12-10-2013

The evolution of floral symmetry across the plant order Lamiales

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A Thesis Submitted to The Graduate School at the University of Missouir-St. Louis in partial fulfillment of the requirements for the degree

Doctor of Philosophy in Biology with an emphasis in Plant Science

November 2013

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ABSTRACT

Bilaterally symmetrical corollas have evolved independently numerous times from radially symmetrical ancestors and are thought to represent adaptation to specific pollinators. However, evolutionary losses of bilateral symmetry have occurred sporadically in different lineages. *CYC2*-like and *RAD*-like are genes needed for the normal development of bilateral symmetry in snapdragon corollas. However, exactly how changes in the floral symmetry patterning genes correlate with the origin and loss of floral bilateral remains poorly known. To address this question, a densely sampled phylogeny of *CYC2*-like genes across the order Lamiales was inferred and calibrated. The expression patterns of these genes in early diverging and higher core clades were also examined.

The phylogeny indicated at least four independent duplications of *CYC2*-like genes in four of the five major lineages of Lamiales around the Cretaceous-Paleogene (K-Pg) boundary, coinciding with the initial diversification of bumble bees and euglossine bees. Losses of *CYC2*-like paralogs were common, but did not correlate with a corresponding loss in floral symmetry. Relaxed positive selection occurred concurrently with retention of duplicate genes. *CYC2*-like paralogs showed differential expression, and asymmetrical expression of individual *CYC2*-like genes in adaxial and lateral petals correlated with the independent origins of floral zygomorphy in core Lamiales. *CYC2*-like genes have duplicated recurrently. However, the retention of *CYC2*-like duplicates was not required for the maintenance of floral zygomorphy.

CYC2-like and *RAD*-like genes were detected broadly in the floral meristem in early diverging Lamiales lineages, but were restricted to adaxial and lateral regions in the core Lamiales. The expression patterns of *CYC2*-like genes have evolved in a stepwise fashion. *CYC2*-like gene was expressed only very early in flower development in Oleaceae, while persistent expression of *CYC2*-like genes in petals originated in the common ancestor of Tetrachondraceae and core Lamiales. Asymmetrical expression in adaxial and lateral petals appeared later within the common ancestor of the core Lamiales. Similarly, expression of *RAD*-like gene in petals appeared in early diverging Lamiales or earlier, while asymmetrical expression in adaxial and lateral petals appeared later within

Plantaginaceae and Gesneriaceae. These data, along with published reports of *CYC2*-like genes expression, show that asymmetrical expression of *CYC2*-like gene is likely a derived condition that correlates with the origins of bilateral symmetry of the corolla. In contrast, the asymmetrical expression of *RAD*-like genes may be unique to the Plantaginaceae and Gesneriaceae lineages and is apparently not required for the development of bilateral symmetry.

To evaluate the possible developmental trajectories and genetic mechanisms underlying independent evolutionary losses of bilaterally symmetry of the corolla, three species of Lamiales with radially symmetrical corollas were compared. Results indicated that each achieved radial symmetry in a different way. Development and expression of *CYC2*-like genes in *Lycopus americanus* were similar to those of their bilaterally symmetrical relatives. However, expanded expression of *CcCYC2A* correlated with a radially symmetrical corolla in *Callicarpa cathayana*. Finally, loss of *CYC2A* and altered expression of *CYC2Bs* may account for the early bilateral symmetry but late radial symmetry in *Mentha longifolia*. Furthermore, expression of *RAD*-like genes, the downstream target of *CYC2*-like genes, was not detected in either *Lycopus americanus* or *Mentha longifolia*, which may further explain the late radial symmetry in these two species. On the other hand, *CcRAD* in *Callicarpa cathayana* resembled the broad expression pattern in floral tissues found in the *CYC2*-like genes.

ACKNOWLEDGEMENTS

I am indebted to so many people, but I particularly wish to acknowledge the efforts and encouragement of my thesis committee, funding sources, my professors, my friends and my family.

First, I am grateful to my advisory committee Elizabeth Kellogg, Peter Stevens, Jill Preston and Bethany Zolman for their insightful advice and feedbacks that helped me develop and complete this dissertation. I am particularly deeply indebted to Elizabeth Kellogg and Peter Stevens for their guidance, patience, continued encouragement and support all along my graduate studies, for inspiring me with their enthusiasm in plant world and sciences, for helping with grant applications, and for helping with revising and editing the thesis all along at all stages of the thesis and papers. Moreover, I want to thank Jill Preston who has been always willing to offer advice, suggestions for improving my thesis and help with complicated and labile nature of floral symmetry patterning network. Likewise, I thank Bethany Zolman for helping me to improve significantly the philosophy of science and the genetic aspects of this thesis.

This work was in part supported by a Doctoral Dissertation Improvement Grant from the National Science Foundation (grant number DEB 1210540 to EAK and JZ), the E. Desmond Lee endowment to the Kellogg Lab, the Jane Harris Scholarship in Tropical Botany from Whitney R. Harris World Ecology Center at the University of Missouri-St. Louis, a Graduate Student Research Award from Botanical Society of America, a Peter Raven Fellowship from the Department of Biology at the University of Missouri-St. Louis, and an Alumni Fellowship from Missouri Botanical Garden.

My dissertation research would not have been possible without access to the living collections from Missouri Botanical Garden and Shaw Nature Reserve, and DNA material from the William L. Brown Center (DNA Bank databases) at Missouri Botanical Garden and Xishuangbanna Tropical Botanical Garden (XTBG, China). For that, I thank the conservation department at Missouri Botanical garden, Phylogenetics and Conservation Biology Group at XTBG, James Trager, Jim Solomon and Alyse Kuhlam for their permission and assistance for sampling. I also particularly want to thank Cynthia

Hong-Wa and George Yatskievych, for helping with sampling the key components of this project.

I am particularly grateful to the following members in the Kellogg lab, past and present, who have trained me in lab work or helped me in one way or another: Matt Estep, Sara Fuentes, John Hodge, Michael McKain, Rosa Ortiz, Renata Reinheimer, Jimmy Triplett, Dilys Vela and Yunjing Wang. I want to thank the biology faculty at the University of Missouri - St. Louis who have broadened my interests beyond molecular plant systematics and evolution. I am also grateful to Maryann Hempen, Patricia Hinton and Kathy Burney-Miller at the Biology Department Office who always provided considerable assistance when needed. I am also very thankful to Mick Richardson and Peter Hoch, Manager of the Graduate Program at the Missouri Botanical Garden, for helping with various administrative matters pertaining to my research.

Friends made life in St. Louis an enjoyable journey. I particularly want to thank Cynthia Hong-wa, Pu Huang, Daniel Layton, Rosa Ortiz, John Pruski, Liang Guo and his family, Geliang Wang and his family, Dilys Vela and Xin Zhong.

Finally, I am greatly grateful to my loving wife, Huiqi Wu and our parents, my brother and his family, Lijuan Ye and her family, Yuyan Zheng for their unconditional and continued support and tireless patience.

CHAPTER 1. EVOLUTION OF *CYC2*-LIKE GENES IN THE ORIGIN AND MAINTENANCE OF FLORAL SYMMETRY IN LAMIALES

Abstract - Duplication, retention and expression of *CYCLOIDEA2* (*CYC2*)-like genes are thought to affect evolution of floral symmetry. However, exactly how changes in the *CYC*-mediated pathway correlate with the origin of floral zygomorphy is poorly known. We infer and calibrate a densely sampled phylogeny of *CYC2*-like genes across the Lamiales and examine their expression in early diverging and higher core clades.

Our results show at least four independent duplications of *CYC2*-like genes in four of the five major lineages of Lamiales around the Cretaceous-Paleogene (K-Pg) boundary. Losses of *CYC2*-like paralogs are common but do not affect floral symmetry. Relaxed positive selection correlates with retention of duplicate genes. *CYC2*-like paralogs show differential expression, and asymmetrical expression of *CYC2*-like in adaxial/lateral petals correlates with the independent origins of floral zygomorphy in core Lamiales.

CYC2-like genes have duplicated recurrently. However, the retention of *CYC2*-like duplicates is not required for the maintenance of floral zygomorphy. The parallel duplications of *CYC2*-like genes are subsequent to the initial diversification of bumble bees and euglossine bees.

Key words: *CYC2*-like gene, gene duplication and retention, floral symmetry, relaxed positive selection, Lamiales

Introduction

Floral zygomorphy has evolved many times during the diversification of angiosperms and is thought to affect the interaction between pollinators and plants, i.e. pollination efficiency (Donoghue *et al.*, 1998; Endress, 1999, 2001; Ree & Donoghue, 1999; Preston & Hileman, 2009). Bees, euglossine bees and bumble bees in particular, are attracted to zygomorphic flowers either through learning or innate preference (Møller, 1995; Neal *et al.*, 1998; Rodríguez *et al.*, 2004; Westerkamp & Claßen-Bockhoff, 2007). Correlational studies of floral zygomorphy and species richness show that floral zygomorphy is associated with several of the most species-rich angiosperm lineages (e.g. core Lamiales, Fabaceae, Orchidaceae), suggesting that floral zygomorphy can greatly accelerate speciation rates (Sargent, 2004). This hypothesis is further supported by some experimental evidence that shows plants with zygomorphic flowers have higher fitness than those with actinomorphic ones in natural conditions (*Erysimum mediohispanicum*, Brassicaceae) (Gómez *et al.*, 2006).

In the model species *Antirrhinum majus* (snapdragon, Plantaginaceae, Lamiales), the development of floral zygomorphy is controlled by a genetic network including two TCP genes, *CYCLOIDEA* (*CYC*) and *DICHOTOMA* (*DICH*) (Luo *et al.*, 1996, 1999), and two MYB transcription factors (Galego & Almeida, 2002; Corley *et al.*, 2005). TCP transcription factors are named for the three founding members of the gene family, *CYC*, *TEOSINTE BRANCHED1* (*TB1*), and *Proliferation Cell Factor* (*PCF*). Within the TCP family, CYC and TB1 (CYC/TB1 or ECE clade) proteins are characterized by a unique ECE (glutamate-cysteine-glutamate) domain (Howarth & Donoghue, 2006) in addition to two highly conserved TCP and R domains. ECE clade genes have duplicated extensively and independently in core eudicots and monocots (Howarth & Donoghue, 2006; Mondragón-Palomino & Theißen, 2009; Bartlett & Specht, 2011; Preston & Hileman, 2012). Phylogenetic analyses have identified three types of ECE clade genes (*CYC1*, *CYC2* or *CYC2*-like, and *CYC3*) that have resulted from two duplications at the base of core eudicots (Howarth & Donoghue, 2006). However, only *CYC/DICH* (*ECE-CYC2* clade) and their orthologs in other core eudicot species have been shown to be involved in floral zygomorphy (Luo *et al.*, 1996, 1999; Feng *et al.*, 2006; Busch & Zachgo, 2007; Broholm *et al.*, 2008; Wang *et al.*, 2008). In snapdragon, flowers of *cyc/dich* double

mutants are actinomorphic, while flowers of *cyc* mutants are semipeloric (weakly-zygomorphic or -actinomorphic) and *dich* mutants are zygomorphic but with less internal asymmetry of the adaxial petals (Luo *et al.*, 1996, 1999). *CYC* and *DICH* are thus partially redundant but not identical. Both genes are expressed exclusively in the adaxial and adjacent lateral regions of the flowers (Luo *et al.*, 1996, 1999). CYC/DICH regulate expression of the MYB transcription factor RADIALIS (RAD), which antagonizes another MYB protein DIVARICATA (DIV) that is thus restricted to the abaxial part of the flower and helps establish adaxial-abaxial asymmetry (Galego & Almeida, 2002; Corley *et al.*, 2005).

A CYC-mediated pathway has apparently been recruited independently for the development of floral zygomorphy in some eudicot lineages, such as Brassicaceae, Fabaceae and Asteraceae (Busch & Zachgo, 2007; Broholm *et al.*, 2008; Wang *et al.*, 2008). Extensive investigation of the phylogeny and expression of *CYC2*-like genes in different lineages has shown that multiple copies of *CYC2*-like genes tend to be maintained in lineages with zygomorphic flowers both in rosids and asterids (Citerne *et al.*, 2000, 2003; Hileman & Baum, 2003; Howarth & Donoghue, 2005; Zhang *et al.*, 2010). In addition, studies in *Arabidopsis* (Brassicaceae, rosids) and *Primulina heterotricha* (Gesneriaceae, asterids) indicate that persistent expression of *CYC2*-like genes in petals in later developmental stages is important for the development and/or maintenance of floral zygomorphy, as in core Lamiales (Cubas *et al.*, 2001; Busch & Zachgo, 2007; Yang *et al.*, 2012).

It remains unclear whether the duplication of *CYC2*-like genes is directly correlated with the origin of floral zygomorphy, how important retention of *CYC2*-like paralogs is for the maintenance of floral zygomorphy, what the most likely selection regime would be for the retention of *CYC2*-like paralogs, and how conservation and diversification of *CYC2*-like gene expression pattern following duplication might have led to evolutionary transitions of floral symmetry. Most previous comparative studies have compared zygomorphic flowers and derived actinomorphy within core Lamiales or floral zygomorphy and actinomorphy between distantly related taxa (e.g. snapdragon versus *Arabidopsis*). Surprisingly, few studies have investigated the duplication, retention and expression of *CYC2*-like genes in lineages with actinomorphic flowers closely related to

those with zygomorphic flowers. Only two previous studies have investigated the selection regime following duplication of *CYC2*-like genes, showing gene retention may be driven by either purifying selection, as in Plantaginaceae-Antirrhineae (Hileman & Baum, 2003), or by relaxed positive selection on one paralog as in Zingiberales (Bartlett & Specht, 2011).

The angiosperm order Lamiales, the focus of this study, includes ca. 12% of eudicot diversity, including about 24,000 species in 24 families; a number of relationships between these families are well resolved (Schäferhoff *et al.*, 2010; *c.f.* McDade *et al.*, 2012). The early diverging grade, including Plocospermataceae, Oleaceae + Carlemanniaceae, and Tetrachondraceae (Fig. 1) contains ca. 3% of the species diversity of Lamiales; these families are successively sister to the core Lamiales (ca. 97%). Within core Lamiales, Gesneriaceae + Calceolariaceae and Plantaginaceae are successive sisters to the remaining taxa, a clade that is sometimes called higher core Lamiales (HCL, *c.f.* Schäferhoff et al., 2010). Lamiales are a rich system for the study of floral evolution; zygomorphic flowers originated early in the clade, and derived actinomorphy evolved separately multiple times within lineages in core Lamiales.

Here we show that multiple parallel duplications of *CYC2*-like genes are not correlated with the floral shift from actinomorphy to zygomorphy early in Lamiales. Expression of *CYC2*-like genes is absent in petals in early diverging lineages Oleaceae; however, asymmetrical expression of *CYC2*-like genes in adaxial/lateral petals has subsequently evolved independently following recurrent gene duplications in major lineages within core Lamiales and these are directly correlated with the origin of floral zygomorphy. We also estimate the ages for major duplications of *CYC2*-like genes and test the selection regime on *CYC2*-like duplicates.

Materials and Methods

Taxon sampling – 92 species from 15 of the 23 families of Lamiales were sampled for this study (Supporting information Table S1). Additional sequences of *CYC2*-like genes of some Calceolariaceae, Gesneriaceae and Plantaginaceae were obtained from GenBank. Species for the study of gene expression were chosen on the basis of previous

phylogenetic findings (Schäferhoff *et al.*, 2010), including taxa from the early diverging lineages like Oleaceae and from higher core Lamiales (HCL), and also representing *CYC2*-like paralogs derived from separate duplication events.

DNA and RNA extraction - Total genomic DNA was extracted from silica-gel dried or fresh leaves from a single plant with DNeasy Plant Mini Kit following the manufacturer's protocol (QIAGEN, USA). Plant material for RNA extraction was collected from a single plant in RNAlater solution (AMBION, USA) and preserved at -20^0 C; total RNA isolation used TRI Reagent® (AMBION, USA). RNA was isolated from young inflorescences, adaxial, lateral, and abaxial petal lobes of floral buds that are one to three days before anthesis, and young leaves for each species.

Gene Isolation– CYC2-like genes were amplified from genomic DNA for most species with various sets of primers (Supporting information, Table S2) using GoTaq® Flexi DNA polymerase kits (PROMEGA, USA) with the annealing temperature Tm-5^0 C. For species in Oleaceae, paired primers CYCF2 and CYCP2R (or LCYCR) were initially used, and then more specific internal primers (CYC126F and CYC693R) were designed. *CYC2*-like genes in *Polypremum procumbens* (Tetrachondraceae) were assembled from four overlapping fragments amplified from RNA using SuperScript® III One-Step RT-PCR System with Platinum®Taq (INVITROGEN, USA). For species of Lamioideae (*Lamium* spp., *Stachys* spp. and *Pogostemon* spp.), cDNA sequences were also amplified to compare with genomic *CYC2*-like sequences to confirm the presence of an intron. All amplified PCR products were cleaned with QIAquick Gel Extraction Kit (QIAGEN, USA) and subcloned to *pGEM*®-T *Vector* (Promega, USA). To obtain all possible paralogs, at least two sets of paired primers were used for the amplification, and at least 12 colonies were screened by PCR for each reaction, and plasmids extracted using 5 PRIME* PerfectPrep* Plasmid 96 VAC Direct Bind Kit (Fisher Scientific, USA). Clones were then sequenced in both directions with BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) and analyzed on an ABI3730 DNA sequencer (Applied Biosystems, USA) at the University of Missouri-St. Louis and/or the Nucleic Acid Facility at Pennsylvania State University.

Motif-based sequence analysis –The unaligned matrix was submitted to Motif-based sequence analysis website (MEME, Bailey & Elkan, 1994, http://meme.nbcr.net/meme/) to search for additional conserved motifs of *CYC2*-like sequences across the order Lamiales.

Phylogenetic analysis – Sequencing trace files were trimmed of vector and assembled from both sequencing directions using Geneious Pro 6.0.5 (BioMatters, New Zealand). Preliminary phylogenetic analyses were conducted with the neighbor-joining algorithm in MEGA 5.10 (Tamura *et al.*, 2011) for all clones. Clones of the same accession that formed a monophyletic clade and shared at least 99.5% identical bases were considered as representing the same genomic sequence and the clone with the shortest branch length was chosen for subsequent analyses. Reduced sequences were translated, aligned with MAFFT Version 7 (Katoh & Standley, 2013) in Seaview (Gouy *et al.*, 2010), and then converted back to nucleotides for later manual refinement with MEGA 5.10 (Tamura *et al.*, 2011). 88 models of molecular evolution were assessed with jModeltest (Darriba *et al.*, 2012) to find the best fit model for phylogenetic inference.

To test whether our new *CYC2*-like sequences fall into the broad CYC2 clade, we created an ECE-CYC dataset that included *CYC* sequences of representatives from major clades of core-eudicots (fabids, malvids, lamiids and campanulids; The Angiosperm Phylogeny Group, 2009). Only the TCP and R domains could be aligned. The final alignment contained 105 sequences and 234 sites. The early diverging eudicot species *Aquilegia coerulea* (Ranunculaceae) was used as outgroup based on the previous study (Howarth & Donoghue, 2006). The GTR+I+G model (-ln*L* = 9876.4542) was selected based on the Akaike Information Criterion (AIC).

A second dataset included only ECE-*CYC2* orthologs in Lamiales. *Helianthus annuus* (Asteraceae, campanulids), *Petunia* x *hybrida* and *Solanum lycopersicum* (both Solanaceae, lamiids) were included as outgroups, and only the conserved sequences of TCP domain to R domain from these species could be aligned with *CYC2*-like sequences from Lamiales for phylogenetic analyses. The aligned matrix consisted of 298 sequences and 1344 sites; ambiguous regions were removed for phylogenetic inference. The GTR+I+G model (-ln*L* = 55756.4228) was chosen based on AIC.

Maximum likelihood analyses were conducted using PhyML (Guindon *et al.*, 2010) http://www.atgc-montpellier.fr/phyml/) and RAxML in CIPRES (Ludwig *et al.*, 2002) with 100 bootstrap replicates. The matrices were partitioned by codon position, and Bayesian inference was performed using MrBayes 3.2.1 (Ronquist *et al.*, 2012) in CIPRES, with the nucleotide substitution model GTR+I+G, 10,000,000 generations and sampling every 1,000 generations. The first 25% of the trees (2,500) were discarded as burn-in. A majority-rule consensus of the remaining trees was produced to assess Bayesian posterior probabilities (PP). Bayesian analysis with enforced monophyly of HCL was also conducted using MrBayes 3.2.1 (Ronquist et al. 2012) in CIPRES with other settings the same as in unconstrained analyses. Trace v1.5 (Rambaut & Drummond, 2009) was used to summarize parameter estimates for a Bayes factor comparison (Kass & Raftery, 1995).

A third dataset was constructed removing sequences that had more than 50% missing data in the TCP domain, and some sequences from densely-sampled clades (Bignoniaceae, Lamiaceae and Acanthaceae). This dataset included 195 Lamiales sequences and was analyzed with RAxML analysis with 100 bootstrap replicates. A fourth reduced dataset included only the 154 Lamiales sequences without any missing data in the TCP domain; this data set was analyzed with RAxML with 100 bootstrap replicates, and was later used for tests of selection.

Molecular dating – To estimate and compare the relative divergence times of major independent duplications, we applied a relaxed-clock Bayesian Markov chain Monte Carlo method as implemented in BEAST v1.7.4 (Drummond *et al.*, 2012) with a Yule tree prior, GTR+I+G substitution model parameters, and both an uncorrelated relaxed lognormal clock (ucld) and an uncorrelated relaxed exponential clock (uced). Fossil calibration points from Oleaceae and Bignoniaceae were applied. The prior age of the *Fraxinus* clade that includes *Olea*, *Osmanthus*, *Syringa, Philyrea*, *Noronhia and Chionanthus* (Oleaceae) was set using a lognormal distribution with mean = 1.5, standard deviation = 0.5, and offset = 37 Myr (i.e., estimated age for *Fraxinus* fossils) (Manchester, 1999), thus setting a hard lower bound to the age of the group of 37 Mya. Likewise, the age of *Catalpa*/*Oroxylum* from Bignoniaceae was set as 28.4 Mya based on fossils of *Catalpa* (Bignoniaceae) (Manchester, 1999). Two additional calibration points were used

for the nodes of Verbenaceae I/Bignoniaceae I and Verbenaceae II/Bignoniaceae II whose prior ages were set using an exponential distribution with mean = 1.0, offset = 49.4 Mya (Nie *et al.*, 2006). The MCMC chain was run for 10,000,000, 20,000,000, 50,000,000 and 100,000,000 generations to make sure that effective sample sizes (ESS) were higher than 200, with tree and parameter values being saved every 1,000th generation. The marginal likelihoods of 2 different clock models, ucld and uced, were compared using a Bayes factor test for best fit (Drummond *et al.*, 2006).

TreeAnnotator was used to summarize the information and calculate the maximum clade credibility phylogenetic tree with the removal of an appropriate burn-in (the first 25% of the samples) after visual inspection in Tracer v1.5 (Rambaut & Drummond, 2009).

