transcriptase PCR (rtPCR)

Frozen tissues were pulverized with the Bio101 FastPrep system and were subsequently extracted using an RNeasy kit with the optional DNase step (Qiagen). When necessary, the samples were subsequently treated with DNase I (NEB), to eliminate all DNA contamination. For most samples, the cDNA was generated with gene-specific primers using the one-step RT-PCR kit (Invitrogen). The rtPCR used the standard protocol suggested with the kit, with a 55 °C annealing temperature. Reactions amplifying *DipsCYC2A* and *DipsCYC2B* were done in 25-µL reactions (half reactions), while *GAPDH* controls were done in 12·5-µL reactions (quarter reactions). The exception was *L. morrowii* from Madison, CT, in which cDNA was generated from RNA using Superscript III (Invitrogen). The genes were subsequently amplified from total cDNA utilizing the following cycling programme: 95 °C for 45 s, 50–56 °C for 1 min and

72 °C for 1 min 30 s, repeated for 39 cycles. Reactions were performed using *Taq* DNA polymerase (Qiagen, Valencia, CA or Promega) in 25 µL, with final concentrations of 2·5 mM MgCl$_2$, 0·5 µM of each primer, 0·8 mM dNTPs and 0·5 × Q Solution (Qiagen). The above PCR parameters were also used to test the total RNA for DNA contamination (data not shown).

To amplify *DipsCYC2A* the following gene-specific primers were used: DierCYC2AF30 (5′-CCAAGGACCCCGAGACC GGAGGGTAAGATTGTCCATTGAAATC) and DierCYC2A R19 (5′-CCCTCGACTCTCTAGCAACAAGATCAATGGC AGATTTATCTGTCTG) for *Diervilla*, and CaprCYC2AF (5′-GRACWCTTGATTGGCTACTCAAC) and LoniCYC2 AR (5′-GATTTATSTGTWGGCTTTCTCAAC) for all four *Lonicera* species. To amplify *DipsCYC2B* the following gene-specific primers were used: DierCYC2BF27 (5′-GGCACACG GTCCTAGGGATCGGAGAGTAAGGTTATCAATGGAG) and DierCYC2BR22 (5′ CGATTCTCTCGAACTACTATC AAATGCATCCCTGATCTTCTCG) for *Diervilla* and Loni CYC2BF (5′-GATGAAAATCAACTGCACTACTGG) and LoniCYC2BR (5′-AGCATCCCTCTTCTCGTTCCCAAC) for all four *Lonicera* species. A *GAPDH* control was also included, using primers from Strand *et al.* (1997). These results were run on a 1 % agarose gel in sodium borate buffer and copies were verified by sequencing. New sequences were submitted to Genbank (JF431004-13).

A modification of the above RNA-isolation protocol was used in *V. plicatum*, which was recalcitrant to extraction due to secondary compounds. Standard methods, such as trizol, urea and SDS (Gough, 1988), or with Qiagen RNeasy kits with either RLT or RLC buffers, were not successful.
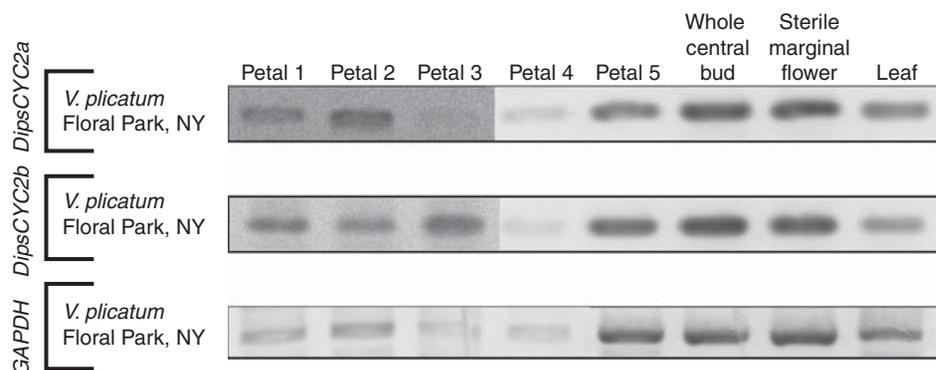
F IG. 3. rtPCR of *V. plicatum* tissues, including each of the five separate petals, whole buds of the central fertile flowers, sterile marginal flowers and leaves. Petals 1–5 are described in Fig. 2. Both *DipsCYC2* copies were present in all tissues, including across the entire corolla.

Modifications such as adding 1–2 % polyvinylpyrrolidone 360000, 2 % beta mercaptoethanol (β-ME), 10 mg mL^{-1} proteinase K and 0·5 % DMSO yielded no RNA. Ultimately, a standard Qiagen RNeasy extraction kit with RLC buffer (including β-ME) and 1 % high molecular weight polyethylene glycol (PEG 20000) was utilized as outlined by Gehrig *et al.* (2000). The use of PEG 20000 provided limited but observable amounts of RNA. Using DNA extracted from DNeasy Qiagen Kit, all members of the CYC2 clade from *V. plicatum* were amplified. Two copies (Genbank numbers: JF431004-5) were uncovered, which were similar in sequence to each of the two previous copies published from *Viburnum rhytidophyllum* (Howarth and Donoghue, 2005). These two copies, *DipsCYC2a* and *DipsCYC2b*, are supported as sister to each other by 0·95 Bayesian posterior probabilities and 81 % parsimony bootstrap, and therefore represent an independent duplication from the two Caprifoliaceae copies, *DipsCYC2A* and *DipsCYC2B* (Howarth and Donoghue, 2005). Primers were designed to specifically amplify each *V. plicatum* copy, *DipsCYC2a* (F: 5'-CAAGGCAAGTAAAACCCTAGAATGG TTG and R: 5'-GCTCATCTTTTTCTCTTTGTTAACGC) and *DipsCYC2b* (F: 5'-CAAACCTAGCAAAACGCTAGA TTGGCTGC and R: 5'-GCTTTACGTTGTCGCCTCAAC AACCCG). rtPCR reaction protocols were as above.

RESULTS

Within the CYC2 clade of ECE-TCP genes, the Adoxaceae ancestrally contains only a single copy. In the Caprifoliaceae, on the other hand, CYC2 was duplicated co-incident with the divergence of the group and a shift to bilateral symmetry. One of the first diverging lineages, the Diervilleae, are weakly bilaterally symmetrical with a 2:3 form. *Lonicera* all have a 4:1 floral form, however, there is variability in how strongly bilaterally symmetrical they are. It was observed that all *Lonicera* species had three different slit sizes. The slit between the ventral and lateral petals is always the deepest, the slit between the lateral and dorsal petals is the next deepest and the single slit between the two dorsal petals is always the most shallow (see Fig. 2I).

In the Adoxaceae, two copies of CYC2 in *V. plicatum*, *DipsCYC2a* and *DipsCYC2b*, which resulted from a duplication independent from those of Caprifoliaceae, were

uncovered. The expression of both of these copies was found in whole buds of the central (fertile) flowers, in sterile marginal flowers (opened but still growing), and in leaves (Fig. 3). Expression was also seen in all five petals. Although expression in a few of these dissections was weak, this can be attributed to minimal quantities of RNA, especially given that expression of *GAPDH* was similarly weak (Fig. 3).

In *D. sessilifolia* of the Caprifoliaceae both *DipsCYC2A* and *DipsCYC2B* are similarly expressed (Fig. 4). Two accessions of *D. sessilifolia* were used, which show the same pattern. Both accessions had mixed ages of flower buds (from the youngest that were dissectible to nearly mature). *DipsCYC2A* and *DipsCYC2B* are both strongly expressed in both dorsal and lateral petal lobes. There is extremely weak, or no, expression in the ventral petals of either gene copy. The mixed age whole buds also exhibited strong expression and there is no expression in leaf tissue.

In all of the *Lonicera* species, by contrast, *DipsCYC2A* and *DipsCYC2B* diverge in expression. *Lonicera morrowii*, *L.* × *bella* and *L. periclymenum* all had a similar pattern of expression across all samples. *DipsCYC2A* was expressed in the dorsal petals, but not in the lateral or ventral petals (Figs 5–7). *DipsCYC2B* was expressed in dorsal and lateral petals but not in the ventral petal. This pattern holds in the mixed ages and the nearly mature bud dissections of *L. morrowii* (Fig. 5) and *L.* × *bella* (Fig. 6), and the mixed ages of *L. periclymenum* (Fig. 7). *Lonicera periclymenum* is strongly bilaterally symmetrical with deep slits between the ventral petal and the upper four petals. There is also strong expression of both *DipsCYC2A* and *DipsCYC2B* in all three taxa in whole bud extractions and in the floral tube. No expression of either gene was detected in leaves in any of the taxa.