Tests for selection - The ratio of nonsynonymous to synonymous nucleotide substitutions (ω = dN/dS) was used to measure selection on protein-coding sequences. If ω is more than 1.0, the sequences of interest are under positive selection, whereas they are under negative selection if ω is less than 1.0 and neutral selection if ω is equal 1.0 (Yang, 2007 and references therein).

To detect shifts in selection following gene duplication, we used branch-based models of selection in which ω varies among different branches in the phylogeny (Yang & Nielsen, 1998; Yang, 1998). We tested four nested hypotheses of various ω values across the phylogeny. The analyses were implemented with CODEML in the program PAML 4.7 (Yang, 2007). We also conducted branch-site random effects likelihood (REL) implemented in HyPHY to infer relaxed positive selection along all branches (Kosakovsky *et al.*, 2005, 2011; Kosakovsky & Frost, 2005b). To identify selection on specific sites, we employed site-models using CODEML in PAML 4.7 (Yang, 2007) and three different likelihood-based methods: single likelihood ancestor counting (SLAC), fixed effects likelihood (FEL) and a fast, unconstrained Bayesian approximation (FUBAR) using the Datamonkey web server (Kosakovsky & Frost, 2005a).

Gene Expression with RT-PCR – Paralog-specific primers (Supporting information, Table S2) were designed for the amplification of *CYC2*-like paralogs using SuperScript® III One-Step RT-PCR System with Platinum®Taq (INVITROGEN, USA). The RT-PCR started with cDNA synthesis for 30 min at 60^0 C, and 30 cycles of regular PCR

amplification. Amplified products were subsequently subcloned to *pGEM*®-T *Vector* (Promega, USA) and sequenced to verify the specificity of primers.

Results

CYC2-*like sequence properties* – All sequences amplified from Oleaceae and core Lamioideae (*Stachys* and *Lamium*) were > 95% identical, so we inferred that they represented a single gene. At least two types of *CYC2*-like sequences were discovered in Tetrachondraceae (with 77.7% sequence similarity) and also in most species within core Lamiales. Most *CYC2*-like sequences contain no intron, but sequences from Lamiaceae-Lamioideae have a 90-bp intron located 3' of the R-domain. At the level of amino acid sequences, two new conserved motifs were discovered by MEME analysis for *CYC2*-like in Lamiales (Fig. 2, Motif 3 and Motif 7) besides the TCP, R, ECE and ending-box domains (Fig. 2). In addition, two new lineage-specific motifs were discovered for the Oleaceae clade (Fig. 2, Motif 5 and Motif 8), and three for core Lamiales members (Fig. 2, Motif 6, Motif 9 and Motif 10). Within the conserved TCP domain, two lineage-specific amino acid replacements were observed in helix 2 (position 50, K-N, in Oleaceae; and position 56, K-E in Oleaceae), a region that is thought to be important for dimerization of TCP protein. Furthermore, all Oleaceae sequences have an amino acid replacement within the characteristic motif ECE (Fig. 2, EGE, ECK or ERK). Within the two aligned matrices, the GC content ranges from 38.80%-56.84% with an average of 45.91% in the ECE-CYC dataset and from 37.41%-55.14% with average of 43.21% in the more restricted *CYC2*-like dataset.

Phylogenetic analyses of ECE-CYC *genes* – *CYC*–like sequences fall into three separate clades (CYC1, CYC2 or CYC2-like, and CYC3) (Fig. 3). The CYC1 clade (Bayesian posterior probabilities: PP 0.74, PhyML Bootstrap support: pBT<50%; RAxML Bootstrap support: rBT<50%) is sister to a clade (PP 0.82, pBT<50%, rBT<50%) that includes the CYC2 and CYC3 clades. All *CYC2*-like sequences amplified from this study are grouped with *CYC/DICH* from *Antirrhinum majus* (Plantaginaceae) in the CYC2 (CYC2-like) clade with high Bayesian posterior probability support and moderate bootstrap support (PP 0.96, pBT 53%, rBT 67%).

Phylogenetic analyses of CYC2*-like genes* – All *CYC2*-like gene sequences from Lamiales form a monophyletic group with high support (PP 1, pBT 99%, rBT 97%) (Fig. 4). *CYC2*-like sequences of Tetrachondraceae plus Oleaceae form a clade (PP 1, pBT 99%, rBT 97%) that is sister to the core Lamiales clade (PP 1, pBT 52%, rBT 50%). However, this sister relationship of Oleaceae and Tetrachondraceae is likely to be caused by rooting with a distantly related taxon *Helianthus annuus* (asterid II/campanulid clade). It is more likely that Oleaceae and Tetrachondraceae are successive sister groups to core Lamiales as shown by multiple chloroplast loci phylogeny of Lamiales (Fig. 1; Schäferhoff *et al.*, 2010).

Two relatively divergent *CYC2*-like sequences (22.3% disparity) of *Polypremum procumbens* (Tetrachondraceae) likely represent paralogs, and form a well-supported monophyletic clade that is sister to the sequences of Oleaceae (PP 1, pBT 99%, rBT 100%). Within Tetrachondraceae and Oleaceae, all sequences of a species are more closely related to each other than to sequences of other species (e.g., *Forsythia*, *Ligustrum* and *Olea*). Within core Lamiales, four well-supported major clades are recognized while the relationships among them remain unresolved (Fig. 4; Gesneriaceae+Calceolariaceae clade PP 1, pBT 89%, rBT 90%; Plantaginaceae clade, PP 1, pBT 88%, rBT 92%; HCL clade I, PP 1 pBT 68%, rBT 76%; and HCL clade II, PP 1, pBT 72%, rBT 77%).

Independent gene duplication events are found in Calceolariaceae (Calceolariaceae I, PP 1, pBT 100%, rBT 100%; Calceolariaceae II, PP 1, pBT 100%, rBT 100%) and Gesneriaceae (Gesneriaceae I, PP 1, pBT 98%, rBT 100%; Gesneriaceae II, PP 0.94, pBT 88%, rBT 95%). In Gesneriaceae I, species in clade A (PP 1, pBT98%, rBT 98%) lack Gesneriaceae II paralogs (*GCYC2*), but an additional duplication has occurred within this clade giving rise to Gesneriaceae Ia (*GCYC1a*, PP 1, pBT 99%, rBT 100%) and Gesneriaceae Ib (*GCYC1b*, PP 1, pBT 75%, rBT 69%).

All *CYC2*-like sequences of Plantaginaceae form a monophyletic group with strong support (PP 1, pBT 88%, rBT 92%). Six well-supported subclades including Antirrhineae I (*DICH*, PP 1, pBT 91%, rBT 94%), Antirrhineae II (*CYC*, PP 1, pBT 91%, rBT 96%), *Veronica* I (PP 1, pBT 100%, rBT 100%), and *Veronica* II (PP 1, pBT 100%, rBT 100%)

are recognized, but the relationships among these clades remain unclear; one or more likely two independent duplications have occurred within the family.

One duplication of *CYC2*-like genes that gave rise to *CYC2A* (PP 1, pBT 68%, rBT 76%) and *CYC2B* (PP 1, pBT 72%, rBT 77%) may have occurred before the diversification of the HCL clade (Fig. 3). We test this hypothesis with phylogenetic constraint analysis using MrBayes by constraining the two HCL clades as sisters, however, the Bayes factor comparison shows that the constrained tree (ln*Lc* = -55163.680) is not more strongly supported by the data (GTR+I+G) than an unconstrained analysis (ln*Lu* = -55163.209) (supporting information Fig. S1). But HCL I and HCL II are supported as sister clades with 73% and 66% RAxML bootstrap support using two reduced datasets that include only Lamiales sequences and less missing data (Supporting information Fig. S2, S3).

As in the Gesneriaceae clade, *CYC2*-like genes have been lost multiple times within HCL I and HCL II, but subsequent duplications have sometimes restored copy number to two. *Thunbergia mysorensis*, *Ruellia* and *Hygrophila* (Acanthaceae), and *Pogostemon* (Lamiaceae) lack *CYC2B* paralogs in HCL II, but maintain two copies of *CYC2A* genes in HCL I, while *Lantana*, *Lippia*, *Verbena* and *Glandularia* (Verbenaceae), and *Mentha* (Lamiaceae) have no *CYC2A* paralogs in HCL I but two *CYC2B* paralogs in HCL II.

Species with actinomorphic flowers (e.g., *Buddleja*, *Callicarpa*, *Tectona*, *Avicennia*; Fig. 4, HCL I and HCL II) retain both paralogs of *CYC2*-like. Conversely, species with conspicuously zygomorphic flowers may have only one type of *CYC2*-like gene as in *Lamium* and *Stachys* (Lamiaceae) and most taxa in Bignoniaceae (with only *CYC2A*).

Molecular dating – Results from 100,000,000 generations were used for discussion as all ESS from these runs are higher than 200. Bayes factor analysis found that the uced model (ln*L* = -57059.1816) was a better fit for the data than the ucld model (ln*L* = -57082.5751, supporting information Fig. S4). Estimates for the most recent common ancestor of Gesneriaceae and Calceolariaceae ranged from 38 to 67 (mean 49.4 Mya) and 26 Mya to 56 Mya (mean 40.1 Mya), respectively (Fig. 5). Mean estimates for *CYC2A* and *CYC2B* were 68.2 Mya and 71.3 Mya, respectively. Mean estimates for the most recent common ancestor of *Polypremum* paralogs was 22.1 Mya.

Tests for selection – Branch-based models of selection using PAML found relaxed positive selection in selection on *CYC2A* immediately following the duplication after the divergence of Plantaginaceae, Gesneriaceae + Calceolariaceae, and HCL (Table 1, H_0 v H_1; Fig. 4, *CYC2A*). HyPHY found three additional branches in Plantaginaceae and the HCL clade (Fig. 4: EPS1-EPS6, and sequences in red) that exhibit significant relaxed positive selection following more recent additional gene duplications. The TCP domain was found to be under strong purifying selection by all methods used. One site in the C-terminus of *CYC2*-like genes is under positive selection.

Expression of CYC2-*like genes with RT-PCR*– Results of gene expression for five species are shown in Fig. 6. In *Syringa vulgaris* (Oleaceae), *CYC2*-like is not expressed in petal lobes. In core Lamiales, one *CYC2*-like paralog is expressed in adaxial and lateral petal lobes while the other paralogs are widely expressed in all petals. This asymmetrical expression of *CYC2*-like in adaxial and lateral petals in core Lamiales is correlated with the floral transition from actinomorphy in early diverging Lamiales to zygomorphy in core Lamiales.

Two species, *Mimulus ringens* (Phrymaceae) and *Schaueria calicotricha* (Acanthaceae), whose two paralogs were derived from a common major duplication event, show conserved orthologous expression patterns. The paralog under relaxed selection (*CYC2A*) (Table 1) is highly conserved and expressed only in adaxial and lateral petal lobes across the higher core Lamiales (Fig. 6, *CYC2A*) while the other paralog is widely expressed in all parts of petals including abaxial petal lobes, though the expression level in abaxial petal lobes varies among different species (Fig. 6, *CYC2B*, *Mimulus* versus *Schaueria*).

Ruellia tweediana (Ruellieae, Acanthaceae) also has two copies of *CYC2*-like genes (Fig. 4), but these both fall into the *CYC2A* clade, which suggests that they have resulted from a separate nested duplication. The expression pattern of these two paralogs in *Ruellia* (Fig. 6, *CYC2A1* and *CYC2A2*) is similar to those in *Schaueria calicotricha* (Fig. 6, *CYC2A* and *CYC2B*; Acanthaceae) but there the paralogs resulted from a much more ancient duplication prior to the diversification of HCL (Fig. 5).

Discussion

Multiple parallel duplications of CYC2*-like genes are not correlated with the origin of floral zygomorphy* - Gene duplication has long been postulated as an important process in the generation of evolutionary novelty (e.g. Ohno, 1970; Force *et al.*, 1999). Phylogenetic investigations in distantly related lineages of eudicots have shown that *CYC2*-like genes have experienced multiple independent duplications, hinting that the duplication of *CYC2*-like is required in the origin of floral zygomorphy (Citerne *et al.*, 2000, 2003; Hileman & Baum, 2003; Zhang *et al.*, 2010; Tähtiharju *et al.*, 2012).

Our phylogenetic and molecular dating analyses of *CYC2*-like genes across Lamiales show that *CYC2*-like genes have duplicated extensively around K-Pg boundary and have been retained repeatedly in lineages with predominantly zygomorphic flowers (Fig. 4, Calceolariaceae, Gesneriaceae, Plantaginaceae and HCL, Fig. 5). The multiple ancient parallel duplications of *CYC2*-like genes in core Lamiales suggest that two copies of *CYC2*-like genes are probably important in the origin of floral zygomorphy in these lineages. Independent additional gene duplications after gene losses in Gesneriaceae, Acanthaceae (*Ruellia*, *Hygrophila*) and Lamiaceae (*Pogostemon*) have restored the two copies of *CYC2*-like genes.

In contrast, actinomorphy does not correlate with number of copies of *CYC2*-like genes, but may instead be correlated with changes in both the regulatory and the amino acid coding regions. Only one *CYC2*-like gene is found in Oleaceae (Fig. 4) and the flowers are predominantly actinomorphic. However, a recent duplication of *CYC2*-like gene is found in *Polypremum procumbens* (Tetrachondraceae), a lineage with actinomorphic flowers that is sister to core Lamiales. Hence, a duplication of *CYC2*-like genes is necessary but not sufficient for the origin of floral zygomorphy. Rather, analyses of the expression of *CYC2*-like genes in Gesneriaceae showed that the acquisition of TCP recognition sites in the regulatory regions is critical to maintain the persistent expression of *CYC2*-like genes in the adaxial and lateral floral parts during later developmental changes thus to establish the bilateral symmetry (Yang *et al.*, 2012). In addition, our motif analyses showed that the pattern of domains suggests that the function of the CYC2-like proteins in Oleaceae and Tetrachondraceae is probably quite different from that of the proteins in core Lamiales (Fig. 2). Therefore, changes in both *cis*-regulatory

domains in *trans* as well are likely to be crucial for the evolution of bilateral symmetry of the corollas.

Retention of CYC2-*like duplicates* – *CYC2*-like paralogs are widely retained following parallel duplications and show differential expression patterns and probably functional divergence (Fig. 1, 4, 6; e.g. Luo *et al.*, 1996, 1999; Yang *et al.*, 2012). Relaxed positive selection may have helped preserve *CYC2*-like duplicates following recurrent duplications (Fig. 4: EPS4 to EPS6; *CYC2A* in HCL, *CCYC1* in Calceolariaceae; Table 1). In HCL, the paralogs that are under positive selection show conserved expression patterns only in the adaxial and lateral petals (*CYC2A* in *Mimulus*, *Schaueria* in HCL; *CYC2A1* in *Ruellia*; Fig. 4, 6). No positive selection is found following the duplications in Gesneriaceae or Plantaginaceae. This agrees with a previous study in snapdragon and its relatives that showed their disparity in the degree of purifying selection on two paralogs may have helped retain the duplicated *CYC* (Hileman and Baum 2003), which then suggests that the preservation of *CYC2*-like duplicates may actually involve multiple selection schemes. Further analyses are needed due to current poorly resolved phylogenetic relationships in Gesneriaceae and Plantaginaceae, common gene losses and subsequent independent duplications, and incomplete sampling in these two families (Fig. 4). Additionally, the sites under positive selection in different lineages (different duplications) may vary, making it difficult, if not impossible, to detect positive selection with global searches.

Losses of CYC2-*like gene are common but have varied effects in shifts in floral symmetry in the HCL clade* - Duplication and retention of *CYC2*-like genes may be important for the origin of floral zygomorphy, but retention of *CYC2*-like paralogs is not necessary for the maintenance of floral zygomorphy, i.e. species having two copies of *CYC2*-like may have actinomorphic flowers (e.g. *Buddleja*, *Callicarpa*, *Tectona*, *Avicennia*), while species having only one copy of *CYC2*-like may either have strongly zygomorphic flowers (e.g. *CYC2A*, *Stachys*, *Lamium*, Bignoniaceae), or weakly actinomorphic flowers (e.g. *CYC2B*, *Mentha*, *Lantana*, *Lippia*, *Verbena*).

Functional analyses in snapdragon and Gesneriaceae show that duplicates of *CYC2*-like are only partially redundant (Luo *et al.*, 1996, 1999; Yang *et al.*, 2012). The flowers of

cyc mutants are weakly actinomorphic while flowers of *dich* mutants are conspicuously zygomorphic (Luo *et al.*, 1996, 1999). The varied pattern of loss of one specific *CYC2*-like paralog in HCL suggests functional divergence of *CYC2*-like paralogs following gene duplication. This hypothesis is further supported by differential expression of *CYC2*-like paralogs, with *CYC2A* being expressed asymmetrically in adaxial and lateral petals while *CYC2B* is expressed in all petals. Indeed, floral zygomorphy could be maintained by only one copy of *CYC2*-like genes as in *dich* mutants through a single positive autoregulatory feedback loop by forming homodimers that positively promote and maintain the expression of *CYC2*-like genes in later developmental stages (Kosugi & Ohashi, 2002; Yang *et al.*, 2012).

Conservation and diversification of CYC2*-like gene expression* **-** The shift from floral actinomorphy to zygomorphy is not correlated with increased copies of *CYC2*-like genes in core Lamiales, but has involved different expression of *CYC2*-like paralogs between early diverging lineages and core Lamiales (Fig. 6). *CYC2*-like genes in Oleaceae are transcribed both in vegetative and reproductive tissues, but not in petal lobes (Fig. 6, *Syringa vulgaris*). Lack of expression of *CYC2*-like genes in later developmental stages in Oleaceae species may represent the ancestral pattern of *CYC2*-like genes in Lamiales, and accounts for floral actinomorphy in the early diverging Lamiales (e.g. Oleaceae). Our RT-PCR data show that expression of *CYC2*-like paralog (*CYC2A*) within HCL across the adaxial and lateral corolla lobes also occurs in *Primulina heterotricha* (*PhCYC1C*, *PhCYC1D*, Gesneriaceae) and snapdragon (*CYC* and *DICH*) despite their different duplication origins, hinting at their conserved roles in determining the adaxial and lateral petals and relatively stronger constraint on these paralogs following parallel gene duplications. This conserved asymmetrical expression pattern of *CYC2*-like paralogs correlates with the development and origin of floral zygomorphy and may have played a critical role in the development of floral zygomorphy in core Lamiales (Fig. 1, 6).

Expression of other *CYC2*-like genes varies with their separate duplication origins in different lineages in the core Lamiales (Luo *et al.*, 1996, 1999; Gao *et al.*, 2008; Preston & Hileman, 2009). *Primulina heterotricha* (Gesneriaceae) has two major types and four copies of ECE-*CYC2* genes (*PhCYC1C*, *PhCYC1D*, *PhCYC2A* and *PhCYC2B*), none of which have transcripts in abaxial petals. *PhCYC1C*, *PhCYC1D* are expressed in adaxial

and partly in lateral petals while in *PhCYC2A* and *PhCYC2B* no paralogs are transcribed (Gao *et al.*, 2008). In *Antirrhinum majus* (Plantaginaceae), both copies of ECE-*CYC2* (*CYC/DICH*) are expressed only in adaxial petals in lateral, not in abaxial petals, and the expression of *DICH* is more confined to adaxial petals (Luo et al. 1996 1999). The expanded expression of *CYC2B* in the abaxial corolla lobes is novel in HCL. The varied expression patterns of *CYC2*-like genes in Gesneriaceae, Plantaginaceae and HCL clade indicates diversification of *CYC2*-like gene expression (e.g. Luo *et al.*, 1996, 1999; Zhou *et al.*, 2008; Preston & Hileman, 2009; Song *et al.*, 2009). Also this varied expression pattern may suggest that *DICH* in Antirrhineae, *CYC2B* in HCL, and *GCYC2* in Gesneriaceae have experienced more relaxed selective pressure (e.g. Hileman & Baum, 2003).

For each pair of duplicate genes, one copy is always expressed only in adaxial and lateral petals. However, the expression pattern of the other copy varies from one duplicate pair to the other. Even after a duplication, the copy that is variably involved in floral development other than floral symmetry has different expression pattern from one species to another. For example, expression of *DICH*, *CYC2B*, *GCYC2* and *GCYC1C* is involved in the development of other parts of the flower but not floral symmetry (Hileman *et al.*, 2003; Song *et al.*, 2009). The situation is even more complex in *Primulina* and *Opithandra* in Gesneriaceae. Neither *PhCYC2A* nor *PhCYC2B* is expressed in petals in either species. However, *PhCYC1C* and *PhCYC1D* are expressed in adaxial and/or lateral petals in *Primulina* (Gao *et al.*, 2008; Yang *et al.*, 2012), but in all petals in *Opithandra* (Song *et al.*, 2009). In snapdragon and its relatives, *CYC* is expressed primarily in adaxial and adjacent lateral floral parts (Luo *et al.*, 1996, 1999; Hileman *et al.*, 2003), but expanded expression of *DICH* in *Mohavea* (Plantaginaceae) is correlated with internal symmetry of adaxial petals and abortion of lateral stamens (Hileman *et al.*, 2003). Likewise, the expression pattern of *CYC2B* in HCL species seems to differ somewhat between *Mimulus* and *Schaueria* (Fig. 6). These varied expression patterns of *CYC2*-like paralogs (*DICH*, *CYC2B*, *GCYC2*) in different lineages may be involved in aspects of floral elaboration other than floral symmetry.

Implications for Paleogene parallel origins of floral zygomorphy in Lamiales after the diversification of core major bee clades – What appears to be a single origin of floral

zygomorphy in Lamiales may actually be underlain by at least four independent duplications of *CYC2*-like genes at different time periods (40.1-71.3Mya) (Fig. 4, 5). The evolutionary transition from floral actinomorphy to zygomorphy early in Lamiales may thus represent multiple cryptic parallel origins of floral zygomorphy.

Floral zygomorphy has long been thought to have evolved independently from actinomorphy under strong selection by specialized pollinators. Zygomorphic flowers conceal and protect stamens within the corolla and thus allow more precise pollination and/or reduce pollen wastage (Neal *et al.*, 1998; Westerkamp & Claßen-Bockhoff, 2007). Zygomorphy is specifically associated with bee pollination, particularly euglossine bees and bumble bees, either through learning or innate preference (Møller, 1995; Neal *et al.*, 1998; Rodríguez *et al.*, 2004; Westerkamp & Claßen-Bockhoff, 2007). Based on our mean age estimates of major parallel duplications of *CYC2*-like genes in core-Lamiales (40.1-71.3Mya), the origin of zygomorphy may coincide with the initial diversification of major core bee lineages, especially those that include euglossine bees and bumble bees (65-75Mya) (Cardinal & Danforth, 2013).

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Tables

Table 1. Parameter estimates under models of variable ω ratios among lineages following duplication of *CYC2*-like genes. The topology and branch-specific ω ratios are labelled in Fig. 4.