Lonicera sempervirens flowers have three different slit depths, like the other species, but differences are minor and the flowers are nearly radial in appearance. In *L. sempervirens*, all three samples showed strong expression in dorsal and lateral petals for *DipsCYC2A*, although all three were weaker in the lateral petals than in the dorsal petals (Fig. 8). There was also strong expression in whole buds at both younger and older stages and in the corolla tube. The same pattern is seen in the mixed bud stages as well as the nearly mature buds (Fig. 8, sample 3).
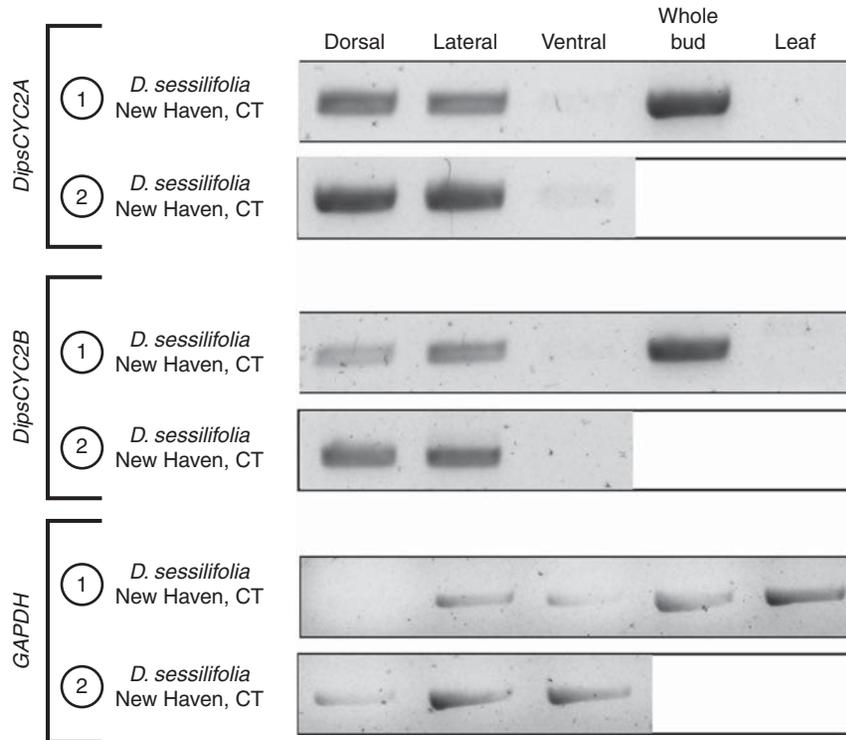
FIG. 4. rtPCR of *Diervilla sessilifolia* tissues from two separate dissections, including dorsal, lateral and ventral corolla lobes. One dissection also included whole bud and leaf tissue. Both *DipsCYC2A* and *DipsCYC2B* are expressed in dorsal and lateral corolla lobes and are absent from ventral lobes. There was also strong expression of both copies in whole buds and absence from leaves. Boxed white areas were not sampled in those dissections.

DipsCYC2B, by contrast, is strongly expressed throughout the flower, although arguably weaker in ventral petals (Fig. 8). *DipsCYC2B* is also strongly expressed in whole-bud dissections and in the corolla tube. Unlike any of the other sampled Caprifoliaceae, *L. sempervirens* showed expression of both *DipsCYC2A* and *DipsCYC2B* in leaf tissue.

DISCUSSION

CYCLOIDEA *duplications*

The *DipsCYC2* genes of Dipsacales all fall into the CYC2 clade of ECE-containing TCP genes (Howarth and Donoghue, 2006). These ECE-TCP genes duplicated twice around the divergence of the Pentapetalae (core eudicots excluding Gunnerales; Cantino *et al.*, 2007), into the paralogous CYC1, CYC2 and CYC3 clades (Howarth and Donoghue, 2006). The predominantly studied copies are orthologues that all fall into the CYC2 clade (including *CYC* and *DICH* paralogues from *Antirrhinum* and *TCP1* from arabidopsis) (Howarth and Donoghue, 2006). There is growing evidence that *CYC*-like genes function similarly across rosids and asterids, with CYC2 members playing a role in dorsal identity of the flower. Although their expression is not examined here, there is evidence that members of the CYC3 clade may also play a role in floral symmetry, especially given that genes in the CYC2 and the CYC3 clades are tandemly duplicated in many lineages, including Caprifoliaceae (Howarth and Donoghue, 2005, 2006). Possible dorsoventral asymmetry to their expression has been shown previously (Howarth and Donoghue, 2006); however, CYC3 members have not yet been explored in any group. CYC1 members have not been implicated in floral symmetry, although they have been shown to play a role in plant branching in arabidopsis (Finlayson, 2007). Outside Pentapetalae, the ECE-containing TCP genes (which are orthologous to the clade containing CYC1, CYC2 and CYC3) appear to have been co-opted in the floral symmetry pathway independently multiple times. Specifically, independent duplications in Papaveraceae have correlative expression with shifts to monosymmetry (Damerval *et al.*, 2007). A paralogous TCP gene has been implicated in patterning the monosymmetry of the *Oryza* palea (Yuan *et al.*, 2009). *TB1*, the orthologue to all three copies in *Zea*, plays a role in both inflorescence branching and stamen abortion (Doebley *et al.*, 1995), the latter also being a recognized function of CYC2 members (Luo *et al.*, 1996).