Hypothesis	ω2a	ω2b	ωcyc	ωdich	ωgcyc1	ωgcyc2	ωc1	ωc2	ωo	-ln*L*	LRT	*P*
H0: ω2a=ω2b=ωcyc=ωdich=ωgcyc1=ωgcyc2=ωc1=ωc2=ωo	0.20625	0.20625	0.20625	0.20625	0.20625	0.20625	0.20625	0.20625	0.20625	33831.046417		
H1: ω2a≠ω2b=ωcyc=ωdich=ωgcyc1=ωgcyc2=ωc1=ωc2=ωo	1.84218	0.20544	0.20544	0.20544	0.20544	0.20544	0.20544	0.20544	0.20544	33827.686253	6.720328	**0.0095****
H2: ω2b≠ω2a=ωcyc=ωdich=ωgcyc1=ωgcyc2=ωc1=ωc2=ωo	0.20599	0.51355	0.20599	0.20599	0.20599	0.20599	0.20599	0.20599	0.20599	33830.619914	0.853006	0.3557
H3: ωcyc≠ωdich=ω2a=ω2b=ωgcyc1=ωgcyc2=ωc1=ωc2=ωo	0.20614	0.20614	0.22131	0.20614	0.20614	0.20614	0.20614	0.20614	0.20614	33831.021659	0.049516	0.8239
H4: ωdich≠ωcyc=ω2a=ω2b=ωgcyc1=ωgcyc2=ωc1=ωc2=ωo	0.20590	0.20590	0.20590	0.41960	0.20590	0.20590	0.20590	0.20590	0.20590	33830.562609	0.967616	0.3253
H5: ωgcyc1≠ωgcyc2=ω2a=ω2b=ωcyc=ωdich=ωc1=ωc2=ωo	0.20631	0.20631	0.20631	0.20631	0.19805	0.20631	0.20631	0.20631	0.20631	33831.039556	0.013722	0.9068
H6: ωgcyc2≠ωgcyc1=ω2a=ω2b=ωcyc=ωdich=ωc1=ωc2=ωo	0.20598	0.20598	0.20598	0.20598	0.20598	0.23809	0.20598	0.20598	0.20598	33830.926953	0.238928	0.625
H7: ωc1≠ωc2=ω2a=ω2b=ωcyc=ωdich=ωgcyc1=ωgcyc2=ωo	0.20731	0.20731	0.20731	0.20731	0.20731	0.20731	0.10905	0.20731	0.20731	33828.909676	4.273482	**0.0387****
H8: ωc2≠ωc1=ω2a=ω2b=ωcyc=ωdich=ωgcyc1=ωgcyc2=ωo	0.20655	0.20655	0.20655	0.20655	0.20655	0.20655	0.20655	0.16934	0.20655	33830.870571	0.351692	0.5532

$\chi^2_{df=1,\ \alpha=0.05} = 3.841$

Figure captions

Fig. 1. Schematic phylogenetic tree of Lamiales. Bar indicates a major floral transition from actinomorphy to zygomorphy. Double-lines shows duplications of *CYC*-like genes within the clade. X indicates absence of expression, single plus sign (+) indicates where only one type of *CYC2*-like gene is expressed, double plus sign (++) indicates where both types of *CYC2*-like genes are expressed (Luo et al 1996 1999; Gao et al. 2008), and question mark (?) indicates no data is available. Abbreviations: HCL, higher core Lamiales; ad, adaxial petal lobes; la, lateral petal lobes; ab, abaxial petal lobes. [1] Although two copies of *CYC2*-like genes are expressed in adaxial petal lobes, these two copies all belong to Gesneriaceae I, the other two *CYC2*-like gene copies in Gesneriaceae II are not expressed (Gao et al. 2008).

Fig. 2. *CYC*-like protein (ECE-CYC2 clade) evolution across the Lamiales.

Fig. 3. Phylogram from Bayesian inference of ECE-*CYC* sequences across the eudicots. Support values (Bayesian posterior probability/PhyML bootstrap/RAxML bootstrap) are labeled above the branches. Two duplications are indicated with grey circles.

Fig. 4. Phylogram from Bayesian inference of *CYC2*-like genes of Lamiales. Support values (Bayesian posterior probability/PhyML bootstrap/RAxML bootstrap) are labeled above the branches. I and II indicated two copies of *CYC2*-like genes resulted from duplications. A refers to clade with additional duplication of *CYC2*-like in Gesneriaceae I. Species in bold in HCL I and HCL II clades have complete actinomorphic flowers. ω are the ratios of dN/dS assigned to those specific branches following *CYC2*-like gene duplications. Asterisk * indicates that *CYC2A* (HCL) and *CYC2*-like paralogs in Calceolariaceae I are under relaxed positive selection following gene duplication using branch-based model in PAML (Yang 2007). EPS1-EPS6 are branches found under relaxed selection using branch-site random effects likelihood (REL) model in HyPHY (Kosakovsky et al. 2005).

Fig. 5. Simplified chronogram of Lamiales: maximum clade credibility tree from the BEAST analysis. Posterior estimates of divergence times were inferred using a uced model, and two fossils as minimum age constraints: an *Fraxinus* (Oleaceae) fossil date of

37 Mya (calibration C1 in Oleaceae), and a *Caltapa* (Bignoniaceae) fossil date of 28.4 Mya (calibration C2 in HCLI). Two additional calibration points derived from fossil information for the nodes: Verbenaceae/Bignoniaceae (I and II, C3 and C4) divergence date of 49.4 Mya.

Fig. 6. Gene expression of *CYC2*-like paralogs using RT-PCR within higher core Lamiales and early diverging Lamiales (*Syringa vulgaris*). Abbreviations: ad, adaxial petal lobes; la, lateral petal lobes; and ab, abaxial petal lobes.

Figures

Fig. 1.

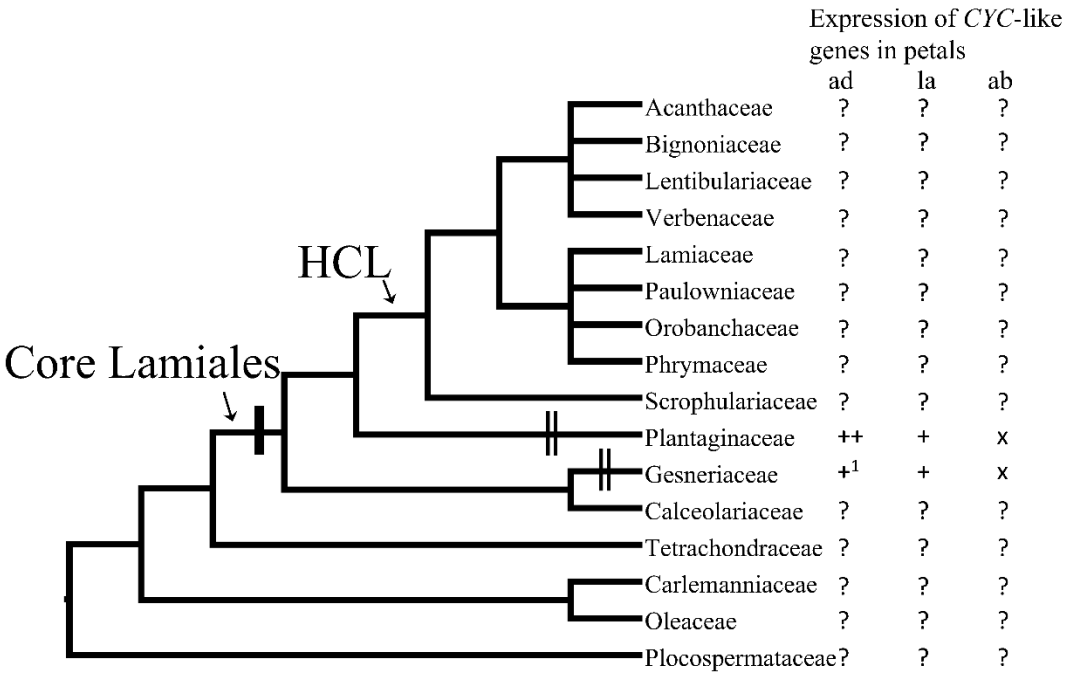


Fig. 2.

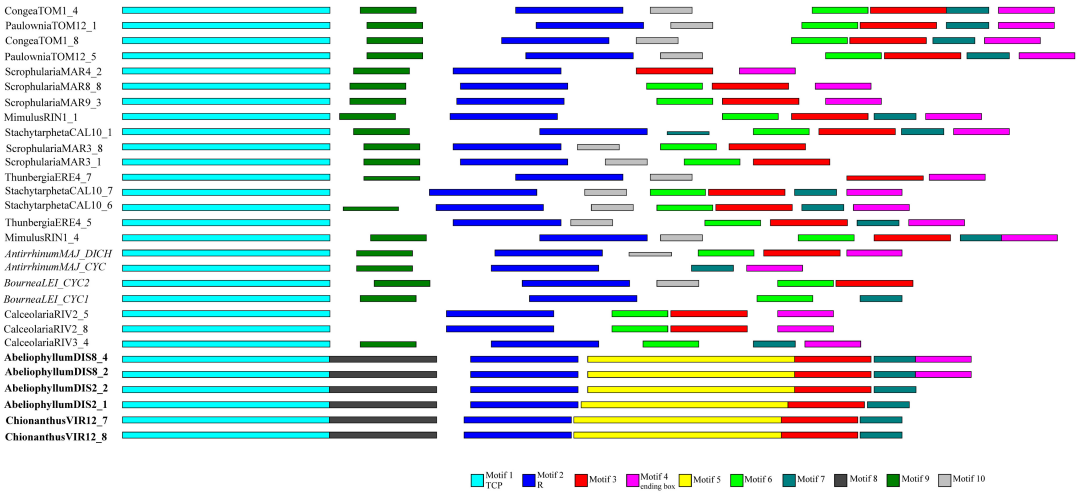
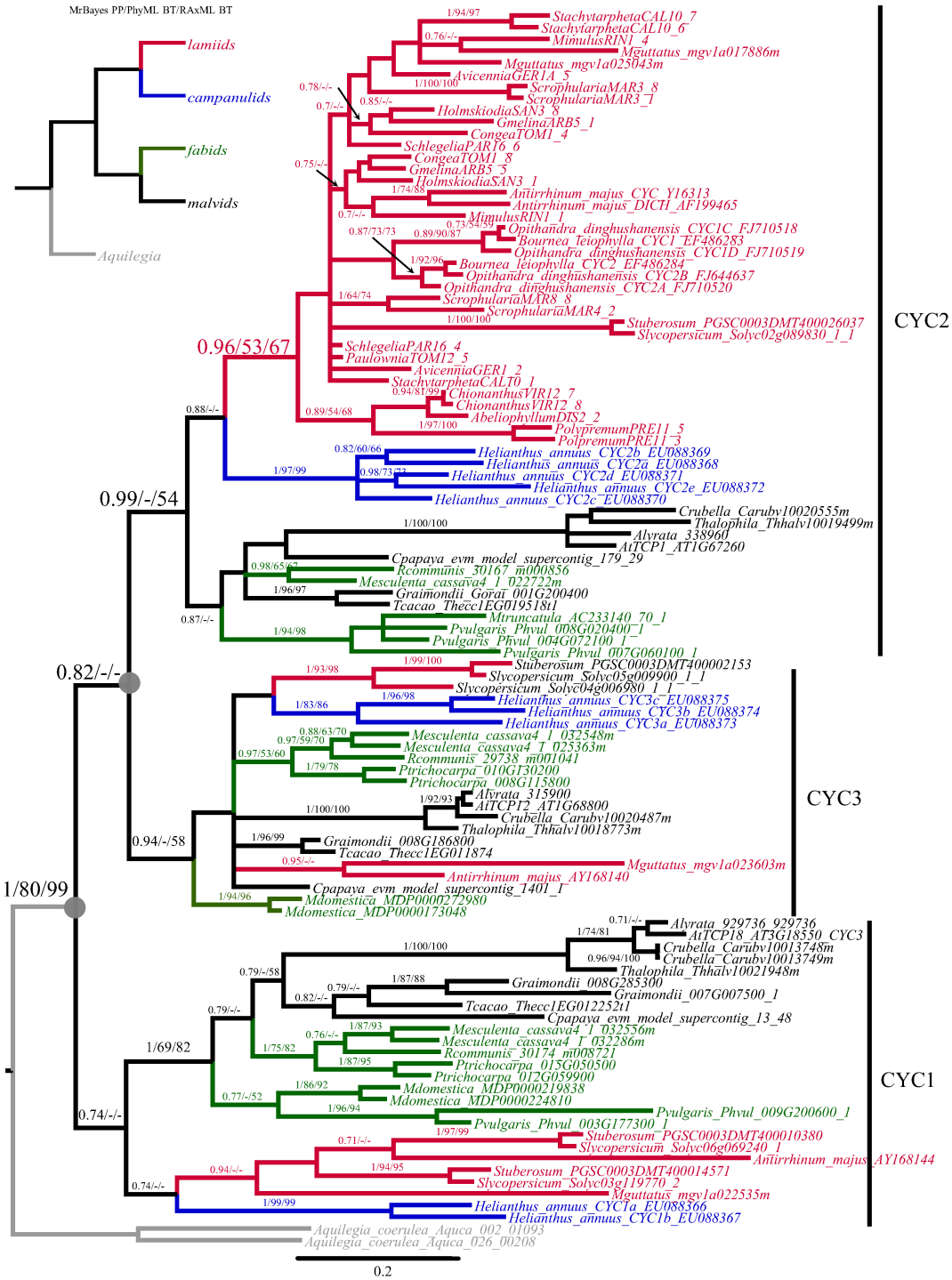

CongeaTOM1_4
PaulowniaTOM12_1
CongeaTOM1_8
PaulowniaTOM12_5
ScrophulariaMAR4_2
ScrophulariaMAR8_8
ScrophulariaMAR9_3
MimulusRIN1_1
StachytarphetaCAL10_1
ScrophulariaMAR3_8
ScrophulariaMAR3_1
ThunbergiaERE4_7
StachytarphetaCAL10_7
StachytarphetaCAL10_6
ThunbergiaERE4_5
MimulusRIN1_4
AntirrhinumMAJ_DICH
AntirrhinumMAJ_CYC
BourneaLEI_CYC2
BourneaLEI_CYC1
CalceolariaRIV2_5
CalceolariaRIV2_8
CalceolariaRIV3_4
AbeliophyllumDIS8_4
AbeliophyllumDIS8_2
AbeliophyllumDIS2_2
AbeliophyllumDIS2_1
ChionanthusVIR12_7
ChionanthusVIR12_8
Motif 1 TCP
Motif 2 R
Motif 3
Motif 4 ending box
Motif 5
Motif 6
Motif 7
Motif 8
Motif 9
Motif 10

Fig. 3.


MrBayes PP/PhyML BT/RAxML BT
lamiids
campanulids
fabids
malvids
Aquilegia
CYC2
CYC3
CYC1
0.96/53/67
0.99/-/54
0.82/-/-
1/80/99
0.94/-/58
1/69/82
0.74/-/-
StachytarphetaCAL10_7
StachytarphetaCAL10_6
MimulusRIN1_4
Mguttatus_mgv1a017886m
Mguttatus_mgv1a025043m
AvicenniaGER1A_5
ScrophulariaMAR3_8
ScrophulariaMAR3_1
HolmskiodiaSAN3_8
GmelinaARB5_1
CongeaTOM1_4
SchlegeliaPAR16_6
CongeaTOM1_8
GmelinaARB5_5
HolmskiodiaSAN3_1
Antirrhinum_majus_CYC_Y16313
Antirrhinum_majus_DICH_AF199465
MimulusRIN1_1
Opithandra_dinghushanensis_CYC1C_FJ710518
Bournea_leiophylla_CYC1_EF486283
Opithandra_dinghushanensis_CYC1D_FJ710519
Bournea_leiophylla_CYC2_EF486284
Opithandra_dinghushanensis_CYC2B_FJ644637
Opithandra_dinghushanensis_CYC2A_FJ710520
ScrophulariaMAR8_8
ScrophulariaMAR4_2
Stuberosum_PGSC0003DMT400026037
Slycopersicum_Solyc02g089830_1_1
SchlegeliaPAR16_4
PaulowniaTOM12_5
AvicenniaGER1_2
StachytarphetaCAL10_1
ChionanthusVIR12_7
ChionanthusVIR12_8
AbeliophyllumDIS2_2
PolypremumPRE11_5
PolpremumPRE11_3
Helianthus_annuus_CYC2b_EU088369
Helianthus_annuus_CYC2a_EU088368
Helianthus_annuus_CYC2d_EU088371
Helianthus_annuus_CYC2e_EU088372
Helianthus_annuus_CYC2c_EU088370
Crubella_Carubv10020555m
Thalophila_Thhalv10019499m
Alyrata_338960
AtTCP1_AT1G67260
Cpapaya_evm_model_supercontig_179_29
Rcommunis_30167_m000856
Mesculenta_cassava4_1_022722m
Graimondii_Gorai_001G200400
Tcacao_Thecc1EG019518t1
Miruncatula_AC233140_70_1
Pvulgaris_Phvul_008G020400_1
Pvulgaris_Phvul_004G072100_1
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Stuberosum_PGSC0003DMT400002153
Slycopersicum_Solyc05g009900_1_1
Slycopersicum_Solyc04g006980_1_1
Helianthus_annuus_CYC3c_EU088375
Helianthus_annuus_CYC3b_EU088374
Helianthus_annuus_CYC3a_EU088373
Mesculenta_cassava4_1_032548m
Mesculenta_cassava4_1_025363m
Rcommunis_29738_m001041
Ptrichocarpa_010G130200
Ptrichocarpa_008G115800
Alyrata_315900
AtTCP12_AT1G68800
Crubella_Carubv10020487m
Thalophila_Thhalv10018773m
Graimondii_008G186800
Tcacao_Thecc1EG011874
Mguttatus_mgv1a023603m
Antirrhinum_majus_AY168140
Cpapaya_evm_model_supercontig_1401_1
Mdomestica_MDP0000272980
Mdomestica_MDP0000173048
Alyrata_929736_929736
AtTCP18_AT3G18550_CYC3
Crubella_Carubv10013748m
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Thalophila_Thhalv10021948m
Graimondii_008G285300
Graimondii_007G007500_1
Tcacao_Thecc1EG012252t1
Cpapaya_evm_model_supercontig_13_48
Mesculenta_cassava4_1_032556m
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Ptrichocarpa_015G050500
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Mdomestica_MDP0000219838
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Slycopersicum_Solyc03g119770_2
Mguttatus_mgv1a022535m
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Helianthus_annuus_CYC1b_EU088367
Aquilegia_coerulea_Aquca_002_01093
Aquilegia_coerulea_Aquca_026_00208
0.2

Fig. 4a.

Fig. 4a MrBayes PP/PhyML BT/RAxML BT

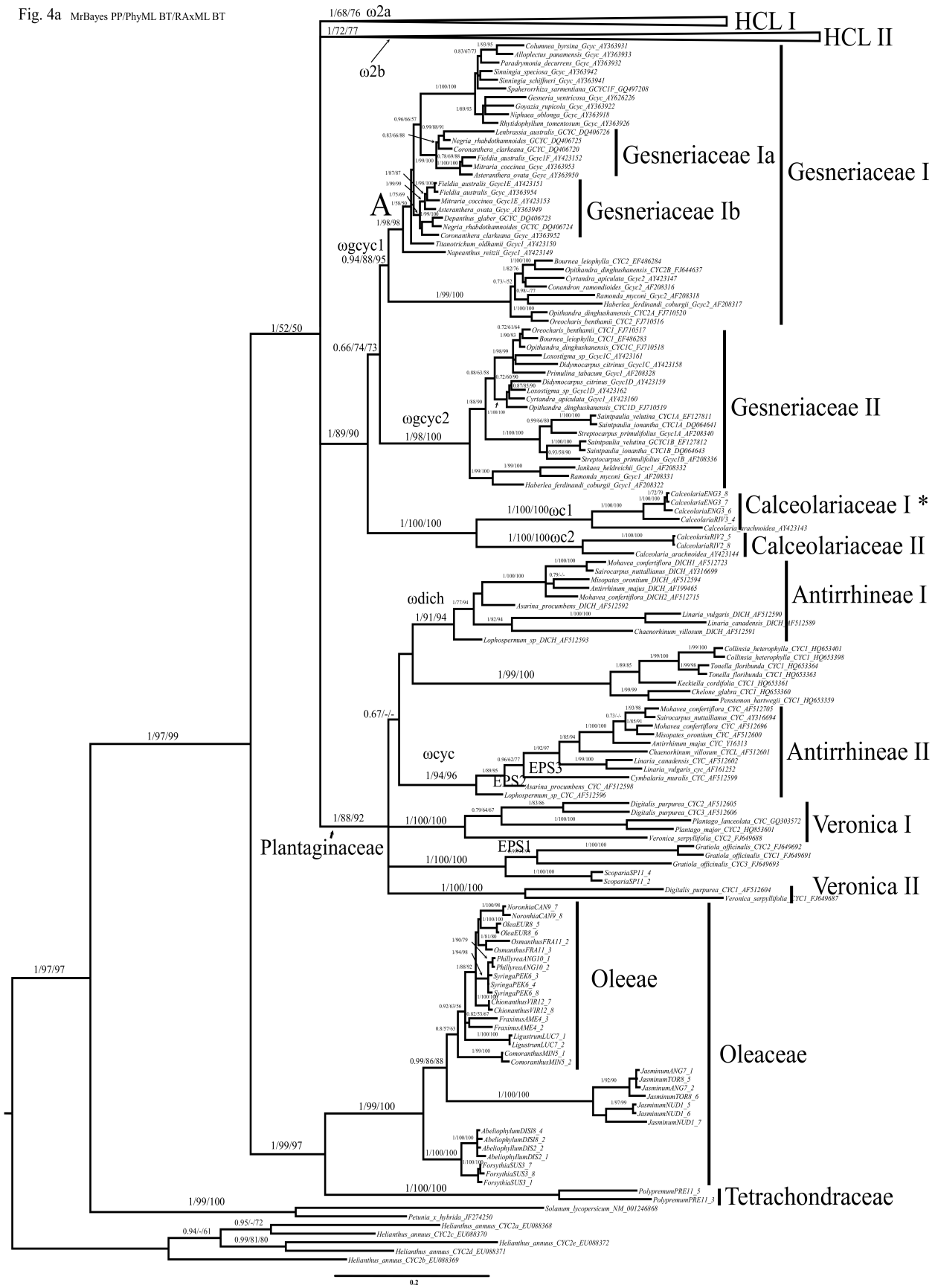


Fig. 4b.

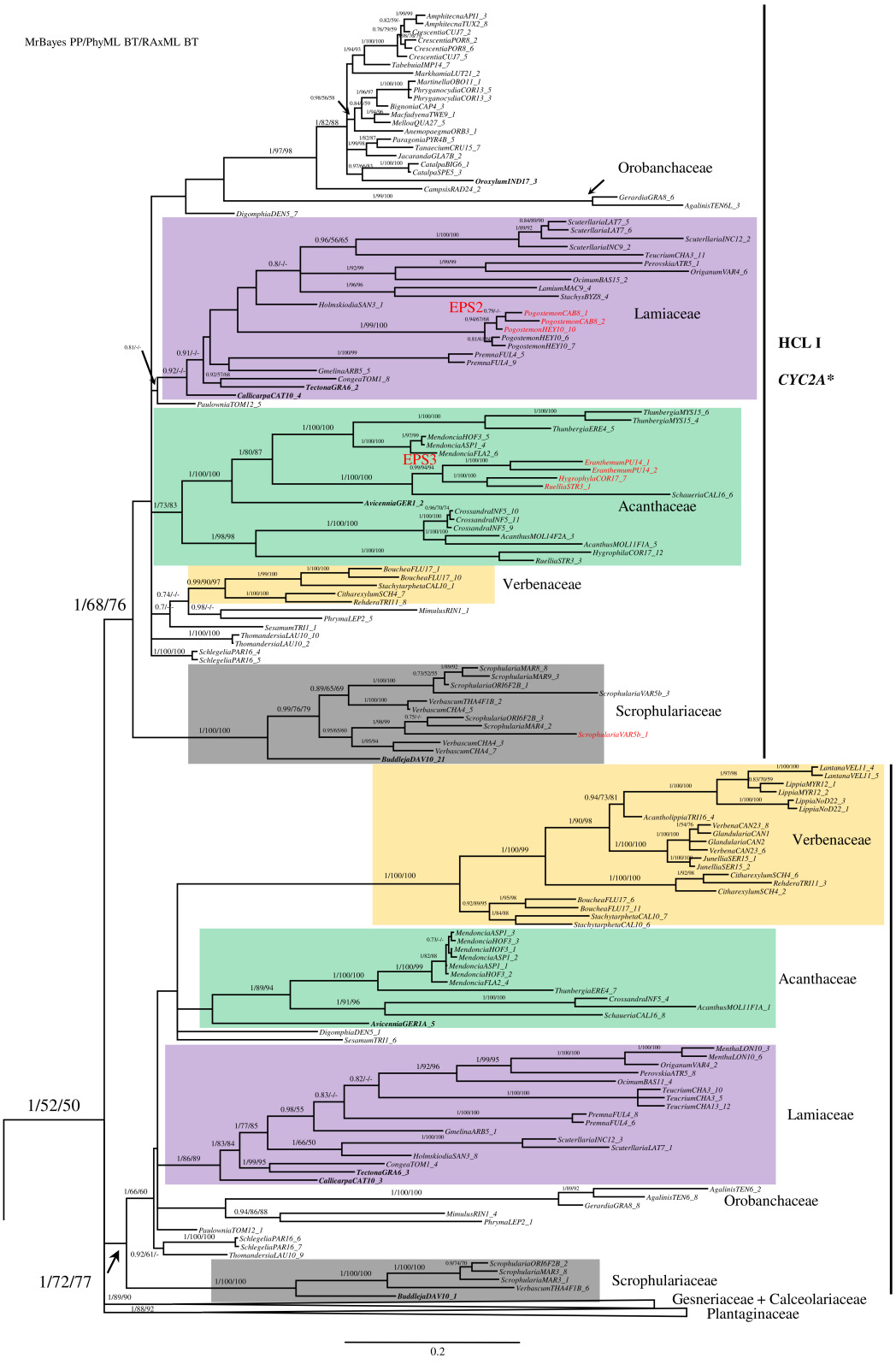

MrBayes PP/PhyML BT/RAxML BT
Orobanchaceae
Lamiaceae
EPS2
HCL I
CYC2A*
EPS3
Acanthaceae
Verbenaceae
1/68/76
Scrophulariaceae
Verbenaceae
Acanthaceae
HCL II
CYC2B
1/52/50
Lamiaceae
Orobanchaceae
1/72/77
Scrophulariaceae
Gesneriaceae + Calceolariaceae
Plantaginaceae
0.2

Fig. 5.

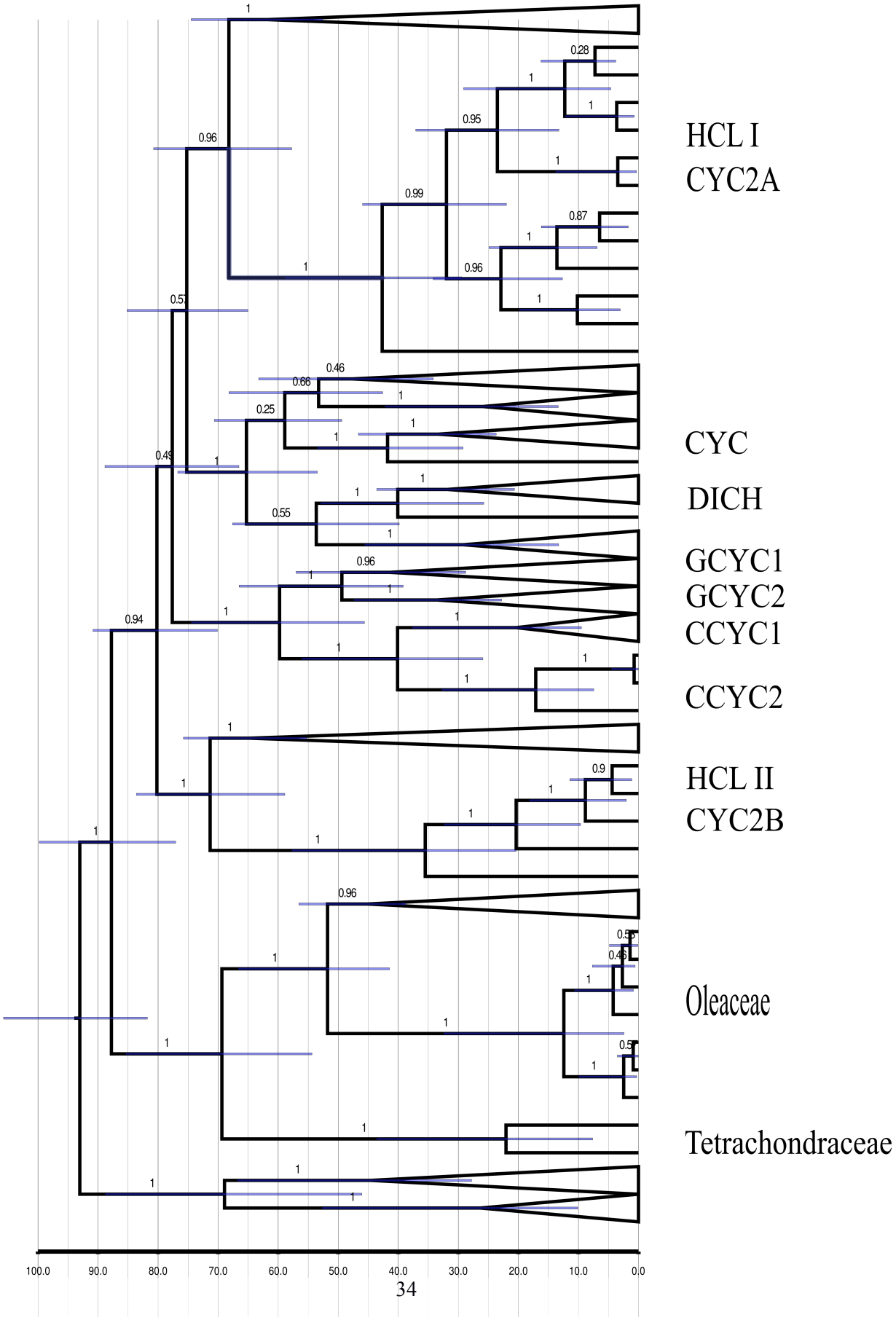
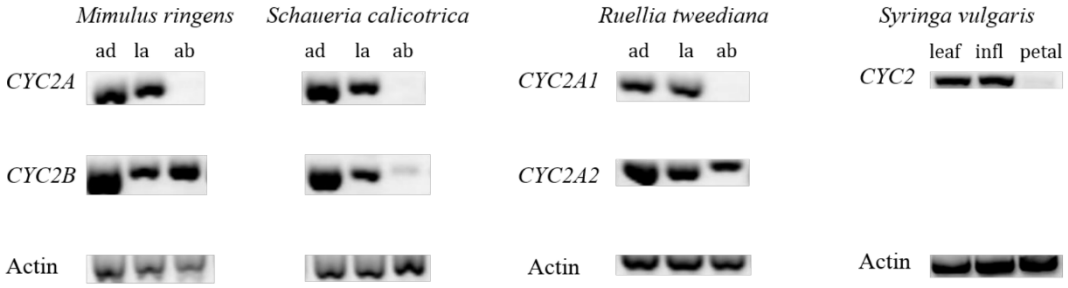

HCL I
CYC2A
CYC
DICH
GCYC1
GCYC2
CCYC1
CCYC2
HCL II
CYC2B
Oleaceae
Tetrachondraceae
100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0

Fig. 6.


Mimulus ringens
ad la ab
CYC2A
CYC2B
Actin
Schaueria calicotrica
ad la ab
Ruellia tweediana
ad la ab
CYC2A1
CYC2A2
Actin
Syringa vulgaris
leaf infl petal
CYC2
Actin

Supporting information

Table S1. Sampled taxa and GenBank accession number;

VOUCHER	TAXON	SOURCE	FAMILY
G. Yatskievych 11-73	*Polypremum procumbens* L.	United States	Tetrachondraceae
CHW SN9	*Abeliophyllum distichum* Nakai	Cult. MBG, United States	Oleaceae
CHW SN2	*Chionanthus virginicus* L.	Cult. MBG, United States	Oleaceae
CIR 1757	*Comoranthus minor* H. Perrier	Madagarscar	Oleaceae
CHW SN11	*Forsythia suspensa* (Thunb.) Vahl	Cult. MBG, United States	Oleaceae
CHW SN6	*Fraxinus americana* L.	Cult. MBG, United States	Oleaceae
2011020	*Jasminum angustifolium* Willd.	Cult. MBG, United States	Oleaceae
CHW SN7	*Jasminum nudiflorum* Lindl.	Cult. MBG, United States	Oleaceae
2011021	*Jasminum tortuosum* Willd.	Cult. MBG, United States	Oleaceae
CHW SN8	*Ligustrum lucidum* W.T. Aiton	Cult. MBG, United States	Oleaceae
CHW 699	*Noronhia candicans* H. Perrier	Cult. MBG, United States	Oleaceae
CHW SN1	*Olea europaea* L.	Cult. MBG, United States	Oleaceae
CHW SN3	*Osmanthus fragrans* (Thunb.) Lour.	Cult. MBG, United States	Oleaceae
CHW SN5	*Phillyrea angustifolia* L.	Cult. MBG, United States	Oleaceae
CHW SN4	*Syringa pekinensis* Rupr.	Cult. MBG, United States	Oleaceae
	Syringa vulgaris L.	Cult. MBG, United States	Oleaceae
2011015	*Ruellia tweediana* Griseb.	MBG	Acanthaceae
2010058	*Ruellia strepens* L.	Shaw Nature Reserve, United States	Acanthaceae
2010066	*Eranthemum pulchellum* Andrews	Cult. MBG, United States	Acanthaceae
2010070	*Schaueria calicotricha* (Link & Otto) Nees	Cult. MBG, United States	Acanthaceae
2010071	*Hygrophila corymbosa* (Blume) Lindau	Cult. MBG, United States	Acanthaceae
2011006	*Acanthus mollis* L.	Cult. MBG, United States	Acanthaceae
2011019	*Crossandra infundibuliformis* (L.) Nees	Cult. MBG, United States	Acanthaceae

2012004	*Thunbergia erecta* (Benth.) T. Anderson	Cult. MBG, United States	Acanthaceae
2012035	*Thunbergia mysorensis* (Wight) T. Anderson	Cult. MBG, United States	Acanthaceae
T.P.Prinzie 131	*Avicennia germinans* (L.) L.	United States	Acanthaceae
A.F. Fuentes - 5307	*Mendoncia aspera* Ruiz & Pav.	Bolivia	Acanthaceae
RAZANATSIMA - 297	*Mendoncia flagellaris* (Baker) Benoist	Madagascar	Acanthaceae
James S. Miller - 9268	*Mendoncia hoffmannseggiana* Nees	Suriname	Acanthaceae
2010051	*Campsis radicans* (L.) Bureau	Shaw Nature Reserve, United States	Bignoniaceae
2012020	*Amphitecna apiculata* A.H. Gentry	Cult. MBG, United States	Bignoniaceae
2012021	*Amphitecna tuxtlensis* A.H. Gentry	Cult. MBG, United States	Bignoniaceae
2012022	*Anemopaegma orbiculatum* (Jacq.) A. DC.	Cult. MBG, United States	Bignoniaceae
2012023	*Bignonia capreolata* L.	Cult. MBG, United States	Bignoniaceae
2012024	*Catalpa speciosa* Warder ex Engelm.	Cult. MBG, United States	Bignoniaceae
2012025	*Catalpa bignonioides* Walter	Cult. MBG, United States	Bignoniaceae
2012026	*Crescentia cujete* L.	Cult. MBG, United States	Bignoniaceae
2012027	*Crescentia portoricensis* Britton	Cult. MBG, United States	Bignoniaceae
2012028	*Macfadyena tweediana* Griseb. ex Lorentz	Cult. MBG, United States	Bignoniaceae
2012029	*Markhamia lutea* (Benth.) K. Schum.	Cult. MBG, United States	Bignoniaceae
2012030	*Martinella obovata* (Kunth) Bureau & K. Schum.	Cult. MBG, United States	Bignoniaceae
2012031	*Melloa quadrivalvis* (Jacq.) A.H. Gentry	Cult. MBG, United States	Bignoniaceae
2012032	*Phryganocydia corymbosa* (Vent.) Bureau ex K. Schum.	Cult. MBG, United States	Bignoniaceae
2012033	*Tabebuia impetiginosa* (Mart. ex DC.) Standl.	Cult. MBG, United States	Bignoniaceae
2012034	*Tanaecium crucigerum* Seem.	Cult. MBG, United States	Bignoniaceae
2012036	*Jacaranda cuspidifolia* Mart. ex A. DC.	Cult. MBG, United States	Bignoniaceae
2012037	*Oroxylum indicum* (L.) Kurz	Cult. MBG, United States	Bignoniaceae
W.D. Stevens - 28076	*Paragonia pyramidata (Rich.) Bureau*	Nicaragua	Bignoniaceae
A.F. Fuentes - 12782	*Calceolaria engleriana* Kraenzl.	Bolivia	Calceolariaceae
A.F. Fuentes - 12761	*Calceolaria rivularis* Kraenzl.	Bolivia	Calceolariaceae
2009001	*Teucrium chamaedrys* L.	Cult. MBG, United States	Lamiaceae

2009004	*Stachys byzantina* C. Koch	Cult. MBG, United States	Lamiaceae
2009005	*Mentha longifolia* (L.) L.	Cult. MBG, United States	Lamiaceae
2009006	*Scutellaria incana* Vent.	Cult. MBG, United States	Lamiaceae
2009007	*Scutellaria lateriflora* L.	Cult. MBG, United States	Lamiaceae
2009014	*Lamium maculatum* L.	Cult. MBG, United States	Lamiaceae
2009008	*Ocimum basilicum* L.	Cult. MBG, United States	Lamiaceae
2009019	*Origanum vulgare* L.	Cult. MBG, United States	Lamiaceae
2009021	*Holmskioldia sanguinea* Retz	Cult. MBG, United States	Lamiaceae
2010005	*Premna fulva* Craib	China	Lamiaceae
2009003	*Perovskia atrplicifolia* Benth.	Cult. MBG, United States	Lamiaceae
2009023	*Pogostemon heyneanus* Benth.	Cult. MBG, United States	Lamiaceae
2010002	*Congea tomentosa* Roxb.	China	Lamiaceae
2010006	*Gmelina arborea* Roxb.	China	Lamiaceae
2010007	*Tectona grandis* fo. Abludens Koord. & Valeton	China	Lamiaceae
2010054	*Callicarpa cathayana* H.T. Chang	Cult. MBG, United States	Lamiaceae
ZW19	*Pogostemon cablin* (Blanco) Benth.	China	Lamiaceae
2010042	*Agalinis tenuifolia* (Vahl) Raf.	Shaw Nature Reserve, United States	Orobanchaceae
2010026	*Paulownia tomentosa* (Thunb.) Steud.	Cult. MBG, United States	Paulowniaceae
Neil W. Snow - 6857	*Sesamum triphyllum* Welw. ex Asch.	Botswana	Pedaliaceae
2010041	*Phryma leptostachya* L.	Shaw Nature Reserve, United States	Phrymaceae
2010043	*Mimulus ringens* L.	Shaw Nature Reserve, United States	Phrymaceae
2010062	*Scoparia sp*	Cult. MBG, United States	Plantaginaceae
2012038	*Schlegelia parviflora* (Oerst.) Monach.	Cult. MBG, United States	Schlegeliaceae
2010076	*Buddleja davidii* Franch.	Cult. MBG, United States	Scrophulariaceae
Mary Merello - 2377	*Scrophularia orientalis* L.	Republic of Georgia	Scrophulariaceae
Mary Merello - 2244	*Scrophularia variegata* M. Bieb.	Republic of Georgia	Scrophulariaceae
Zhong - 2011013	*Scrophularia marilandica* L.	Shaw Nature Reserve, United States	Scrophulariaceae

Robert M. King - 13931	*Verbascum thapsus* L.	United States	Scrophulariaceae
2011018	*Verbascum chaixii* Vill.	Cult. MBG, United States	Scrophulariaceae
Adam F. Bradley - 1052	*Thomandersia laurifolia* (T. Anderson ex Benth.) Baill.	Gabon, Haut-Ogooue	Thomandersiaceae
2010045	*Lippia nodiflora* Cham.	Shaw Nature Reserve, United States	Verbenaceae
2010050	*Verbena canadensis* (L.) Britt.	Shaw Nature Reserve, United States	Verbenaceae
2010079	*Glandularia canadensis* (L.) Nutt.	Cult. MBG, United States	Verbenaceae
W.D. Stevens - 30011	*Citharexylum schottii* Greenm.	Nicaragua	Verbenaceae
A. Araujo M. - 2112	*Bouchea fluminensis* (Vell.) Moldenke	Bolivia	Verbenaceae
W.D. Stevens - 27497	*Stachytarpheta calderonii* Moldenke	Nicaragua	Verbenaceae
W.D. Stevens - 29615	*Lantana velutina* M. Martens & Galeotti	Nicaragua	Verbenaceae
W.D. Stevens - 29194	*Lippia myriocephala* Schltdl. & Cham.	Nicaragua	Verbenaceae
Charlotte M. Taylor - 11541	*Junellia seriphioides* (Gillies & Hook.) Moldenke	Chile	Verbenaceae
Charlotte M. Taylor - 11607	*Acantholippia trifida* (Gay) Moldenke	Chile	Verbenaceae
W.D. Stevens - 27257	*Rehdera trinervis* (S.F. Blake) Moldenke	Nicaragua	Verbenaceae

Table S2. Primers used in this study. Prefix Olea- refers to primers used specifically for Oleaceae; likewise, PolPRE- to the species *Polypremum procumbens*. Other primers were used for all species in core Lamiales and some Oleaceae. Asterisks (*) indicate that that primer is paired with CYCP2R.

Phylogenetic analysis	Primer Sequence: 5' to 3'	reference
Forward		
CYCF1	AAA GAY CGV CAC AGC AA	(Vieira *et al.*, 1999)
CYCF2	AAR GAY MGV CAY AGC AA	
Olea-CYC126F	AACCCTYAATTGGCTGCTTA	
PolPRE-CYC110F	THGAYAARCCRAGYAAAAC	
PolPRE-CYC562F	GCR AGR GCD AGR GCD AGR GVR AG	
PolPRE-CYC27F	TGGCTGCTHACAAAWTCAAG	
PolPRE-CYC57F	CAGGAGCTTAAGGAGAAGAAACAA	
PolPRE-CYC87F	CATGCTGGCWGYAAGACCAAT	
PolPRE-CYC178F	TGGGRGCAGATTCAAARAGG	
Reverse		
CYCP2R	AAT TGA TGA ACT TGT GCT GAT	Vieira et al. 1999
LCYC1R	ATG AAC TTG TGC TGA TTC	
Olea-CYC693R	CGTGCTGAAATCCCAATTTT	
PolPRE-CYC562R	CTY BCY CTH GCY CTH GCY CTY GC	
PolyT	GACTCGACTCGACATCGATTTTTTTTTTTTTTT	
Reverse-Transcription PCR		
Forward		
SchCALCYC2BF*	ATCATCACGCCTACGAATCC	
SchCALCYC2AF2	TTCTGCAGACCAAACAAAAGG	
RueSTRCYC2A1F	GACCTTCAAGAAACGCTTGG	

RueSTRCYC2A2F	AGAGCAAGGGCTAGGGAAAG
MimRINCYC2AF *	AGCAGCCTATCGTCCATTGT
MimRINCYC2BF2	ACAAAGGAGGAGGAGCATCA
Olea287F	TTTCCAATKTGAGCCCCTC
Reverse	
SchCALCYC2AR2	GTGATATACGCCCATGGTGAC
RueSTRCYC2A1R	AGCTGCTTGAGGCACATTTT
RueSTRCYC2A2R	TCAGCAGCAGCAGTAGCATT
MimRINCYC2BR 2	GGCTGAAATCCCAAGATTGA

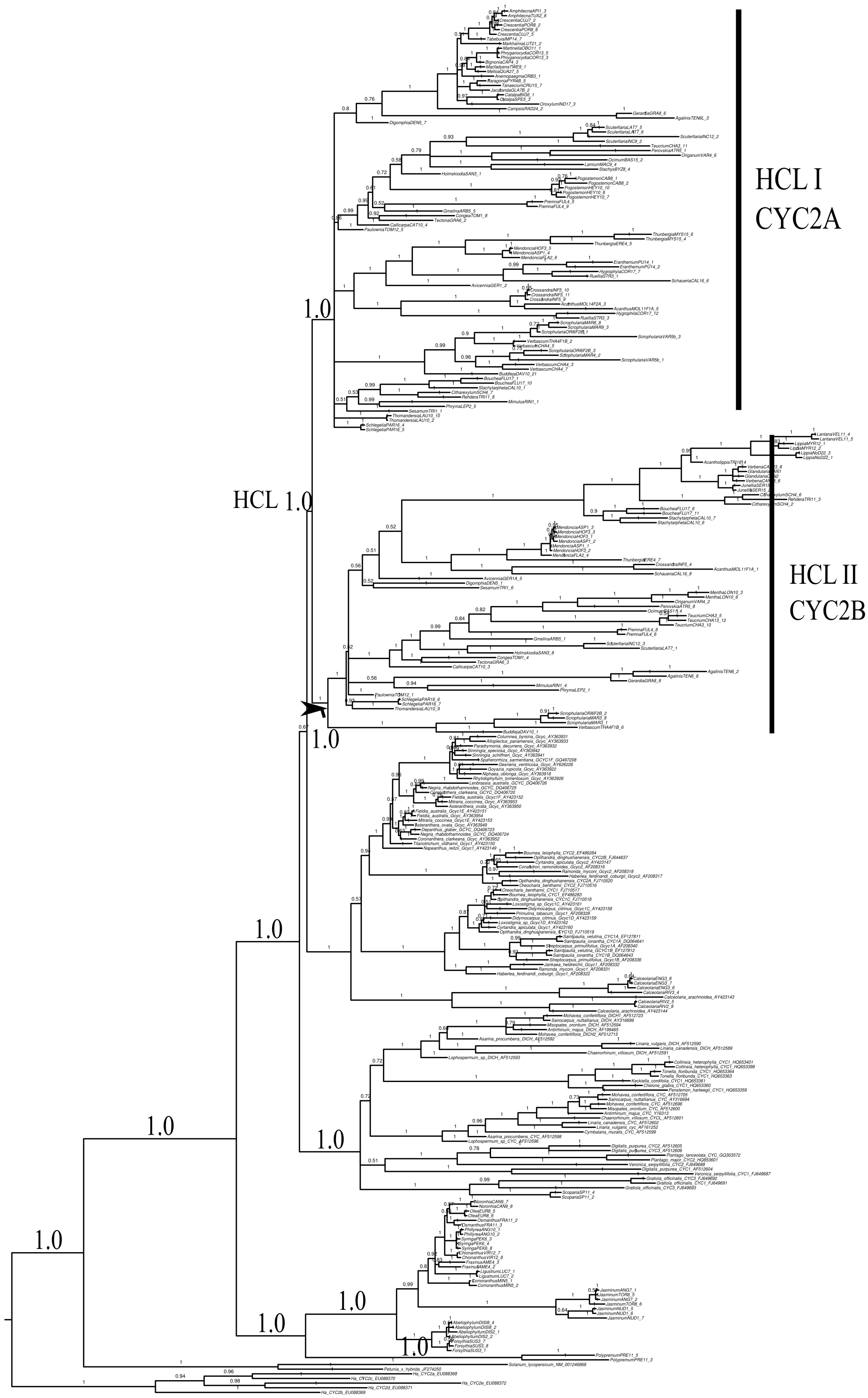
Fig. S1. Bayesian phylogeny of constrained dataset with enforcing monophyly of HCL;

Fig. S2. Phylogeny of *CYC2*-like genes (only 195 Lamiales sequences included) rooted with Oleaceae using RAxML with 100 bootstrap replicates;

Fig. S3. Phylogeny of *CYC2*-like genes (only 154 Lamiales sequences included) rooted with Oleaceae using RAxML with 100 bootstrap replicates;

Fig. S4. Results from BEAST dating analyses using uncorrelated relaxed log-normal clock (ucld);

Fig. S1.