Duplications in the *CYC2* gene clade have been found in nearly every group examined, spanning core eudicots (Citerne *et al.*, 2000, 2003; Fukuda *et al.*, 2003; Gübitz *et al.*, 2003; Hileman and Baum, 2003; Zhang *et al.*, 2003; Ree *et al.*, 2004; Smith *et al.*, 2004; Feng *et al.*, 2006; Chapman *et al.*, 2008; Wang *et al.*, 2008). In Dipsacales, the bilaterally symmetrical Caprifoliaceae have been explicitly compared with the radially symmetrical Adoxaceae and evidence of a duplication near the divergence of the Caprifoliaceae has been shown (Howarth and Donoghue, 2005). Other duplications within the Caprifoliaceae resulted in two copies of *DipCYC2B* in Morinaceae (which may be concurrent with a shift to calyx monosymmetry) and multiple
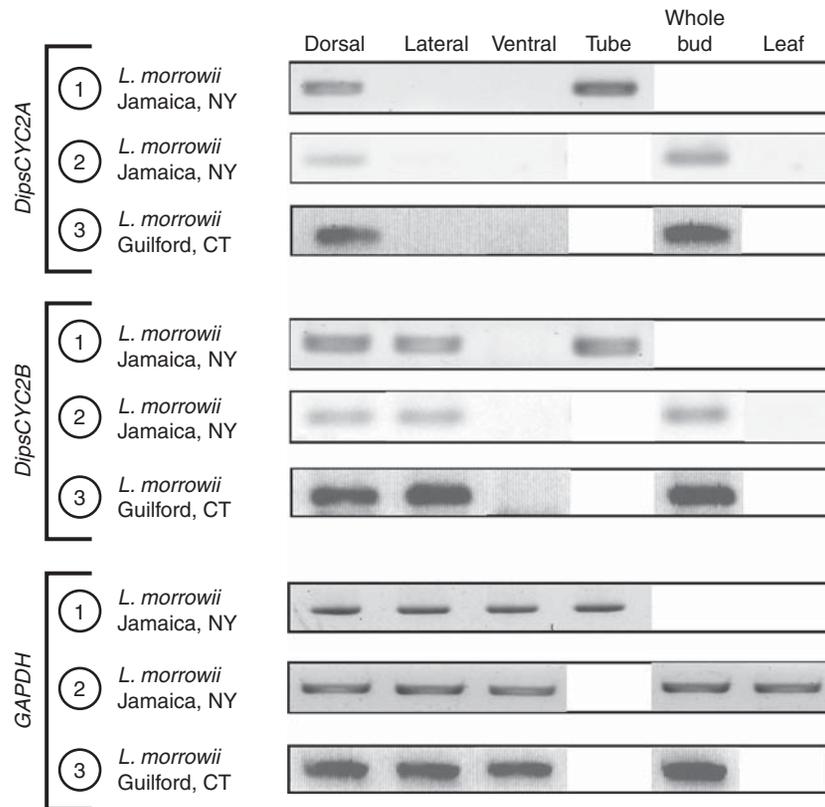
FIG. 5. rtPCR of *Lonicera morrowii* tissues from three separate dissections from two separate collection sites. All collections included dorsal, lateral and ventral dissections of corolla lobes. Dissection 1 also contained corolla tube tissue. Dissection 2 contained whole bud and leaf tissue. Dissection 3 contained whole bud tissue. *DipsCYC2A* was expressed in dorsal lobes, but not in lateral or ventral lobes. It was also strongly expressed in corolla tubes and whole buds. *DipsCYC2B* was expressed in dorsal and lateral corolla lobes but was absent from ventral lobes. *DipsCYC2B* was also found in corolla tube tissue and whole buds. Both *DipsCYC2A* and *DipsCYC2B* were absent in leaves. Boxed white areas were not sampled in those dissections.
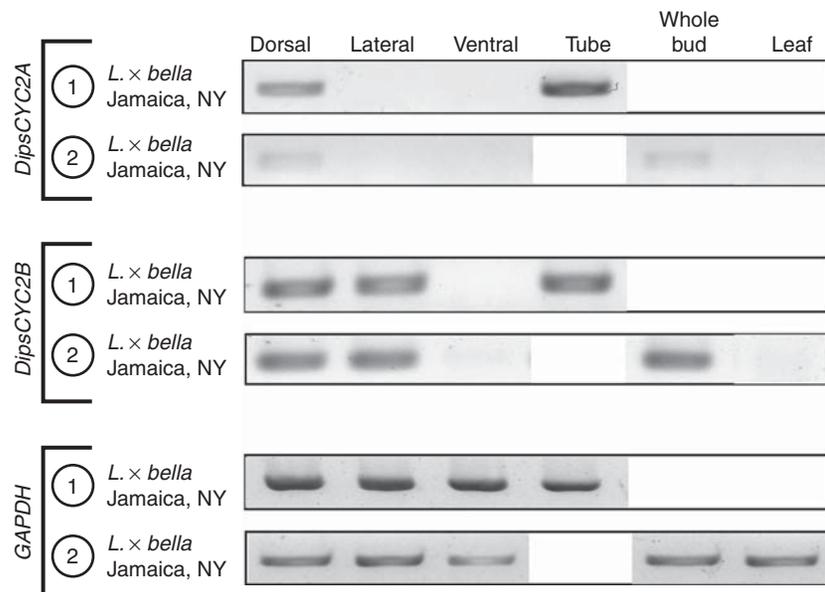
FIG. 6. rtPCR of *Lonicera* × *bella* tissues from two separate dissections. All collections included dorsal, lateral and ventral dissections of corolla lobes. Dissection 1 included corolla tube tissue. Dissection 2 included whole bud and leaf tissue. *DipsCYC2A* was expressed in dorsal lobes, but not in lateral or ventral lobes. It was also expressed in corolla tubes and whole buds. *DipsCYC2B* was expressed in dorsal and lateral corolla lobes but was absent from ventral lobes. *DipsCYC2B* was also found in corolla tube tissue and whole buds. Both *DipsCYC2A* and *DipsCYC2B* were absent in leaves. Boxed white areas were not sampled in those dissections.