HCL I
CYC2A
HCL II
CYC2B
HCL 1.0
1.0
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1.0
1.0
1.0
1.0
1.0
1.0
1.0
1.0

Fig. S2.

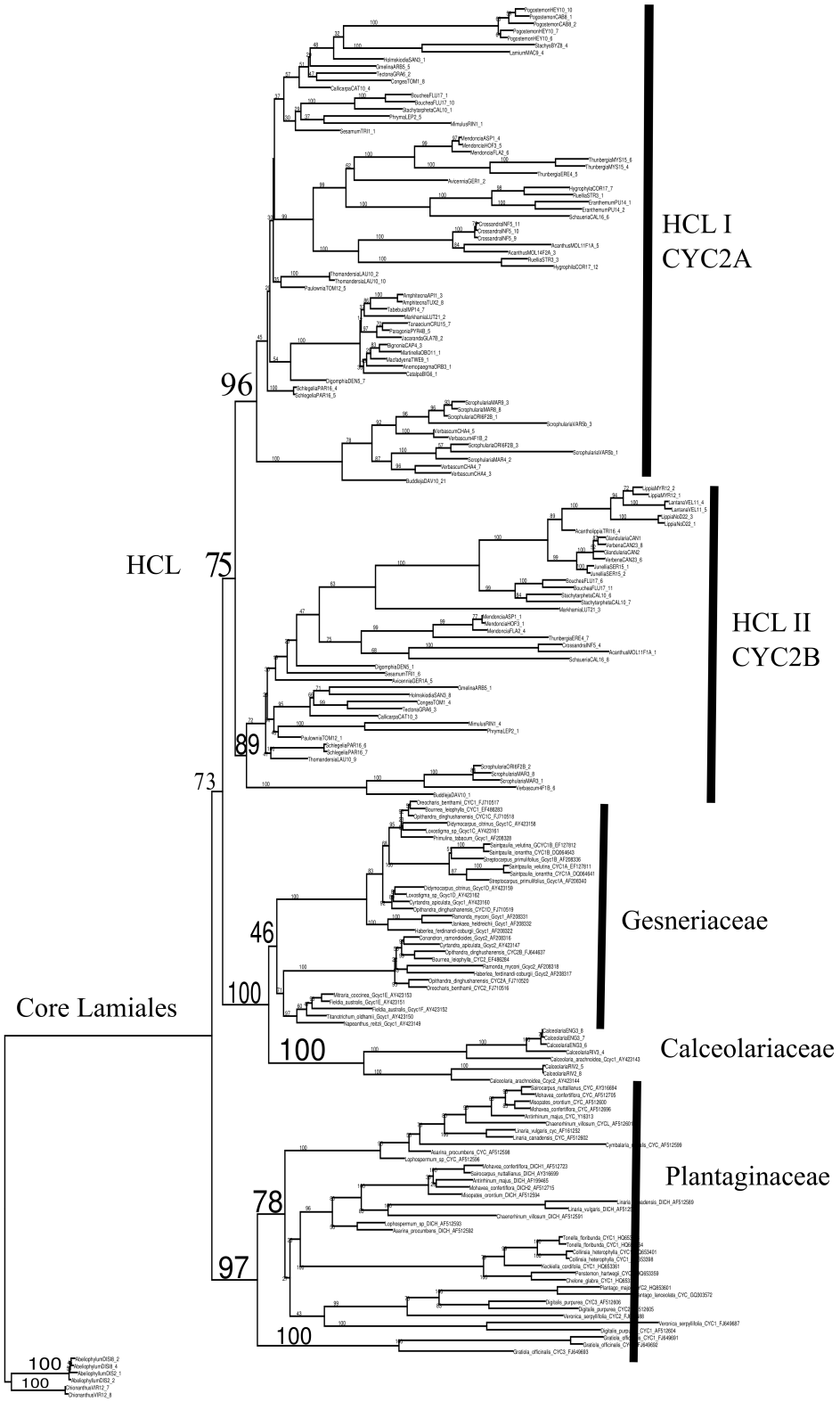

HCL I
CYC2A
HCL II
CYC2B
HCL
Gesneriaceae
Calceolariaceae
Plantaginaceae
Core Lamiales

Fig. S3.

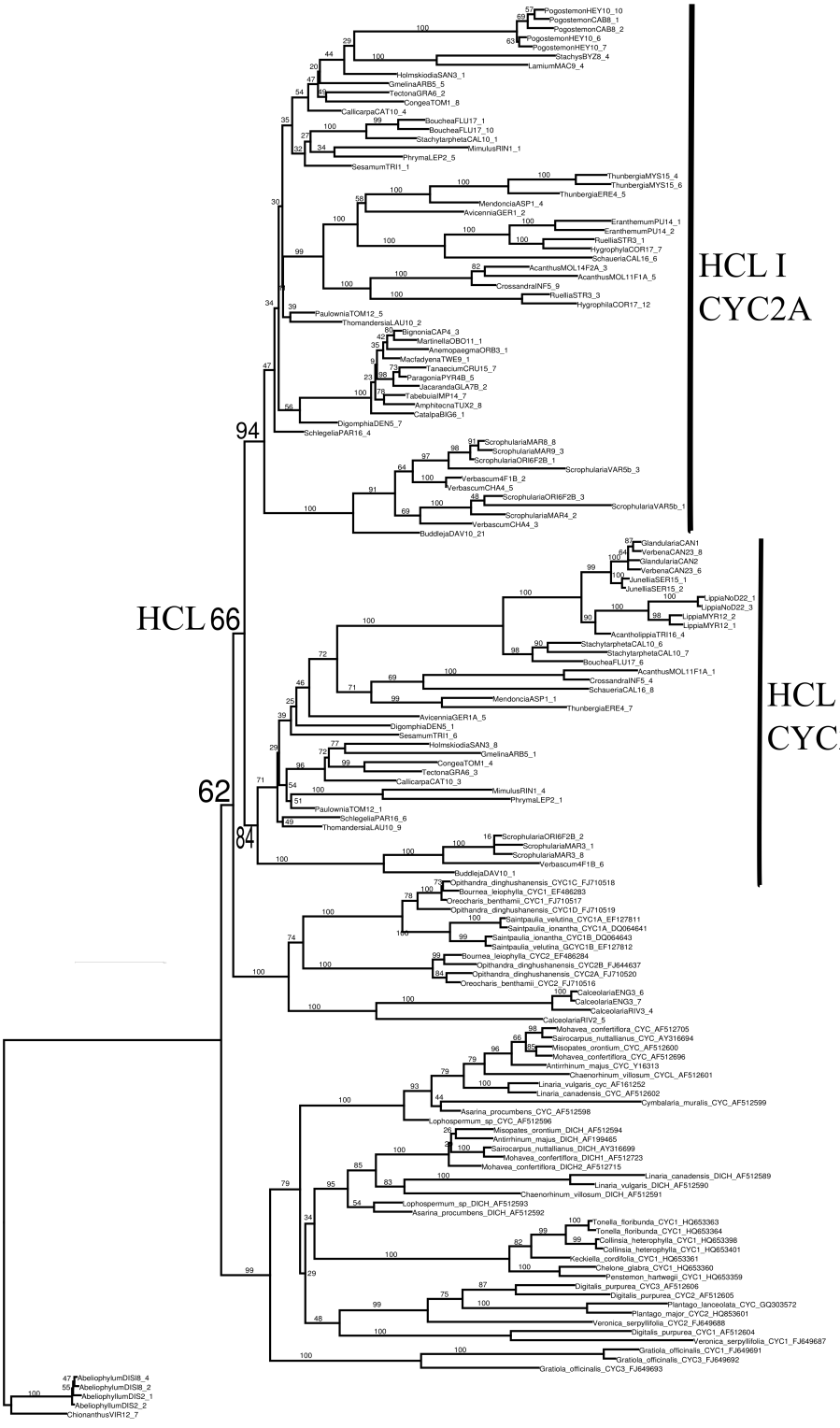
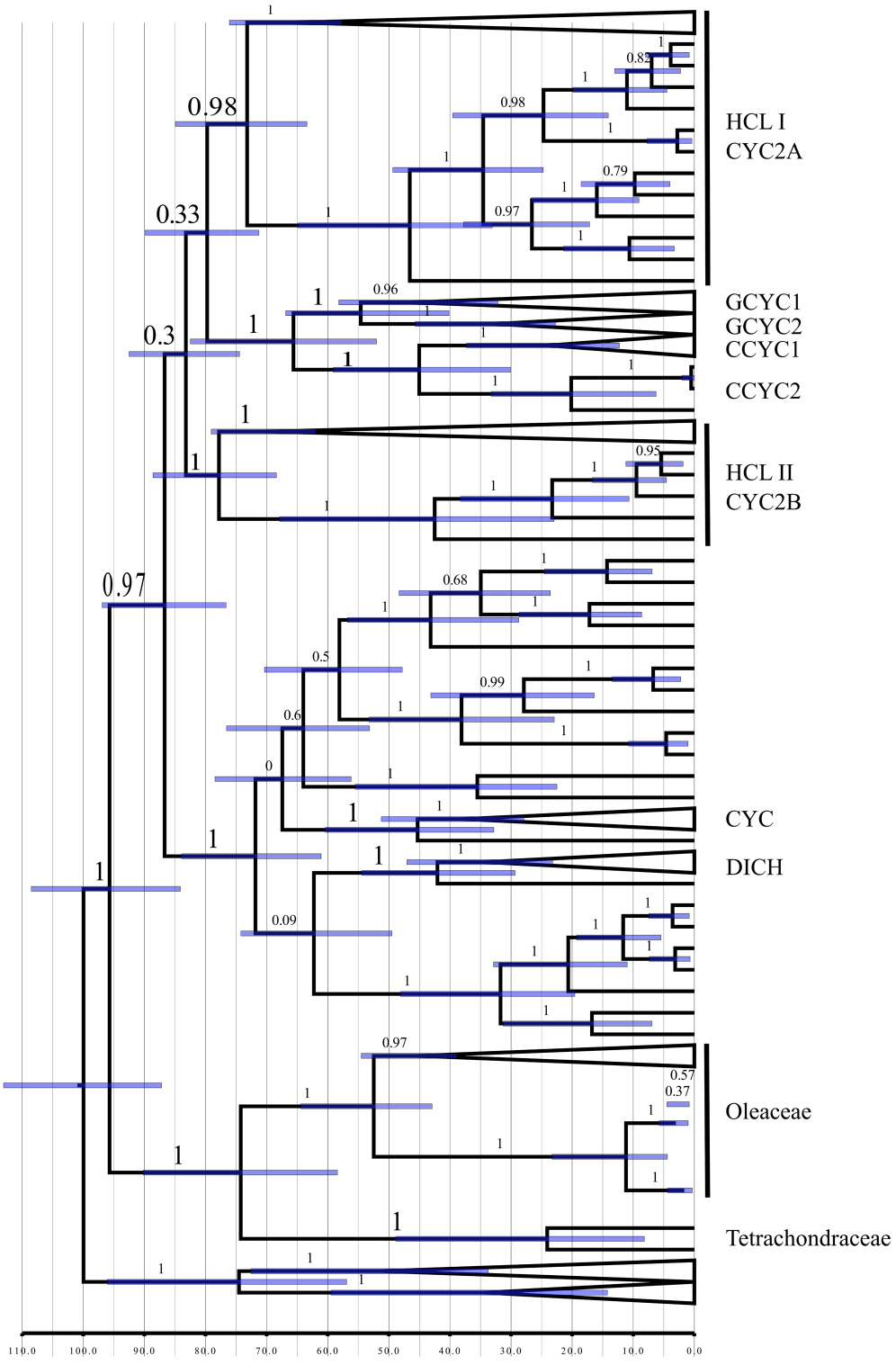

HCL I
CYC2A
HCL II
CYC2B
HCL 66
62
94
84
PogostemonHEY10_10
PogostemonCAB8_1
PogostemonCAB8_2
PogostemonHEY10_6
PogostemonHEY10_7
StachysBYZ8_4
LamiumMAC9_4
HolmskiodiaSAN3_1
GmelinaARB5_5
TectonaGRA6_2
CongeaTOM1_8
CallicarpaCAT10_4
BoucheaFLU17_1
BoucheaFLU17_10
StachytarphetaCAL10_1
MimulusRIN1_1
PhrymaLEP2_5
SesamumTRI1_1
ThunbergiaMYS15_4
ThunbergiaMYS15_6
ThunbergiaERE4_5
MendonciaASP1_4
AvicenniaGER1_2
EranthemumPU14_1
EranthemumPU14_2
RuelliaSTR3_1
HygrophylaCOR17_7
SchaueriaCAL16_6
AcanthusMOL14F2A_3
AcanthusMOL11F1A_5
CrossandraINF5_9
RuelliaSTR3_3
HygrophilaCOR17_12
PaulowniaTOM12_5
ThomandersiaLAU10_2
BignoniaCAP4_3
MartinellaOBO11_1
AnemopaegmaORB3_1
MacfadyenaTWE9_1
TanaeciumCRU15_7
ParagoniaPYR4B_5
JacarandaGLA7B_2
TabebuiaIMP14_7
AmphitecnaTUX2_8
CatalpaBIG6_1
DigomphiaDEN5_7
SchlegeliaPAR16_4
ScrophulariaMAR8_8
ScrophulariaMAR9_3
ScrophulariaORI6F2B_1
ScrophulariaVAR5b_3
Verbascum4F1B_2
VerbascumCHA4_5
ScrophulariaORI6F2B_3
ScrophulariaVAR5b_1
ScrophulariaMAR4_2
VerbascumCHA4_3
BuddlejaDAV10_21
GlandulariaCAN1
VerbenaCAN23_8
GlandulariaCAN2
VerbenaCAN23_6
JuneliaSER15_1
JuneliaSER15_2
LippiaNoD22_1
LippiaNoD22_3
LippiaMYR12_2
LippiaMYR12_1
AcantholippiaTRI16_4
StachytarphetaCAL10_6
StachytarphetaCAL10_7
BoucheaFLU17_6
AcanthusMOL11F1A_1
CrossandraINF5_4
SchaueriaCAL16_8
MendonciaASP1_1
ThunbergiaERE4_7
AvicenniaGER1A_5
DigomphiaDEN5_1
SesamumTRI1_6
HolmskiodiaSAN3_8
GmelinaARB5_1
CongeaTOM1_4
TectonaGRA6_3
CallicarpaCAT10_3
MimulusRIN1_4
PhrymaLEP2_1
PaulowniaTOM12_1
SchlegeliaPAR16_6
ThomandersiaLAU10_9
ScrophulariaORI6F2B_2
ScrophulariaMAR3_1
ScrophulariaMAR3_8
Verbascum4F1B_6
BuddlejaDAV10_1
Opithandra_dinghushanensis_CYC1C_FJ710518
Bournea_leiophylla_CYC1_EF486283
Oreocharis_benthamii_CYC1_FJ710517
Opithandra_dinghushanensis_CYC1D_FJ710519
Saintpaulia_velutina_CYC1A_EF127811
Saintpaulia_ionantha_CYC1A_DQ064641
Saintpaulia_ionantha_CYC1B_DQ064643
Saintpaulia_velutina_GCYC1B_EF127812
Bournea_leiophylla_CYC2_EF486284
Opithandra_dinghushanensis_CYC2B_FJ644637
Opithandra_dinghushanensis_CYC2A_FJ710520
Oreocharis_benthamii_CYC2_FJ710516
CalceolariaENG3_6
CalceolariaENG3_7
CalceolariaRIV3_4
CalceolariaRIV2_5
Mohavea_confertiflora_CYC_AF512705
Sairocarpus_nuttallianus_CYC_AY316694
Misopates_orontium_CYC_AF512600
Mohavea_confertiflora_CYC_AF512696
Antirrhinum_majus_CYC_Y16313
Chaenorhinum_villosum_CYCL_AF512601
Linaria_vulgaris_cyc_AF161252
Linaria_canadensis_CYC_AF512602
Cymbalaria_muralis_CYC_AF512599
Asarina_procumbens_CYC_AF512598
Lophospermum_sp_CYC_AF512596
Misopates_orontium_DICH_AF512594
Antirrhinum_majus_DICH_AF199465
Sairocarpus_nuttallianus_DICH_AY316699
Mohavea_confertiflora_DICH1_AF512723
Mohavea_confertiflora_DICH2_AF512715
Linaria_canadensis_DICH_AF512589
Linaria_vulgaris_DICH_AF512590
Chaenorhinum_villosum_DICH_AF512591
Lophospermum_sp_DICH_AF512593
Asarina_procumbens_DICH_AF512592
Torenia_floribunda_CYC1_HQ653363
Torenia_floribunda_CYC1_HQ653364
Collinsia_heterophylla_CYC1_HQ653398
Collinsia_heterophylla_CYC1_HQ653401
Keckiella_cordifolia_CYC1_HQ653361
Chelone_glabra_CYC1_HQ653360
Penstemon_hartwegii_CYC1_HQ653359
Digitalis_purpurea_CYC3_AF512606
Digitalis_purpurea_CYC2_AF512605
Plantago_lanceolata_CYC_GQ303572
Plantago_major_CYC2_HQ853601
Veronica_serpyllifolia_CYC2_FJ649688
Digitalis_purpurea_CYC1_AF512604
Veronica_serpyllifolia_CYC1_FJ649687
Gratiola_officinalis_CYC1_FJ649691
Gratiola_officinalis_CYC3_FJ649692
Gratiola_officinalis_CYC3_FJ649693
AbeliophyllumDISI8_4
AbeliophyllumDISI8_2
AbeliophyllumDIS2_1
AbeliophyllumDIS2_2
ChionanthusVIR12_7

Fig. S4.


HCL I
CYC2A
GCYC1
GCYC2
CCYC1
CCYC2
HCL II
CYC2B
CYC
DICH
Oleaceae
Tetrachondraceae
110.0
100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0

CHAPTER 2. STEPWISE EVOLUTION OF COROLLA SYMMETRY *CYC2*-LIKE AND *RAD*-LIKE GENE EXPRESSION PATTERNS IN LAMIALES

Abstract – *CYC2*-like and *RAD*-like genes are needed for the development of corolla bilateral symmetry in snapdragon. However, what changes in *CYC2-like* and *RAD-like* genes correlate with the origin of corolla bilateral symmetry early in Lamiales remain poorly known. The asymmetrical expression of *CYC2*-like and *RAD*-like genes during floral meristem development could be ancestral or derived in Plantaginaceae. We therefore focus primarily on the expression of *CYC2*-like and *RAD*-like genes in both early diverging Lamiales and core Lamiales lineages. Our results show that the expression of *CYC2*-like and *RAD*-like genes is detected broadly in the floral meristems in early diverging Lamiales lineages with radially symmetrical corolla, and is restricted to adaxial and lateral regions only in core Lamiales. The expression pattern of *CYC2*-like genes has evolved in stepwise fashion, in that *CYC2*-like genes are expressed only very early in development in Oleaceae; persistent expression of *CYC2*-like in petals originated in the common ancestor of Tetrachondraceae and core Lamiales, and asymmetrical expression in adaxial/lateral petals appeared later, in the common ancestor of the core Lamiales. Likewise, expression of *RAD*-like in petals appeared in early diverging Lamiales or earlier; asymmetrical expression in adaxial/lateral petals then appeared in Plantaginaceae and Gesneriaceae. These data and published reports of *CYC2*-like genes show that asymmetrical expression of *CYC2*-like is likely derived and correlates with the origins of corolla bilateral symmetry. In contrast, the asymmetrical expression of *RAD*-like genes may be unique to Plantaginaceae and Gesneriaceae lineages and is apparently not required for development of bilateral symmetry in general.

Key words - *CYC2*-like gene, *RAD*-like gene, Lamiales, co-aysmmetrical expression, floral symmetry

Introduction

Bilaterally symmetrical corollas have evolved many times during the diversification of flowering plants and are associated with specialized pollination and increased species richness (Donoghue *et al*., 1998; Endress, 1999, 2001; Ree & Donoghue, 1999; Preston & Hileman, 2009). Most members of Lamiales have flowers that are bilaterally symmetrical and characterized by a single axis of symmetry. The bulk of Lamiales (core Lamiales, CL; Fig. 1), accounting for ca. 9% of angiosperm diversity, are characterized by having two-lipped (bilabiate) flowers with prominent bilateral symmetry particularly in the petals. The typical two-lipped flower has three distinctive types of petal: two upper (adaxial or dorsal) petals, two lateral petals, and a single lower (abaxial or ventral) petal. Corolla bilateral symmetry originated early in Lamiaes, with species in the early diverging grades (EDG; e.g. Plocospermataceae, Oleaceae, Tetrachondraceae) having radially symmetrical corolla and the majority of core Lamiales having predominantly bilaterally symmetrical corollas.