copies of *DipCYC2B* in various Valerianaceae (Howarth and Donoghue, 2005; G. Boyden and D. G. Howarth, unpubl. res.) and Dipsacaceae (S. Carlson and M. J. Donoghue, unpubl. res.). Previously these genes have been cloned from *Diervilla* and *Weigela* (Diervilleae) and from *Leycesteria*, *Triosteum*, *Symphoricarpos* and multiple species of *Lonicera* but no evidence for additional duplications of *DipsCYC2A* or *DipsCYC2B* has been found within these groups (Howarth and Donoghue, 2005). Therefore, in this study, it was possible to examine shifts in gene expression following duplication, focusing directly on the location in the phylogeny where gene duplication and divergence occurred and where there were relevant shifts in flower morphology.
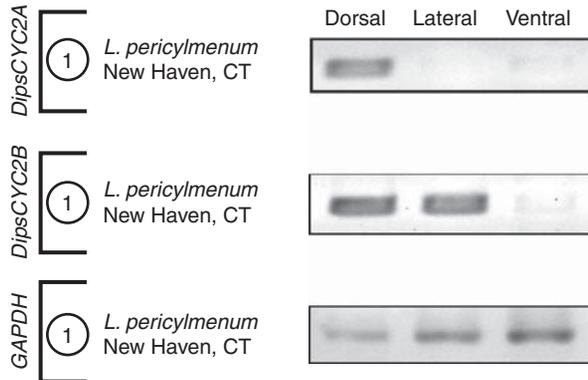

FIG. 7. rtPCR of a single dissection of *Lonicera periclymenum*. A single dissection from *L. periclymenum* included dorsal, lateral and ventral corolla lobes. *DipsCYC2A* was expressed in dorsal lobes, but not in lateral or ventral lobes. *DipsCYC2B* was expressed in dorsal and lateral corolla lobes but was absent from ventral lobes.

Flower symmetry evolution

Studies of floral symmetry have generally focused on species with derived morphologies, nested well within bilaterally symmetrical groups. *Antirrhinum*, *Mohavea* and *Linaria* are all nested within the bilaterally symmetrical Antirrhineae (Luo *et al.*, 1996; Cubas *et al.*, 1999; Hileman *et al.*, 2003; Oyama and Baum, 2004). *Lupinus*, *Lotus* and *Pisum* are typical papilionoid legumes, and are nested well within this major clade (Citerne *et al.*, 2006; Feng *et al.*, 2006; Wang *et al.*, 2008). *Helianthus*, *Gerbera* and *Senecio* are highly derived Asteraceae containing both radial disk and bilateral ray flowers (Broholm *et al.*, 2008; Chapman *et al.*, 2008;

FIG. 8. rtPCR of *Lonicera sempervirens* tissues from three separate dissections. All collections included dorsal, lateral, and ventral dissections of corolla lobes. Dissection 1 also included three differently aged whole buds (10-, 25- and 40-mm-long buds), an early inflorescence, and leaf tissue. Dissection 2 included corolla tube tissue. Dissection 3 included a whole young bud (10 mm). *DipsCYC2A* was expressed in dorsal and lateral lobes, and was absent from ventral lobes. By contrast, *DipsCYC2B* was expressed across all corolla lobes. Both copies were also found in all stages of flower buds, in corolla tube tissue, and weakly in leaves. Boxed white areas were not sampled in those dissections.

Kim *et al.*, 2008). *Iberis* is nested within a small bilaterally symmetrical clade of Brassicaceae (Busch and Zachgo, 2007). Zhang *et al.* (2010) compared the expression of *CYC2*-like genes of outgroups to morphological shifts in old-world and new-world Malpighiaceae. A few studies have examined radially symmetrical species that are nested within bilaterally symmetrical clades, such as in *Bournea*, *Cadia*, *Plantago* and *Tengia* (Citerne *et al.*, 2006; Zhou *et al.*, 2008; Reardon *et al.*, 2009; Pang *et al.*, 2010). The present study attempts to understand the independent evolution of bilaterally symmetrical flowers within the Dipsacales, focusing specifically on the primary gene duplication event subtending the Caprifoliaceae, and subsequent evolutionary shifts in gene expression associated with flower form in its early-diverging lineages.

Character state reconstructions using likely outgroups (Paracryphiaceae) support the view that the first Dipsacales had radially symmetrical flowers (Donoghue *et al.*, 2003; Winkworth *et al.*, 2008*b*; Tank and Donoghue, 2010). The transition to bilateral symmetry occurred along the line leading to Caprifoliaceae, after divergence from its radially symmetrical sister group, Adoxaceae. Concurrent with that morphological transition there was a duplication in the *CYC2*-like gene lineage, yielding *DipsCYC2A* and *DipsCYC2B* (Fig. 1). Most Adoxaceae have only a single form of a *CYC2*-like gene, *DipsCYC2* (Howarth and Donoghue, 2005). However, the present analyses confirm that a separate duplication occurred within *Viburnum*, resulting in two *CYC2* copies, *DipsCYC2a* and *DipsCYC2b*, both of which are expressed throughout the corolla (Fig. 3). In other radially symmetrical flowers, *CYC2* genes are either expressed across the entire corolla (as in *Viburnum*) or are entirely absent. Elatinaceae, *Cadia* and *Tengia* show *CYC* expression across the entire corolla (Citerne *et al.*, 2006; Pang *et al.*, 2010; Zhang *et al.*, 2010), whereas in *Antirrhinum* peloric mutants, *Bournea* (late expression), Centroplacaceae and *Plantago*, floral *CYC* expression is lacking (Luo *et al.*, 1996; Zhou *et al.*, 2008; Reardon *et al.*, 2009; Zhang *et al.*, 2010). Based on the discovery that *CYC2* genes are expressed across the entire corolla in *Viburnum*, and that they are widely expressed in *Diervilla*, we hypothesize that the first Dipsacales expressed *DipsCYC2* throughout the corolla.

Within Caprifoliaceae, the first split separated Diervilleae from all of the rest (see Donoghue *et al.*, 2003). Diervilleae contains two genera, *Diervilla* and *Weigela*, which are both weakly bilaterally symmetrical with the 2:3 floral form, and marked by a morphologically distinctive ventral corolla lobe (Fig. 2). The present expression data for *Diervilla* indicate that *DipsCYC2A* and *DipsCYC2B* are expressed in a similar pattern to each other; both are strongly expressed in the dorsal and lateral petals, and are nearly completely absent from the ventral petal (Fig. 4). Based on this observation we hypothesize that the expression of Caprifoliaceae *DipsCYC2* orthologues shifted out of the ventral petal. This shift could have occurred before or after the duplication (Fig. 9).

Within the large clade including the remainder of the Caprifoliaceae, the basal split separated the Caprifolieae lineage from all the rest (Figs 1 and 9). While the relationships are still uncertain, *Heptacodium* (with a 2:3 floral form) appears as sister to the Caprifolieae in most analyses (reviewed

in Winkworth *et al.*, 2008*a*), and the best-supported result to date shows *Triosteum* as sister to the other three genera, with *Leycesteria* and *Symphoricarpos* forming a clade that is sister to *Lonicera* (Theis *et al.*, 2008; Smith, 2009). These data suggest that the 4:1 form may have evolved independently in *Lonicera* and *Triosteum*, implying that shifts in the type of bilateral symmetry are labile in Caprifolieae. Even within *Lonicera*, the degree of separation between the ventral petal and the four upper petals varies greatly.

In the species examined that have a clearly recognizable 4:1 form (*L. periclymenum*, *L. morrowii* and *L. × bella*), *DipsCYC2A* is expressed in only the dorsal petals, whereas *DipsCYC2B* is expressed in both dorsal and the lateral petals (Figs 5–7). In view of the observation that these genes are expressed in the dorsal and the lateral petals in *Diervilla*, we hypothesize that *DipsCYC2A* expression became further restricted in *Lonicera* to the dorsal petals (Fig. 9). This is a more parsimonious explanation than the expansion of *DipsCYC2B* expression. As the present sample includes representatives of the two major lineages within *Lonicera* (Theis *et al.*, 2008; Smith, 2009), it is inferred that the restriction of *DipsCYC2A* occurred at some point prior to the origin of crown group *Lonicera*. It is likely, therefore, that the expression pattern of the nearly radial *L. sempervirens* is derived, with *DipsCYC2A* in dorsal and lateral petals and *DipsCYC2B* in all petals (Fig. 8). In this case, it would appear that both *DipsCYC2A* and *DipsCYC2B* shifted their expression ventrally.