The genetic basis of corolla bilateral symmetry has been studied in detail in the model species *Antirrhinum majus* (snapdragon, Plantaginaceae, Lamiales) (Luo *et al*., 1996, 1999; Galego & Almeida, 2002; Corley *et al*., 2005). Development of corolla bilateral symmetry is controlled by a genetic network including two TCP (*TEOSINTE BRANCHED1*, *CYCLOIDEA* [*CYC*] and *Proliferation Cell Factor*) genes *CYC* and *DICHOTOMA* (*DICH*) (Luo *et al*., 1996, 1999), and two MYB transcription factors *RADIALIS* (*RAD*) and *DIVARICATA* (*DIV*) (Galego & Almeida, 2002; Corley *et al*., 2005). *CYC* and *DICH* have originated from a recent *CYC2*-like gene duplication in the tribe Antirrhineae and are functionally partially redundant (Luo *et al*., 1996, 1999; Hileman & Baum, 2003; Feng *et al*., 2006; Busch & Zachgo, 2007; Broholm *et al*., 2008). Expression of both genes is restricted to the adaxial and lateral regions of the flowers (Luo *et al*., 1996, 1999). Flowers of *cyc/dich* double mutants are radially symmetrical, while flowers of *cyc* mutants are weakly-bilaterally symmetrical or -radially symmetrical, and *dich* mutants are conspicuously bilaterally symmetrical but with less internal asymmetry of the adaxial petals (Luo *et al*., 1996, 1999). Similarly, the *Arabidopsis CYC2*-like gene (*TCP1*) is also expressed asymmetrically but transiently in adaxial regions at the floral meristem stage, even though flowers at maturity in *Arabidopsis* are

radially symmetrical. This early asymmetrical adaxial expression pattern is thus hypothesized as the ancestral expression condition of *CYC2*-like for both rosids and asterids (Costa *et al*., 2005; Rosin & Kramer, 2009). In contrast to *CYC/DICH*, the asymmetrical expression pattern of *TCP1* does not persist during later stages of flower development, which may then account for radial symmetry of the corolla as in *Arabidopsis* (Cubas *et al*., 2001; Busch & Zachgo, 2007). Studies comparing core Lamiales and *Arabidopsis* have further shown that the acquisition of TCP II recognition sites in the 5'-regulatory regions of *CYC2*-like genes has helped form positive regulatory feedback loops and thus maintain *CYC2*-like gene expression during later floral developmental stages. This persistent expression of *CYC2*-like genes is therefore critical for the development of bilateral symmetry of the corolla at maturity (Cubas *et al*., 2001; Costa *et al*., 2005; Busch & Zachgo, 2007; Yang *et al*., 2012).

CYC/DICH affect corolla symmetry mostly through their downstream target *RAD*, a transcription factor in snapdragon (Corley *et al*., 2005). *RAD* mutants have an almost radially symmetrical corolla and *RAD* expression is up-regulated by CYC/DICH. The RAD protein antagonizes DIV that is thus confined to the abaxial part of the flower, and helps establish adaxial-abaxial asymmetry (Galego & Almeida, 2002; Corley *et al*., 2005). In contrast to *Antirrhinum*, *RAD*-like genes in *Arabidopsis* are not expressed in petals or in the adaxial domains of floral meristems, rather, *RAD*-like genes are expressed in the development of embryos (Costa *et al*., 2005; Baxter *et al*., 2007). The apparently distinct roles of *RAD*-like genes in *Arabidopsis* and snapdragon suggest that either they have been co-opted to interact with *CYC2*-like genes in snapdragon flowers, or that the floral function of *RAD*-like was lost in *Arabidopsis*.

To summarize, the asymmetrical expression of *CYC2*-like and *RAD*-like genes during floral meristem development could either be ancestral or derived in Plantaginaceae. It is also unclear whether the persistent expression of *CYC2*-like genes in petals correlates with the origin of corolla bilateral symmetry in core Lamiales, and whether the origin of corolla bilateral symmetry requires coupling of *CYC2*-like and *RAD*-like gene expression. We therefore examined *CYC2*-like and *RAD*-like gene expression in both early diverging Lamiales and core Lamiales lineages, following the well-supported phylogeny of the order Lamiales (Schäferhoff *et al*., 2010). We show that *CYC2*-like and *RAD*-like genes

are expressed throughout the floral meristem in early diverging Lamiales lineages, and are confined to adaxial and lateral regions only in core Lamiales. The *CYC2*-like expression pattern has evolved in stepwise fashion, in that *CYC2*-like genes are expressed only very early in development in Oleaceae; persistent expression of *CYC2*-like in petals originated in the common ancestor of Tetrachondraceae and the remainder of the Lamiales, and asymmetrical expression in adaxial/lateral petals appeared later, in the common ancestor of the core Lamiales. Likewise, expression of *RAD*-like in petals appears in early diverging Lamiales or earlier; asymmetrical expression in adaxial/lateral petals then appeared in Plantaginaceae and Gesneriaceae. These data and published reports show that asymmetrical expression of *CYC2*-like gene is likely derived and correlates with the origins of bilateral symmetry. In contrast, the asymmetrical expression of *RAD*-like genes may be unique to Plantaginaceae and Gesneriaceae lineages and is apparently not required for development of bilateral symmetry in general. Finally, we find no evidence for a role of *CYC2*-like gene in the development of staminodes, but suggest a possible role of *RAD*-like gene in the development of ovules.

Materials and Methods

Plant Materials

On the basis of the infered evolutionary history of the *CYC2*-like genes (Zhong and Kellogg, submitted) (Figure 1), we sampled two early diverging Lamiales (EDL) species and one species from higher core Lamiales (HCL) for the gene expression study. We also collected nine additional species for phylogenetic analyses of *RAD*-like genes (Supporting information, Table S1). *Ligustrum lucidum* (Oleaceae, EDL lineages) is cultivated in temperate greenhouse at the Missouri Botanical Garden (MBG) in Missouri, *Polypremum procumbens* (Tetrachondraceae) was collected in southern Missouri and is growing in the greenhouse at the University of Missouri-St. Louis and *Mimulus ringens* (Phrymaceae) of the higher core Lamiales (HCL) is from Shaw Nature Reserve (MBG) in Missouri. Materials for RNA extraction were collected in RNAlater (AMBION, USA) and preserved at -20^0C; developing inflorescences and flowers for *in situ* RNA hybridization were fixed in FAA (100 ml: 50 ml 95% Ethanol, 10 ml 37% Formaldehyde,

5 ml Glacial Acetic Acid, 35 ml diethylpyrocarbonate-treated distilled-H_2O (DepC-dH_2O), and dehydrated in a graded series of ethanol. Total RNA was isolated using TRI Reagent® (AMBION, USA). Dehydrated materials further went through a graded series of Histo-Clear (National Diagnostics, USA) before being embedded in Paraplast (Fisher Scientific, USA) for sectioning.

Gene isolation and phylogenetic analyses of *RAD*-like genes

RAD-like genes were amplified with the reverse transcriptase polymerase chain reaction (RT-PCR) from RNA using degenerate primers (Supporting Information, Table S2) designed from alignments of published *RAD*-like sequences from Genbank. To infer the evolutionary history of *RAD*-like genes across Lamiales, our *RAD*-like gene sequences were aligned as amino acids with those of eudicots from Phytozome (http://www.phytozome.net/), GenBank (http://www.ncbi.nlm.nih.gov/) and the 1KP database (http://218.188.108.77/Blast4OneKP/) using MAFFT incorporated in Seaview (Gouy *et al*., 2010). Aligned amino acid sequences were then converted back to nucleotides for phylogenetic analyses. Maximum likelihood analyses were conducted using PhyML (Guindon *et al*., 2010) (http://www.atgc-montpellier.fr/phyml/) and RAxML in CIPRES (Ludwig *et al*., 2002) with 100 bootstrap replicates under the GTR+I+G model selected by jModeltest (Darriba *et al*., 2012).

***In situ* RNA hybridization**

Paralog-specific forward primers were designed from previous genomic sequences (Zhong and Kellogg, unpublished data). Paralog-specific forward primers were paired with a universal Poly-T primer (5'-GACTCGACTCGACATCGATTTTTTTTTTTTTTTT-3'), and were used to amplify parts of the coding region and 3'-untranslated region (3'-UTR) of both *CYC2*-like paralogs and *RAD*-like genes. Gene (paralog)-specific forward and reverse primers (Supporting information, Table S2) were then designed and used to amplify the 3'-UTR and a small portion of the coding region (less than 100 bps). RT-PCR used SuperScript® III One-Step RT-PCR System with Platinum®Taq (INVITROGEN, USA) and started with cDNA synthesis for 30 min at 60^0 C, and 35 cycles of regular PCR amplification. Amplified products were subsequently subcloned into *pGEM®-T Vector* (Promega, USA)

and sequenced to verify the identity and direction of the inserts. Sense and anti-sense probes were ca. 150-400 bps long and were generated by in vitro transcription using MEGAscript® T7/SP6 kits (AMBION, USA) and labeled by Digoxigenin-UTP (ROCHE, USA).

Expression of *CYC2*-like and *RAD*-like genes was examined using *in situ* RNA hybridization following previous protocols for plant material (Malcomber & Kellogg, 2004). The only difference was that purified probes that were shorter than 300 bps were used directly for *in situ* RNA hybridization without hydrolysis. *In situ* hybridization results were inspected and visualized using a compound microscope (OLYMPUS BX40) at the Missouri Botanical Garden. Images were adjusted for brightness, contrast and color balance using GIMP 2.8.

Results

Phylogenetic analyses of *RAD*-like genes - Trees were rooted with *Aquilegia coerulea* as the outgroup. All our amplified *RAD*-like sequences were grouped with the snapdragon *RAD* sequence with moderate maximum likelihood bootstrap support (68%) (Fig. 2). Unlike *CYC2*-like genes, most species in Lamiales have only one copy of *RAD*-like gene except *Polypremum procumbens* (Tetrachondraceae), a sister taxon to core Lamiales, that has two copies.

Expression of *CYC2*-like genes - To determine stage- and tissue-specific expression patterns of *CYC2*-like gene and *RAD*-like, we conducted *in situ* RNA hybridizations on several different species from both early diverging and core Lamiales lineages.

Ligustrum lucidum (Oleaceae, EDL) has a radially symmetrical corolla and maintains only one *CYC2*-like gene copy (*LlCYC*). *LlCYC* is expressed early in the floral meristem, and is not limited to the adaxial region but is widely expressed throughout the floral meristem (Fig. 3a, b). During initiation and development of floral parts, *LlCYC* gene transcripts are slightly detected in petals, stamens, and gynoecia but not in sepals or subtending leafy bracts (Fig. 3c). However, *LlCYC* expression is gradually down-

regulated during flower development in petals, with no detectable expression during style elongation and later developmental stage (Fig. 3d, e).

Polypremum procumbens (Tetrachondraceae, EDL) also has a radially symmetrical corolla and is sister to core Lamiales that have bilaterally symmetrical corollas. Unlike *Ligustrum lucidum*, this species maintains two copies of *CYC2*-like genes (*PpCYC2A* and *PpCYC2B*). Similar to *LlCYC* expression, *PpCYC* transcripts are not localized in adaxial regions of floral meristems but are strongly detected throughout the floral meristem and in sepals, but not in subtending leafy bracts (Fig. 4, a-d). *PpCYCs* are also expressed in stamens and gynoecia, and in ovules (Fig. 4, c). The major difference in expression of *CYC2*-like genes in *Ligustrum* (*LlCYC*) and *Polypremum* (*PpCYC2A* and *PpCYC2B*) is that the expression of *PpCYC2A* and *PpCYC2B* is maintained during style elongation and later developmental stages (Fig. 4, d).

Mimulus ringens (Phrymaceae, CL) has a bilaterally symmetrical corolla and has two *CYC2*-like genes (*MrCYC2A* and *MrCYC2B*). During the floral meristem stages, *MrCYC2A* is expressed in the adaxial region (Fig. 5a), whereas *MrCYC2B* is expressed throughout the floral meristem (Fig. 5g). After floral organ initiation, the expression of *MrCYC2A* is found exclusively in the adaxial and partly in the lateral but not in the abaxial petals (Fig. 5 b, c), while *MrCYC2B* expression persists in all petals (Fig. 5h, i).

Expression of *RAD*-like genes - Expression of *Ligustrum lucidum RAD*-like gene (*LlRAD*) is similar to that of the *LlCYC* gene from early to mid-stages of flower development with gene transcripts being detected in all petals and stamens but not in subtending leafy bracts (Fig. 6, a, b, c). However, unlike *LlCYC*, *LlRAD* expression is detected in the early but not late development of sepals, while *LlRAD* expression persists during the later stages of flower development in petals, stamens and gynoecia (Fig. 6, d, e). Unlike *Ligustrum lucidum*, *Polypremum procumbens* has two copies of *RAD*-like genes (*PpRAD*), but these two *PpRAD* genes are also strongly detected in all petals, stamens and ovules during the flower development, and in the early development of sepals but not in subtending leafy bracts as that in *Ligustrum* (*LlRAD*) (Fig. 7).

Transcripts of the *RAD*-like gene from *Mimulus ringens* (*MrRAD*) are apparently not confined to adaxial/lateral regions as that observed for *MrCYC2A* but are detected in all petals during flower development from early to late stages (Fig. 8, a-d).

Evolution of co-asymmetical *CYC2*-like and *RAD*-like expression in Lamiales - To investigate the evolution of *CYC2*-like and *RAD*-like gene expression within Lamiales, we used maximum parsimony character reconstruction methods in the context of the most recent well-resolved Lamiales phylogeny (Fig. 9) (Schäferhoff *et al*., 2010). We added our data in early diverging Lamiales species *Ligustrum lucidum*, *Polypremum procumbens* and higher core Lamiales species *Mimulus ringens* with published expression patterns of snapdragon *CYC*, *DICH* and *RAD* (Luo *et al*., 1996, 1999; Corley *et al*., 2005), *CYC2*-like and *RAD*-like genes in *Gratiola* and *Veronica* (Preston *et al*., 2009), *CYC1C*, *CYC1D*, *CYC2A* and *CYC2B* of *Primulina heterotricha* (Gao *et al*., 2008; Yang *et al*., 2012).

Discussion

Adaxial/abaxial asymmetry of floral organs in Plantaginaceae and Gesneriaceae is the result of differential expression of duplicated *CYC2*-like genes along the adaxial/abaxial axis of the flower, their persistent expression in late development, and the co-option of *RAD*-like genes to roles in floral development (Luo *et al*., 1996, 1999; Hileman & Baum, 2003; Corley *et al*., 2005; Yang *et al*., 2012). *CYC2*-like genes specify adaxial identity to floral parts and are important for the development of bilateral symmetry of the corolla at maturity; they are expressed in adaxial organs early in floral meristem development and their expression persists during later developmental stages (Cubas *et al*., 2001; Busch & Zachgo, 2007, 2009; Yang *et al*., 2012). In addition, *CYC2*-like genes influence corolla symmetry via their downstream *RAD*-like targets, which help determine lateral petal identity. The RAD-like protein also antagonizes the transcription factor DIV, resulting in abaxial petal identity (Galego & Almeida, 2002; Corley *et al*., 2005).

Many studies have shown that changes in the number, expression, and interaction of developmental genes have likely been involved in the evolution of plant form (Theissen *et al*., 2000; Hileman *et al*., 2003; Hileman & Irish, 2009; Preston *et al*., 2011; Zhang *et*

al., 2013). However, our phylogenetic analyses of *CYC2*-like genes showed that duplications of *CYC2*-like genes are not correlated with the evolution of bilateral symmetry of the corolla (Zhong and Kellogg, unpublished data). Here we primarily focus on the expression of *CYC2*-like and *RAD*-like genes in early diverging Lamiales and test whether and how the expression and interaction of developmental genes may have contributed to the origin of bilateral symmetry in core Lamiales.

Evolution of *CYC2*-like gene expression - Our data show stepwise evolution of *CYC2*-like gene expression patterns. *CYC2*-like genes are expressed only in the early but not late developmental stages in petals in the early diverging lineage Oleaceae (Fig. 3); subsequently, persistent expression in all petals is followed in Tetrachondraceae, the sister to core Lamiales (Fig. 4), and is then followed by asymmetrical and persistent expression in adaxial/lateral petals in lineages within core Lamiales (Fig. 5) (Luo *et al*., 1996, 1999; Gao *et al*., 2008; Yang *et al*., 2012). This restricted expression of *CYC2*-like gene in adaxial/lateral petals correlates with the origin of bilateral symmetry of the corolla in core Lamiales.

We find no evidence to support the hypothesis that asymmetrical expression of *CYC2*-like gene during floral meristem stages is ancestral for Lamiales (Costa *et al*., 2005; Rosin & Kramer, 2009). Instead, *CYC2*-like gene expression is diffuse in all petals, stamens and the gynoecium during early developmental stages in early diverging Lamiales. Previous studies hypothesized that the ancestral expression pattern of *CYC2*-like gene for rosids and asterids might be restricted to the adaxial floral region during floral meristem stages (Costa *et al*., 2005; Rosin & Kramer, 2009). However, sampling was limited mostly to species with bilaterally symmetrical corollas. Very few species with radially symmetrical corollas have been sampled and their *CYC2*-like gene expression remains poorly known; exceptions are *Arabidopsis* and species embedded within clades with predominantly bilaterally symmetrical corollas. Our expression data in early diverging Lamiales show that broad expression of *CYC2*-like genes throughout the floral meristem is ancestral for Lamiales. Recent extended examination across Brassicaceae shows that adaxial expression of *CYC2*-like genes is absent in many sampled Brassicaceae species with bilaterally symmetrical corollas, suggesting that the transient asymmetrical expression of *TCP1* observed in *Arabidopsis* may be derived

independently from asymmetrical expression in other lineages (Busch & Zachgo, 2007; Busch *et al*., 2012).

Our findings in Tetrachondraceae indicate that the persistent expression of *CYC2*-like genes could have preceded the origin of bilateral symmetry of the corolla. The transient expression of *TCP1* (*CYC2*-like) in the adaxial floral meristem in *Arabidopsis* during early developmental stages and the formation of positive regulatory feedback loops in Gesneriaceae indicate that persistence of the adaxial expression pattern in petals during later developmental stages is critical for the development and evolution of bilateral symmetry (Cubas *et al*., 2001; Costa *et al*., 2005; Yang *et al*., 2012). The formation of positive regulatory feedback loops is key to the persistence of *CYC2*-like gene expression in petals at maturity, which requires the acquisition of TCP binding sites in 5'-regulatory regions of *CYC2*-like genes (Kosugi & Ohashi, 2002; Yang *et al*., 2012). It may be that a similar change in the 5'-regulatory regions of *CYC2*-like genes in Tetrachondraceae also has led to the persistent expression of *CYC2*-like genes in this sister species of core Lamiales, but without the development of bilaterally symmetrical corolla.

Expression evolution of *RAD*-like gene in Lamiales - Unlike *CYC2*-like genes, *RAD*-like genes have not duplicated extensively. Among most Lamiales only one copy of *RAD*-like is preserved, except in *Polypremum procumbens* (Tetrachondraceae), which has two copies (Fig. 2). *RAD*-like gene expression throughout the development of ovules is conserved across all sampled Lamiales, but its expression in adaxial and lateral petals is not conserved across core Lamiales.

RAD-like genes are expressed in all petals in early diverging Lamiales; their bilaterally symmetrical expression in core Lamiales correlates with the emergence of bilateral symmetry of the corolla. However, neither of the homologs of *RAD* in *Arabidopsis* (*AtRL1* and *AtRL2*) is expressed in petals. *AtRL1* is detected in total mRNA extracted from 20-day-old whole plants but it is not clear where it is expressed, whereas *AtRL2* is found in the funiculus of ovules and in embryos (Baxter *et al*., 2007). The evolution of the expression of *RAD*-like genes in petals may have preceded the diversification of Lamiales, lamiids, or even asterids.

Our data also support a possible role of *RAD*-like genes in the development of ovules as indicated in the expression analysis in *Arabidopsis* (Baxter *et al*., 2007).

Evolution of co-asymmetrical expression of *CYC2*-like and *RAD*-like genes - Our results show that co-asymmetrical expression in adaxial and lateral petals of *CYC2*-like and *RAD*-like genes may be unique to Plantaginaceae and Gesneriaceae (Fig. 8) (Corley *et al*., 2005; Zhou *et al*., 2008; Preston *et al*., 2009). In snapdragon, CYC/DICH affect corolla bilateral symmetry mostly through their downstream targets RAD (Corley *et al*., 2005). Expression of the snapdragon *CYC/DICH* genes in *Arabidopsis* and expression patterns of *Arabidopsis RAD*-like genes indicate that the co-option of interacting CYC2 and RAD proteins in shaping corolla symmetry may have emerged in asterids but not in rosids (Corley *et al*., 2005; Costa *et al*., 2005; Baxter *et al*., 2007; Preston & Hileman, 2009). Our data suggest that it is very unlikely that *CYC2*-like transcription factors induce the expression of *RAD*-like gene during development of petals in early diverging Lamiales because the timing of expression of the two sets of genes are so different. In *Ligustrum* (Oleaceae), expression of *LlCYC* in petals is barely detectable during late developmental stages, whereas *LlRAD* is consistently strongly expressed at these stages. In addition, the co-asymmetrical expression of *CYC2*-like and *RAD*-like genes in adaxial/lateral petals is not found in *Mimulus ringens* (Phrymaceae).

Acknowledgements

We thank George Yatskievych, Missouri Botanical Garden, and Shaw Nature Reserve for their assistance in sampling. This work was supported by a Doctoral Dissertation Improvement Grant from the National Science Foundation (grant number DEB 1210540 to EAK and JZ), the Jane Harris Scholarship in Tropical Botany from Whitney R. Harris World Ecology Center at the University of Missouri-St. Louis, and a Graduate Student Research Award from Botanical Society of America.

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Figure captions

Fig. 1. Duplication patterns of *CYC2*-like genes and floral transition from radial to bilateral symmetry early in Lamiales. Double lines show duplications of *CYC2*-like gene. Bar depicts floral transition from radial to bilateral symmetry; Abbreviations, CL, core Lamiales; HCL, higher core Lamiales.

Fig. 2. Phylogeny of *RAD*-like sequences; sequences in the shaded box are from species in Lamiales. Abbreviations: adaxial, ad; abaxial, ab; lateral, l; bracts, b; sepal, s; petal, p; stamen, st; carpel, c; g, gynoecium adaxial sepal, ads; abaxial sepal, abs; lateral sepal, ls; adaxial petal; abaxial petal, abp; lateral petal, lp; lateral stamen, lst; abaxial stamen, abst.

Fig. 3. RNA *In situ* hybridization expression of *CYC2*-like gene of *Ligustrum lucidum* (Oleaceae) flower at different developmental stages. (a)-(e) anti-sense; (f)-(g) sense; Abbreviations: adaxial, ad; abaxial, ab; lateral, l; bracts, b; sepal, s; petal, p; stamen, st; carpel, c; g, gynoecium adaxial sepal, ads; abaxial sepal, abs; lateral sepal, ls; adaxial petal; abaxial petal, abp; lateral petal, lp; lateral stamen, lst; abaxial stamen, abst.

Fig. 4. RNA *In situ* hybridization expression of *CYC2*-like genes during *Polypremum procumbens* (Tetrachondraceae) flower development. (a)-(d) anti-sense; (e)-(g) sense; Abbreviations: adaxial, ad; abaxial, ab; lateral, l; bracts, b; sepal, s; petal, p; stamen, st; carpel, c; g, gynoecium adaxial sepal, ads; abaxial sepal, abs; lateral sepal, ls; adaxial petal; abaxial petal, abp; lateral petal, lp; lateral stamen, lst; abaxial stamen, abst.