Hypothesized shifts

It has been argued that the diversification of plants relates to gene duplication and the subsequent adaptation of the duplicate genes (Flagel and Wendel, 2009; Van de Peer *et al.*, 2009). When a gene duplicates, its primary fate is to return to one copy (Lynch and Conery, 2000). One way that both gene copies can be maintained, however, is by undergoing subfunctionalization, in which the two duplicates take on different, complementary, roles (Lynch and Force, 2000). An alternative is when one paralogous copy undergoes neofunctionalization, taking on a brand new role relative to the original gene (Ohno, 1970). Accordingly, duplication can promote the rapid modification of expression patterns (Adams *et al.*, 2003). Here, it has been shown that *DipsCYC2a* and *DipsCYC2b* are expressed throughout the corolla in the radially symmetrical flowers of *Viburnum* (Adoxaceae) and that a first step toward bilateral symmetry entailed a shift in both duplicates out of the ventral region of the corolla. Subsequently, during the divergence of the Caprifolieae, we hypothesize that one of the duplicates, *DipsCYC2A*, became further restricted in its expression to the two dorsal corolla lobes. At this point, *DipsCYC2A* and *DipsCYC2B* diverged in their zones of expression. We view this as a form of subfunctionalization, in which one copy retains the ancestral expression pattern, and one becomes restricted in its expression to just a portion of the ancestral zone. However, judging by their coupled expansion in expression in *L. sempervirens*, the expressions of the two gene copies appear not to be completely decoupled from one another (Fig. 9).
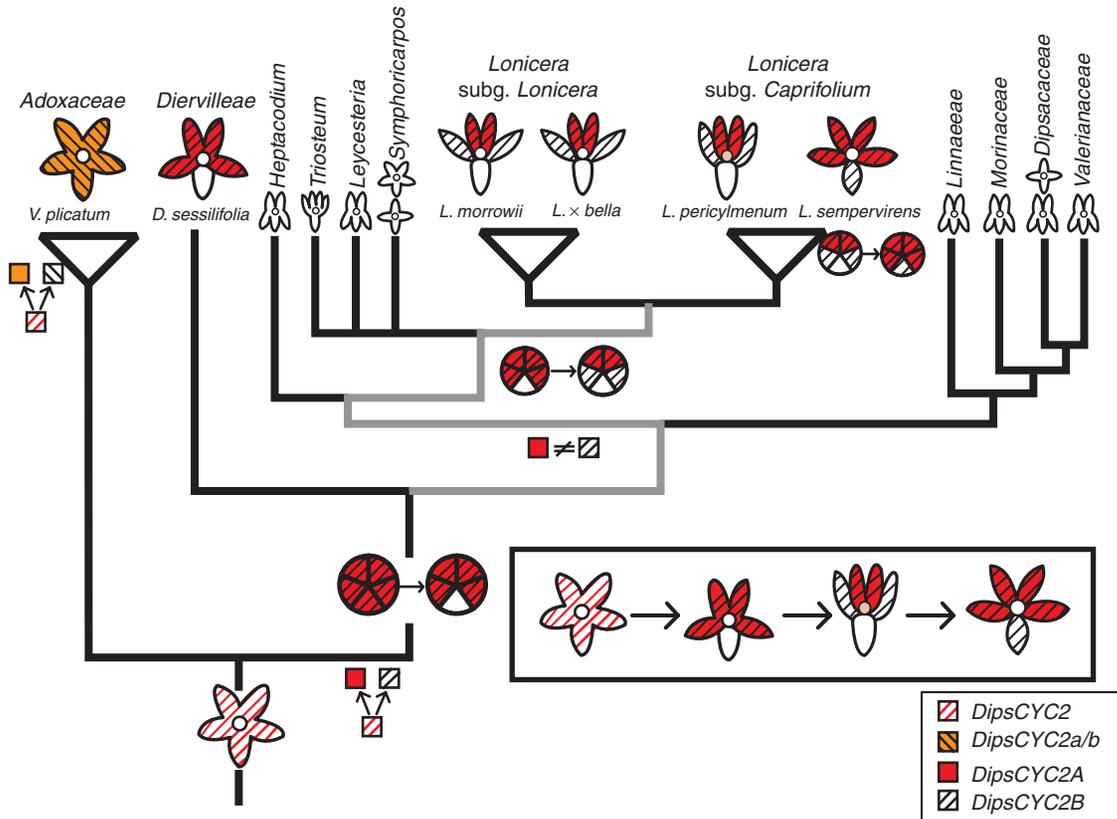
FIG. 9. A model for transitions in gene expression in Diervilleae and *Lonicera* placed on a phylogeny and in the order that we hypothesize that they occurred (boxed). Striped orange lines indicate expression of both *DipsCYC2a* and *DipsCYC2b* in *V. plicatum*. Red shading shows expression of *DipsCYC2A* and black striped lines show the expression of *DipsCYC2B* in Caprifoliaceae. The first step occurred around the divergence of Caprifoliaceae and the duplication event leading to two copies. This transition is correlated with the loss of expression in the ventral corolla lobe (Diervilleae). The second transition involved the decoupling of *DipsCYC2A* and *DipsCYC2B* expression, with *DipsCYC2A* only in dorsal corolla lobes, and *DipsCYC2B* in dorsal and lateral lobes (*Lonicera*). This transition could have happened anywhere along the grayed nodes. A third shift occurred in the nearly radial *L. sempervirens*, in which both copies shifted ventrally.

The major transitions in the evolution of bilateral symmetry involve the individualization of distinct zones of the corolla along the dorsoventral axis. Within Caprifoliaceae, the first step entailed the individuation of the ventral corolla lobe from the other four corolla lobes. This is seen at the morphological level (differentiation of the ventral corolla lobe in Diervilleae) and genetically (the loss of expression of both *DipCYC2* copies in the ventral lobe). The second step resulted in the individuation of three separate corolla zones: ventral, lateral and dorsal. We hypothesize that this was underlain by the decoupling of the two *DipsCYC2* copies, such that one became expressed only in dorsal tissue while the other maintained expression in the lateral and dorsal tissues. This partial subfunctionalization may have set the stage for the two dorsal petals to operate independently of the two lateral petals.

Nearly all bilaterally symmetrical groups examined have multiple copies of *CYC2*-like genes [one clear exception is *Iberis* (Busch and Zachgo, 2007), which falls into a small bilaterally symmetrical group of the radially symmetrical Brassicaceae]. Caprifoliaceae, Malpighiaceae and Asteraceae all have duplications in *CYC2*-like genes that correlate with shifts in floral symmetry (Howarth and Donoghue, 2006; Chapman *et al.*, 2008; Zhang *et al.*, 2010). In bilaterally

symmetrical flowers in which the expression of multiple copies has been documented, one copy is nearly always dorsally restricted with respect to the other copy. It is perhaps not surprising that duplicate gene copies are showing evidence of partial subfunctionalization, with one copy expanding or restricting expression with respect to the other. What is striking, however, is that these shifts in expression are happening again and again in the same way in widely separated plant groups. In *Lonicera*, it is found that the expression of *DipsCYC2A* is restricted to just the dorsal petals, while that of *DipsCYC2B* remains in dorsal and lateral petals. A similar pattern has been found in Malpighiaceae, with at least one gene duplicate expressed in just the dorsal petal, and at least one gene duplicate expressed in both the dorsal and lateral petals (Zhang *et al.*, 2010). In Papilionoid legumes, the same pattern exists in *Pisum* and *Lotus* (Feng *et al.*, 2006; Wang *et al.*, 2008). In *Pisum*, *PsCYC2* (*LST1*) is expressed only in the dorsal petal and mutations of this gene result in dorsal petals that are lateralized. *PsCYC3* (*K*) is expressed in both dorsal and lateral petals and mutations result in lateral petals that are ventralized. Mutants at both loci result in fully ventralized flowers. Differential expression in petals has also been documented in *Opithandra* (Gesneriaceae) in which one gene copy is restricted to just the dorsal petals, while a

separate duplicate is expressed throughout the corolla (Song *et al.*, 2009). Even in *Antirrhinum*, in which *CYC* and *DICH* are both restricted to the dorsal petals, *DICH* is restricted to the dorsal portion of the dorsal lobes, while *CYC* is expanded over most of the dorsal portion of the corolla (Luo *et al.*, 1996). These data, taken together, imply that differential expression of duplicated *CYC2* genes may play a role in setting up petal identity with respect to the axis; i.e. whether petals will have dorsal, lateral or ventral identity.

Alternatively, the decoupling of the expression of *CYC2* duplicates may play a role in the orientation of the lateral petals. Three plants groups have been shown to have one *CYC2* duplicate expressed in dorsal petals and a second *CYC2* duplicate expressed in dorsal and lateral petals: *Lonicera*, *Pisum* (Wang *et al.*, 2008) and Malpighiaceae (Zhang *et al.*, 2010). All three of these groups also share an uncommon morphology in which the lateral petals are oriented more dorsally than they would be in a radially symmetric flower. Most bilaterally symmetrical groups exhibit the 2:3 form, with the two lateral petals oriented ventrally. It is possible, therefore, that *CYC2* members play a role in the orientation of the lateral petals, possibly through the expression of fewer copies in the lateral petals than in the dorsal petals. This could be controlled through either differential growth, changes in slit depth that can determine petal orientation, or a gradient of expression.

In summary, we hypothesize that the first Dipsacales had radially symmetrical flowers with ubiquitous corolla expression of *CYC2*. Following the split between the Adoxaceae and the Caprifoliaceae, *CYC2* was duplicated along the Caprifoliaceae branch and both copies lost expression in the ventral corolla lobe (Fig. 9). This shift in expression was accompanied by a shift in the morphology of the ventral corolla lobe relative to the dorsal and lateral lobes. Subsequently, a shift in *DipsCYC2A* expression along the line leading to *Lonicera* (partial subfunctionalization) resulted in a distinction between dorsal and lateral petal identities, and may have affected corolla lobe orientation, yielding the 4:1 morphology. Coupled ventral expansion of both *DipsCYC2* paralogues in *L. sempervirens* was associated with reversion to a more radially symmetrical flower. We hypothesize that the more dorsally restricted the expression of *CYC2* paralogues in the corolla, the more extreme the resulting bilateral symmetry.

ACKNOWLEDGEMENTS

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