Fig. 5. RNA *In situ* hybridization expression of *CYC2*-like genes during *Mimulus ringens* (Phrymaceae) flower development. (a)-(c) CYC2A anti-sense; (d)-(f) CYC2A sense; (g)-(i) CYC2B anti-sense; (j)-(k) CYC2B sense; Abbreviations: adaxial, ad; abaxial, ab; lateral, l; bracts, b; sepal, s; petal, p; stamen, st; carpel, c; g, gynoecium adaxial sepal, ads; abaxial sepal, abs; lateral sepal, ls; adaxial petal; abaxial petal, abp; lateral petal, lp; lateral stamen, lst; abaxial stamen, abst.

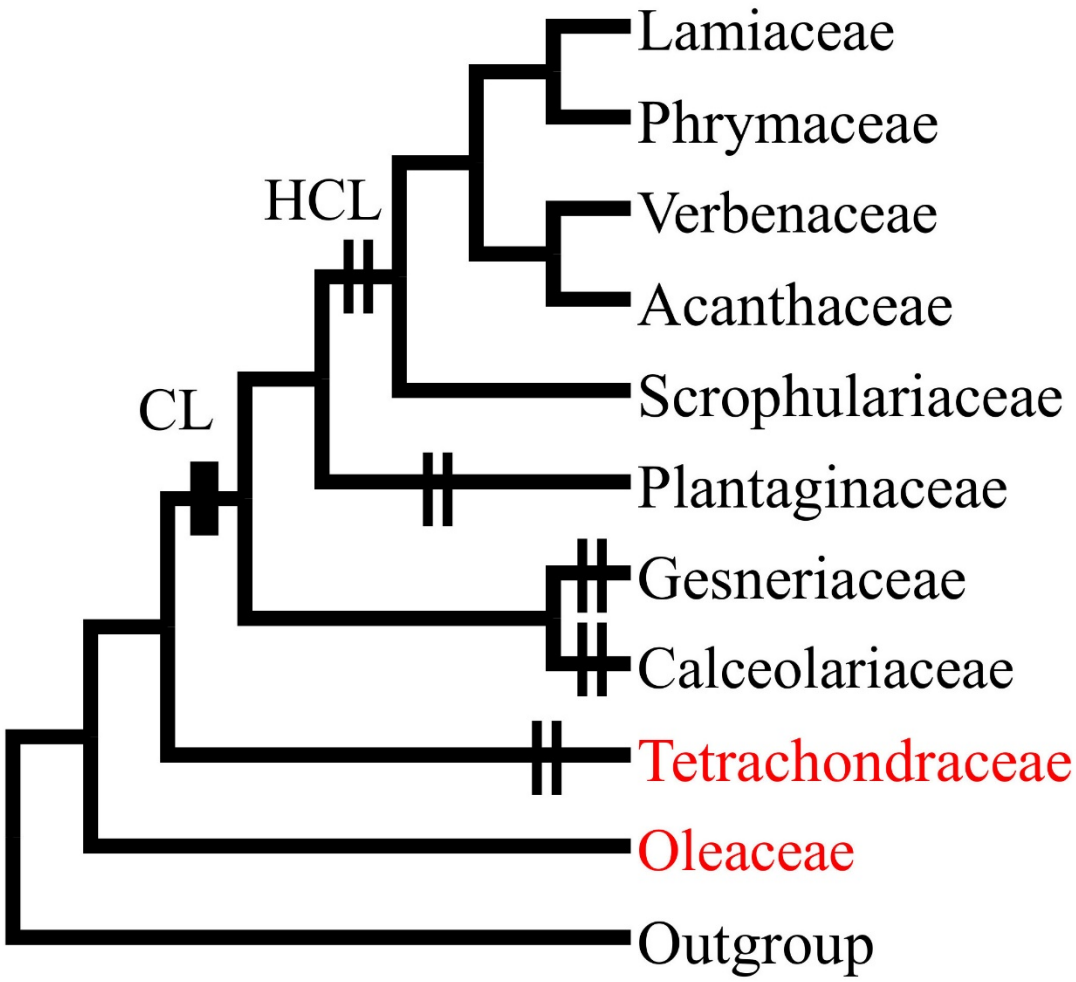
Fig. 6. RNA *In situ* hybridization expression of *RAD*-like gene during *Ligustrum lucidum* (Oleaceae) flower development. (a)-(e) anti-sense; (f)-(i) sense; Abbreviations: adaxial, ad; abaxial, ab; lateral, l; bracts, b; sepal, s; petal, p; stamen, st; carpel, c; g, gynoecium adaxial sepal, ads; abaxial sepal, abs; lateral sepal, ls; adaxial petal; abaxial petal, abp; lateral petal, lp; lateral stamen, lst; abaxial stamen, abst.

Fig. 7. RNA *In situ* hybridization expression of *RAD*-like gene during *Polypremum procumbens* (Tetrachondraceae) flower development. (a)-(c) anti-sense; (d)-(f) sense; Abbreviations: adaxial, ad; abaxial, ab; lateral, l; bracts, b; sepal, s; petal, p; stamen, st; carpel, c; g, gynoecium adaxial sepal, ads; abaxial sepal, abs; lateral sepal, ls; adaxial petal; abaxial petal, abp; lateral petal, lp; lateral stamen, lst; abaxial stamen, abst.

Fig. 8. RNA *In situ* hybridization expression of *RAD*-like gene during *Mimulus ringens* (Phrymaceae) flower development. (a)-(d) anti-sense; (e)-(f) sense; Abbreviations: adaxial, ad; abaxial, ab; lateral, l; bracts, b; sepal, s; petal, p; stamen, st; carpel, c; g, gynoecium adaxial sepal, ads; abaxial sepal, abs; lateral sepal, ls; adaxial petal; abaxial petal, abp; lateral petal, lp; lateral stamen, lst; abaxial stamen, abst.

Figures

Fig. 1.



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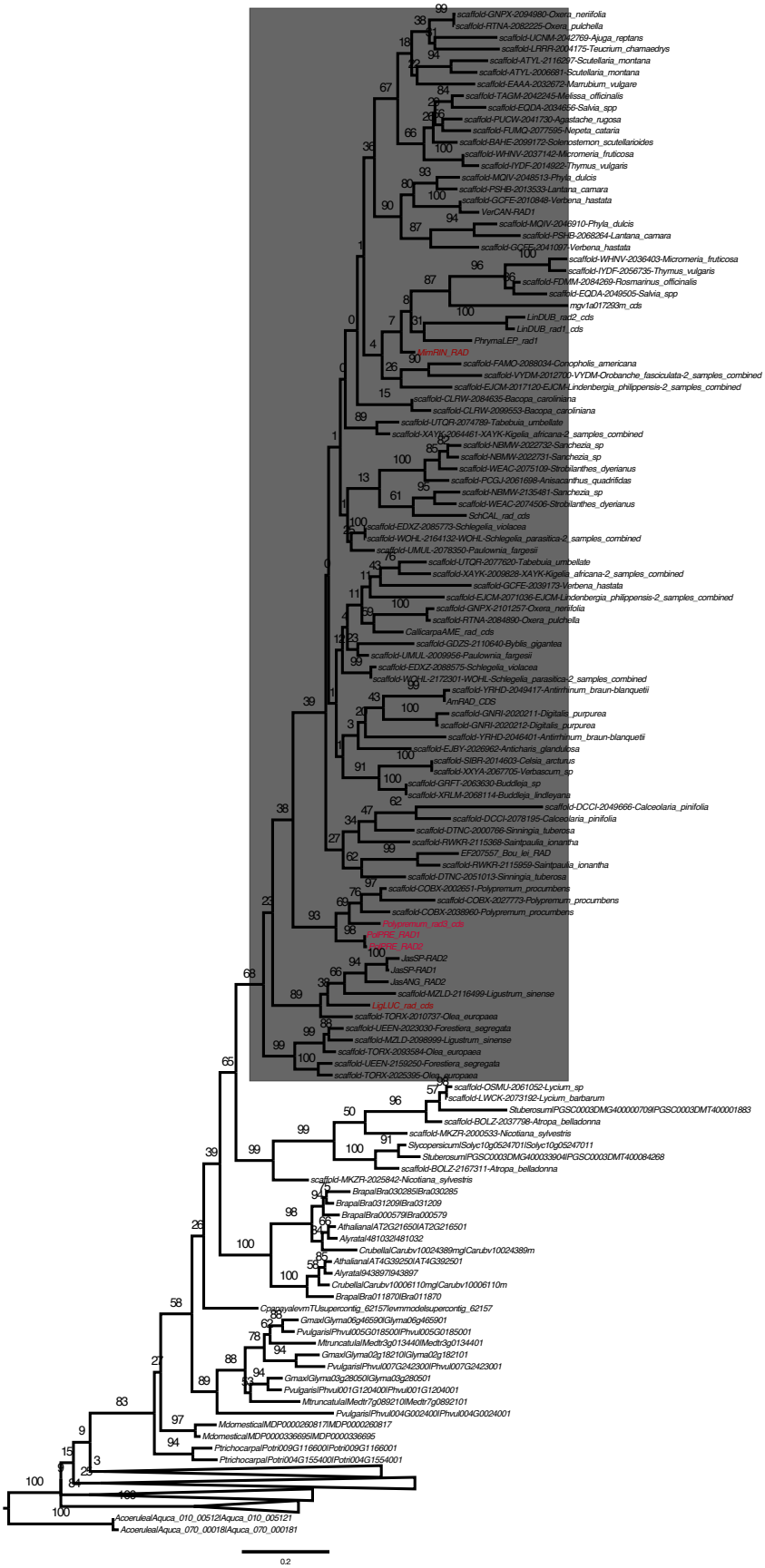
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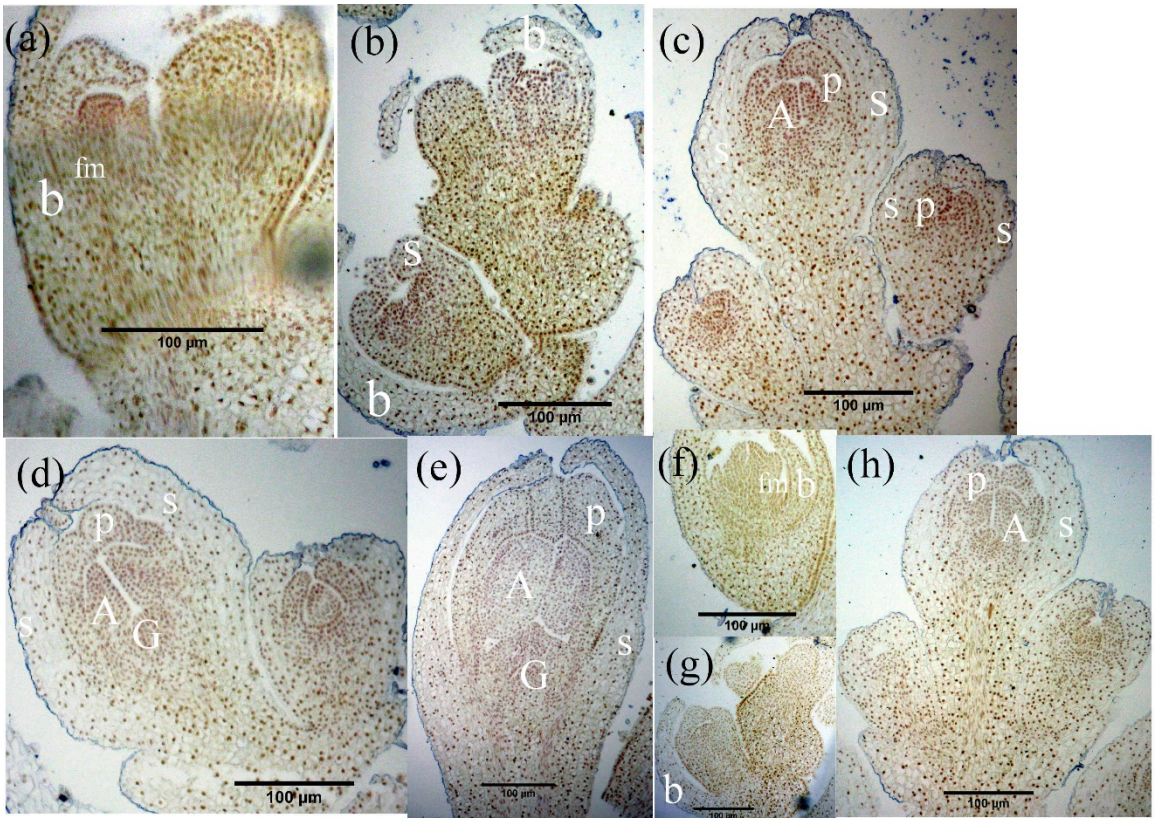
Fig. 3.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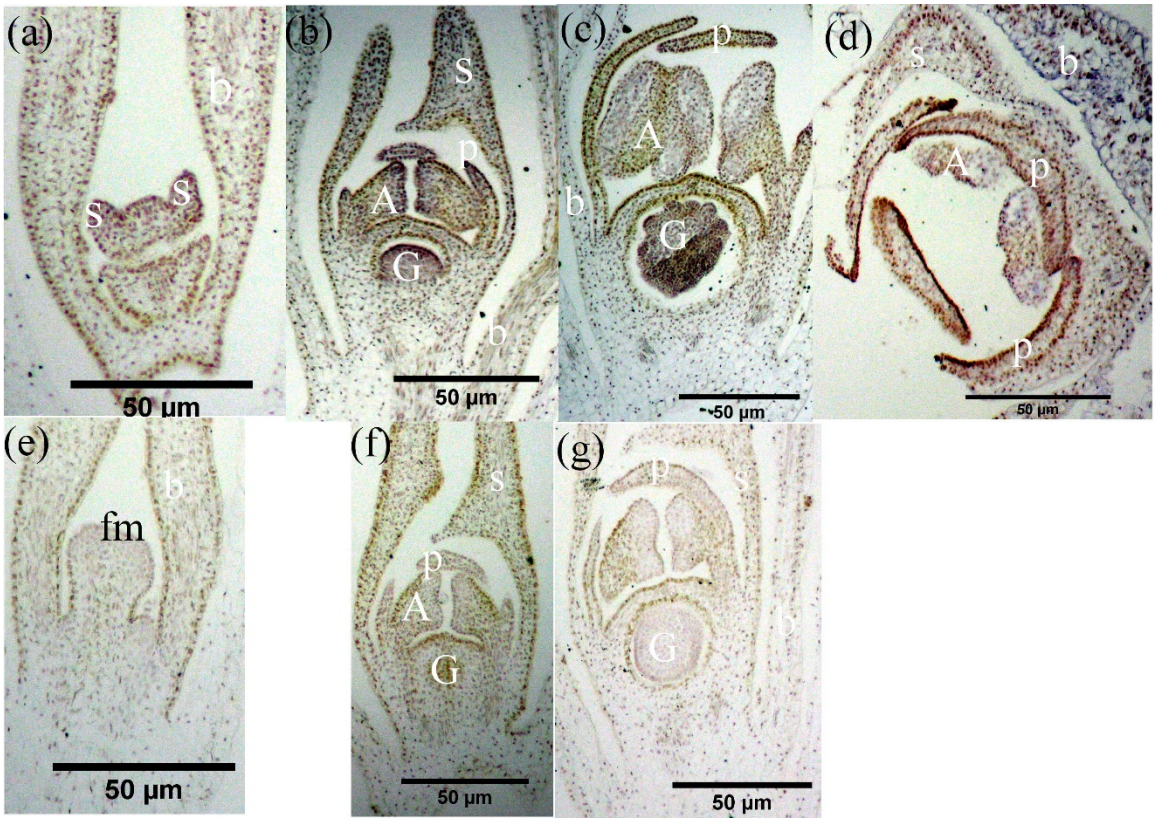
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Fig. 5.

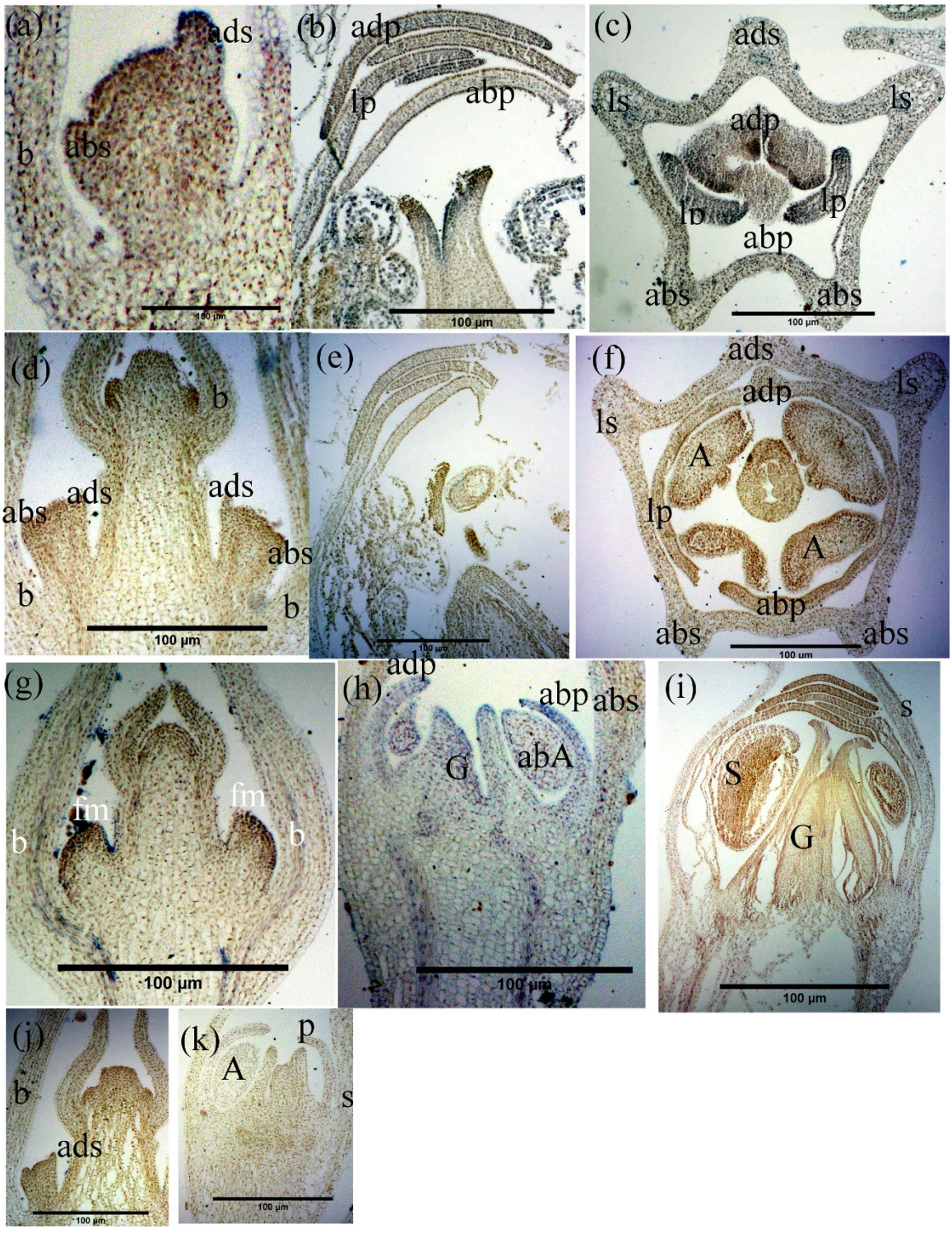
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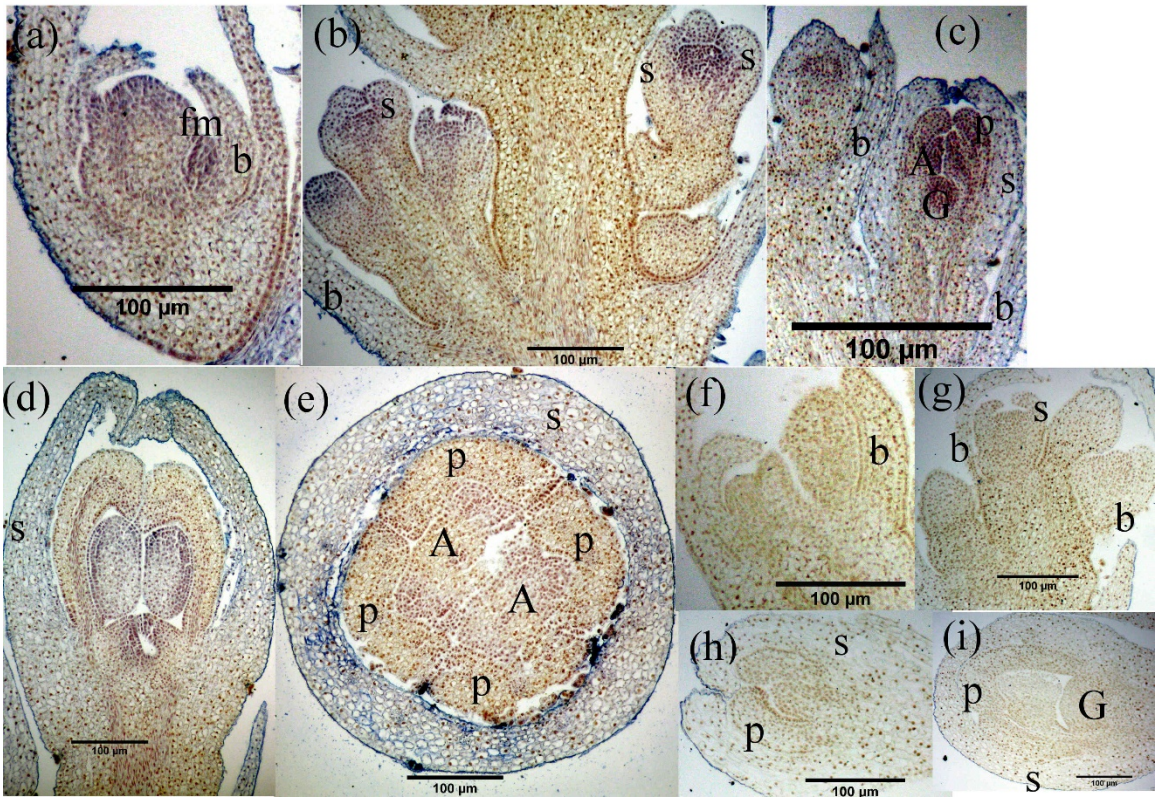
Fig. 6.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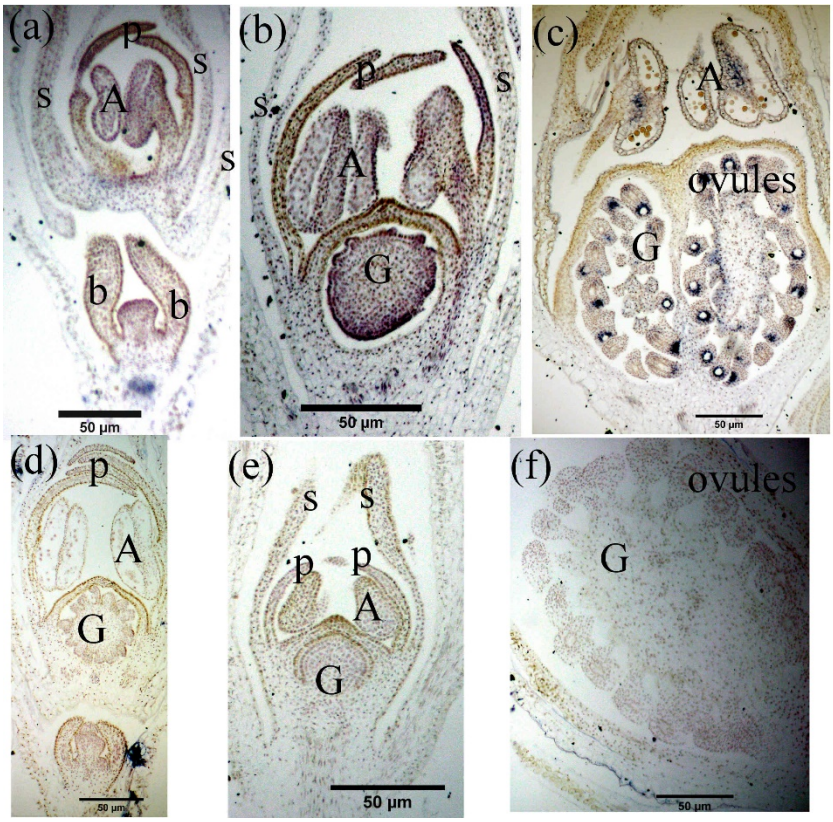
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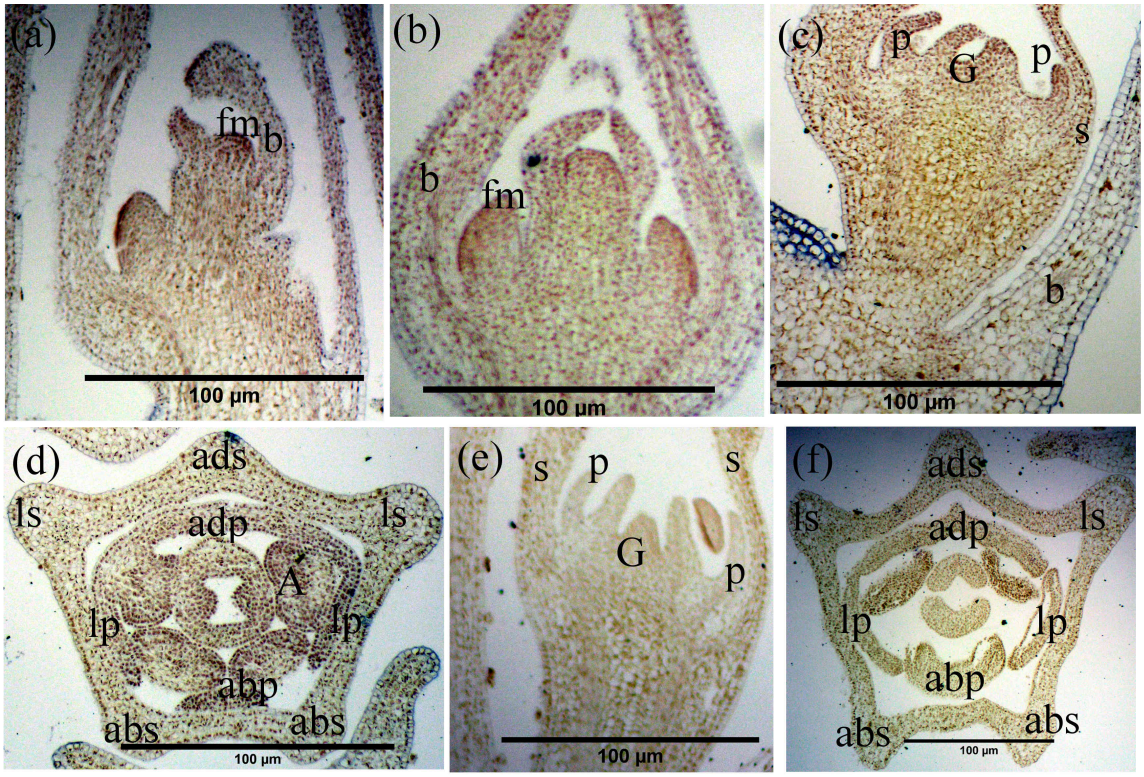
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Supporting information

Table S1. Species sampled for the amplification of *RAD-like* genes.

VOUCHER	TAXON	SOURCE	FAMILY
2010070	*Schaueria calicotricha* (Link & Otto) Nees	Cult. MBG, United States	Acanthaceae
2009020	*Callicarpa americana* L.	Cult. MBG, United States	Lamiaceae
2011014	*Lindernia dubia* (L.) Pennell	Shaw Nature Reserve, United States	Linderniaceae
2011020	*Jasminum angustifolium* Willd.	Cult. MBG, United States	Oleaceae
CHW SN8	*Ligustrum lucidum* W.T. Aiton	Cult. MBG, United States	Oleaceae
2011021	*Jasminum tortuosum* Willd.	MBG	Oleaceae
2010041	*Phryma leptostachya* L.	Shaw Nature Reserve, United States	Phrymaceae
2010043	*Mimulus ringens* L.	Shaw Nature Reserve, United States	Phrymaceae
G. Yatskievych 11-73	*Polypremum procumbens* L.	United States	Tetrachondraceae
2010050	*Verbena canadensis* (L.) Britt.	Shaw Nature Reserve, United States	Verbenaceae

Table S2. Primers used in this study for the amplification of *RAD-like* genes and locus-specific probes for *CYC2-like* and *RAD-like* genes.

	Primer sequence: 5' to 3'
***For RAD-like* gene probes**	
Forward	
LigLUC-RAD-202F	AGCCAAGAAAGYGATATAACGTCAG
LigLUC-RAD-227F	GGAACAAAGGATGAGGAATCTAAG
PolPRE-RAD-169F	CCCWTCCCCAACTACAGGRC
PolPRE-RAD-146F	ACYACATTGAGRGTGGTCAT
MimRIN_Rad214F	AAATGGTAACATGATCATCAAGAGG
MimRIN_Rad174F	AAGTGCCCTTCCCCAACTAC
Reverse	
LigLUC-RAD-342R	TCACTTTTCATATATTGCTTCCTCC
LigLUC-RAD-337R	TTCATATATTGCTTCCTCCACAGA
PolPRE_RAD_3UTRL	TGAACATKTTAMAGACAAGAAGGAA
PolPRE_RAD_3UTRs	GATCGAGAGGTAAATCAAAGSG
PolPRE-RAD-385R	ATGCTTCCTCCATAATTTAAAACCTA
MimRIN_Rad506R	TTCCATTTGACATTGAAGATTAAGA
MimRIN_Rad483R	AGATAAATGTGAAGGAAYTCAACTGC
PolyT	GACTCGACTCGACATCGATTTTTTTTTTTTTTTT
For *CYC2-like* gene probes	
Forward	
LigLUC334F	TTCTAACGGTCACAACATTTGC
LigLUC489F	CAAACTGCAATTCTTCACCAAA
PolPRE_CYC2_363F	ACCTGATGCAACCGAGAACT
PolPRE_CYC2A_421F	GCCCTATTTGACCAGCGTTA
PolPRE-CYC2B-643F	GTCAAAAGGCACATCGAACAT
MimRINCYC2A_700F	TCACAATCCAACCTCTGTGC
MimRINCYC2B_764F	GGGATATGGTGAGCAGCTTTAC
Reverse	
PolPRE-CYC2A-604R	TTCCAAAAGCCAACAGGTTC
PolPRE-CYC2B-748R	GTTTCATGCCTTGGAGGTCA
MinRINCYC2_867R	CCAGACAAAGTATTACATCCTCTGA
PolyT	GACTCGACTCGACATCGATTTTTTTTTTTTTTTT
***RAD-like* gene amplification**	

Forward	
RAD40F	TGG WCC GCS AAG GAR AAC AAR G
RAD41F	GGW CCG CSA AGG ARA ACA ARG
RAD46FA	GCS AAG GAR AAC AAR GMS TTC GA
RAD46FB	GCS AAG GAR AAC AAR GMS TTC G
RAD44FA	CC GCS AAG GAR AAC AAR GMS TTC G
RAD44FB	CC GCS AAG GAR AAC AAR GMS TT
RAD49F	AAGGARAACAARGMSTTYG
RAD88FA	GACAARGACAYNCCNGANMGKTGG
RAD88FB	GACAARGACAYNCCNGANMG
RAD149F	ARG AAG TKA AGA RRC AYT AYG
RAD152F	AAG ARR CAY TAY GAA RTT CT
RAD154F	GTK AAG ARR CAY TAY GAA RTT C
RAD217F	CCCWTYCCYAAYTACAGGRC
Reverse	
RAD232R	CWC SRG KDG TMM TRT AST TVG G
RAD152R	TCATARTGYCTCTTVACYTCYT
RAD154R	TCR TAR TGY YTC TTN ACY TC
PolyT	GACTCGACTCGACATCGATTTTTTTTTTTTTTT

CHAPTER 3. CONVERGENT EVOLUTION OF MATURE RADIALLY SYMMETRICAL COROLLAS IS DUE TO DISTINCT DEVELOPMENTAL GENETIC TRAJECTORIES IN THE LAMIALES

Abstract - Bilaterally symmetrical corollas have evolved multiple times independently from radially symmetrical ancestors and are thought to represent adaptations to pollinators. However, evolutionary losses of bilateral symmetry have occurred sporadically in different evolutionary lineages, either by modification of bilaterally symmetrical corollas in late development, or early establishment of radial symmetry. Here, we integrate phylogenetic, developmental and gene expression approaches to evaluate the possible developmental trajectories and genetic mechanisms underlying independent evolutionary losses of bilaterally symmetrical corollas. We compare three species of Lamiales with radially symmetrical corollas and find that each reaches radial symmetry in a different way. In particular, the development and expression pattern of *CYC2*-like genes in *Lycopus americanus* are similar to those of their bilaterally symmetrical relatives, expanded expression of *CcCYC2A* correlates with the radially symmetrical corolla in *Callicarpa cathayana*, and loss of *CYC2A* and altered expression of *CYC2Bs* may account for the early bilateral symmetry but late radial symmetry in *Mentha longifolia*. Furthermore, expression of *RAD*-like genes, the downstream target of *CYC2*-like genes are not detected in *Lycopus americanus* or *Mentha longifolia*, which may further explain the late radial symmetry in these two species. By contrast, *CcRAD* in *Callicarpa cathayana* resembles the broad expression pattern in floral tissues as *CYC2*-like genes.

Key words: Convergence, Corolla symmetry, *CYC2*-like genes, *RAD*-like genes, Lamiaceae

Introduction

The repeated evolution of similar features among distantly related lineages has commonly been considered evidence of adaptation to similar selective pressures. However, increasing evidence shows that convergent features can arise through developmental constraints that may produce biased phenotypic variation upon which natural selection can act. To address how complex traits are repeatedly gained and lost, we focus on the evolution of floral symmetry in the plant order Lamiales, a group that accounts for about 9% of angiosperm diversity. Most species of Lamiales have two-lipped (bilabiate) corolla with a single plane of symmetry (bilateral symmetry, monosymmetry or zygomorphy). The typical two-lipped flower has three distinctive types of petal: two upper (adaxial or dorsal), two lateral, and one lower (abaxial or ventral). The androecium of typical two-lipped flowers is also bilaterally symmetrical, with two pairs of stamens of different lengths (didynamous stamens). However, within core Lamiales, a number of unrelated species or genera have almost or completely radially symmetrical (actinomorphic or polysymmetrical) flowers. These atypical flowers were previously considered to be retained from radially symmetrical ancestors while recent phylogenetic analyses indicate it is more likely that they were derived from bilaterally symmetrical flowers (e.g. Donoghue *et al.*, 1998; Endress, 2011).

Genes required for the normal development of bilaterally symmetrical flowers have been identified in *Antirrhinum majus* L. (Plantaginaceae, Lamiales). Two closely related TCP genes *CYCLOIDEA* (*CYC*) and *DICHOTOMA* (*DICH*) (Luo *et al.*, 1996, 1999) demarcate the adaxial part of the flower and affect the development of floral organs along the adaxial-abaxial axis. Flowers of *cyc/dich* double mutants of snapdragon are radially symmetrical. These two genes are expressed exclusively in the adaxial and adjacent lateral regions of the flowers (Luo *et al.*, 1996, 1999). CYC/DICH affect floral bilateral symmetry mostly through their downstream target, RADIALIS (RAD), a MYB transcription factor (Corley *et al.*, 2005). *RAD* mutants also have almost radially symmetrical flowers, and functional analyses have indicated that *RAD* expression is up-regulated by CYC/DICH. Phylogenetic analyses of *CYC2*-like genes and *RAD*-like genes across the Lamiales show that most members within higher core Lamiales (HCL) have

two copies of *CYC2*-like genes (*CYC2A* and *CYC2B*) but have only one copy of *RAD*-like genes. In addition, *CYC2A* and *CYC2B* result from a common duplication event after the divergence of Plantaginaceae and HCL and exhibit relatively conserved expression pattern across the higher core Lamiales in that the expression of *CYC2A* is confined to the adaxial/lateral region of the flower, whereas *CYC2B* is expressed broadly in floral tissues (Zhong and Kellogg, unpublished data).

The mechanisms underlying convergent evolutionary losses of bilateral symmetry remain largely unknown, though much progress has been made in Gesneriaceae, Plantaginaceae and Malpighiales (Zhou *et al.*, 2008; Reardon *et al.*, 2009; Zhang *et al.*, 2010; Preston *et al.*, 2011). The development of derived radially symmetrical mature flowers in Gesneriaceae and Plantaginaceae show that most mature radially symmetrical flowers exhibit an early asymmetrical developmental pattern along the adaxial-abaxial flower axis (Zhou *et al.*, 2008; Preston *et al.*, 2009). For example, a flower that is radially symmetrical at maturity may have been bilaterally symmetrical early in development (Zhou *et al.*, 2008). However, it remains poorly known whether early asymmetrical developmental pattern along the adaxial-abaxial axis is true for all derived radially symmetrical flowers at maturity within core Lamiales. Thus, In order to evaluate the developmental and genetic mechanisms that underlie independently derived radial symmetry, we integrate phylogenetic, developmental and gene expression approaches focusing on three Lamiaceae species (*Callicarpa cathayana*, *Lycopus americanus* and *Mentha longifolia*) in which radially symmetrical flowers have evolved independently (Fig. 1) (Donoghue *et al.*, 1998).

Materials and Methods

Plant Materials

Three Lamiaceae species - *Callicarpa cathayana*, *Lycopus americanus* and *Mentha longifolia* - were sampled; all have radially symmetrical flowers at maturity. Developing inflorescences and flowers were collected from the greenhouse at the Missouri Botanical Garden (MBG) or Shaw Nature Reserve in Missouri. Materials were collected in RNAlater (AMBION, USA) for RNA extraction or fixed in FAA (50 ml: 25 ml 95%

Ethanol, 5 ml 37% Formaldehyde, 2.5 ml Glacial Acetic Acid, 17.5 ml diethylpyrocarbonate-treated distilled-H_2O (Depc-dH_2O), and dehydrated in a graded series of ethanol. Dehydrated materials further went through a graded series of Histo-Clear (National Diagnostics, USA) and a subset of tissues was embedded in Paraplast for *in situ* RNA hybridization.

Phylogenetic analyses of *CYC2*-like genes in Lamiaceae

Plant leaf material from single individual plants was collected for 10 Lamiaceae species (Supporting information, Table S1). *CYC2*-like genes were isolated with multiple sets of degenerate primer pairs as described in a previous study (Zhong and Kellogg, unpublished data). Phylogenetic analyses were conducted using RAxML (Ludwig *et al.*, 2002) as described by previous study (Zhong and Kellogg, unpublished data).

Scanning Electron Microscope

FAA-fixed inflorescence tissues were dissected as necessary to reveal internal floral organs, and dried with a Tousimis Critical Point Dryer. Specimens were mounted on stubs, sputter-coated with Argon using a Tousimis Sputter Coater, and examined using a scanning electron microscope (Hitachi S-2600H) at Washington University-St. Louis. Photographs were adjusted for brightness, contrast and color balance using GIMP 2.8.

***In situ* RNA hybridization**

Total RNA was isolated using TRI Reagent (AMBION, USA). Paralog-specific forward primers were designed from previously published genomic sequences (Zhong and Kellogg, unpublished data). Paralog-specific forward primers were paired with a universal Poly-T primer (5'- GACTCGACTCGACATCGATTTTTTTTTTTTTTT-3') and were used to amplify parts of the coding region and the 3'-Untranslated Region (3'-UTR) of both *CYC2*-like paralogs and *RAD*-like genes from each species. Gene- (paralog) specific forward and reverse primers (Supporting information, Table S2) were then designed and used to amplify only the 3'-UTR and a small portion of the coding region (less than 100 bps). The reverse transcriptase PCR (RT-PCR) used SuperScript® III One-Step RT-PCR System with Platinum®Taq (INVITROGEN, USA) and started with cDNA synthesis for 30 minutes at 60^0 C, and 35 cycles of regular PCR amplification. Amplified

products were subsequently subcloned to *pGEM*®-T *Vector* (Promega, USA) and sequenced to verify the identity and direction of the inserts. Sense and anti-sense probes were ca. 200-400 basepairs (bps) long and were generated by in vitro transcription with MEGAscript® T7/SP6 kits (AMBION, USA) and labeled by Digoxigenin-UTP (ROCHE, USA) to facilitate later antibody binding for visualization (Malcomber & Kellogg, 2004). Expression of *CYC2*-like and *RAD*-like genes was examined using *in situ* RNA hybridization following previous protocols for plant material (Malcomber & Kellogg, 2004). The only difference was that purified probes were used directly for *in situ* RNA hybridization without hydrolysis. *In situ* hybridization results were inspected and visualized using OLYMPUS BX40 at the MBG. Images were adjusted for the brightness, contrast and color balance using GIMP 2.8.

Results

Floral development

Flowers of *Lycopus americanus* are bilaterally symmetrical during the early developmental stages, and initiate five sepals and petals but only four stamens (Fig. 3, a-f). The two adaxial petals are partly fused at early developmental stages and appear as single petal during later developmental stages (Fig. 3, d-f). The corolla shifts from early bilateral to late nearly radial symmetry. In addition, the paired abaxial stamens appear to be well developed during early stages but this development is arrested after anthesis (Fig. 3. d-f).

The flowers of *Mentha longifolia* are also bilaterally symmetrical at the early developmental stages of calyx initiation (Fig. 4a). In contrast, petal symmetry changes from almost radially symmetrical (Fig. 4b) at very early developmental stages to clearly bilaterally symmetrical in later flower development (Fig. 4c, d) to nearly radially symmetrical before anthesis (Fig. 4e, f). In addition, petal number changes during organ initiation and subsequent developmental stages. Five petal primordia are initiated (Fig. 4, b), but the two adaxial petals subsequently fuse and appear as a single adaxial petal during later developmental stages (Fig. 4, c, d, e). Furthermore, the adaxial stamen is likely initiated but not fully developed and becomes arrested at a very early stage (Fig. 4,

b versus c, d, f). The stamens seem to be morphologically identical before anthesis (Fig. 4, f).

In contrast to the other two species, flowers of *Callicarpa cathayana* appear to be truly tetramerous with four sepals, four petals and four stamens initiated and developed. The calyx is bilaterally symmetrical in very early development (Fig. 5, a) but shifts to radially symmetry before petal initiation (Fig. 5, b). The corolla appears to be radially symmetrical through petal initiation to late developmental stages (Fig. 5, c-f). Aestivation of petals differs between *Mentha* and *Callicarpa*, and is descending-cochlear (i.e. the adaxial corolla lobes are outside the others in bud) versus ascending-cochlear (i.e. the abaxial corolla lobes are outside the others), respectively (Fig. 4, e versus 5, e). Thus despite their similarity in corolla symmetry at maturity, the four-petaled radially symmetrical corollas of *Mentha* and *Callicarpa* differ structurally and developmentally.

Phylogenetic analyses of *CYC2*-like genes in Lamiaceae

Two major clades of *CYC2*-like genes (*CYC2A* and *CYC2B*) in Lamiaceae are strongly supported (Fig. 2; pp=1.0). *Callicarpa cathayana* and *Lycopus americanus* both have *CYC2A* and *CYC2B*, whereas no *CYC2A* was amplified for *Mentha longifolia*, instead, two copies of *CYC2*-like genes isolated from *Mentha longifolia* both fall into the *CYC2B* clade.

Expression of *CYC2*-like genes

To determine stage- and tissue-specific expression patterns of *CYC2*-like genes and test if changes in gene expression pattern correlate with floral transitions from bilateral to radial symmetry, we conducted *in situ* RNA hybridizations on all three species.

Lycopus americanus CYC2A (*LaCYC2A*) is expressed in adaxial sepals early in sepal initiation (Fig. 6). However, the asymmetrical expression of *LaCYC2A* is not detected in later stages of flower development with apparent *LaCYC2A* transcripts being detectable in all petals (Fig. 6). In contrast to early asymmetrical expression of *LaCYC2A*, *Lycopus americanus CYC2B* (*LaCYC2B*) expression is found widely throughout the floral meristem at the very early developmental stages but not in subtending leafy bracts (Fig.

6). After floral organ initiation, *LaCYC2B* is later expressed in all sepals, petals, abaxial stamens and gynoecium but not in bracts (Fig. 6).

The *Mentha longifolia CYC2*-like gene *MlCYC2B1* is expressed widely during sepal initiation (Fig. 7). However, *MlCYC2B1* expression is subsequently down-regulated in later stages (Fig. 7). In contrast to the early expression of *MlCYC2B1* during sepal initiation, the other copy of *Mentha longifolia CYC2*-like genes *MlCYC2B2* is not detectable in flowers in any developmental stages and appears to be expressed in the development of vascular tissues (Fig. 7).

Callicarpa cathayana (Lamiaceae) has radially symmetrical flowers and maintains two copies of *CYC2*-like gene. Both copies of *Callicarpa cathayana CYC2*-like genes (*CcCYC2A* and *CcCYC2B*) are strongly expressed at the floral meristem stages, and are not limited to adaxial/lateral region of the flowers but are widely transcribed throughout floral meristem (Fig. 8). During initiation and development of floral parts, *CcCYC2A* and *CcCYC2B* gene transcripts are found in sepals, petals, stamens, gynoecia but not in subtending leafy bracts (Fig. 8). The expression of *CcCYC2s* persists in petals, stamens and gynoecia during later stages of flower development but not in sepals (Fig. 8).

Expression of *RAD*-like genes

No *RAD*-like gene transcripts were isolated from extracted total RNA from *Lycopus americanus* and *Mentha longifolia* developing inflorescences despite using multiple different degenerate primers derived from the conserved MYB domain.

The *Callicarpa cathayana RAD*-like gene (*CcRAD*) resembles the expression of *CcCYC2s*. The *CcRAD* expression is found throughout the meristem and is not asymmetrical. After organ initiation, *CcRAD* is evidently expressed in sepals, petals, stamens, and gynoecia, but not in subtending leafy bracts (Fig. 9). The expression of *CcRAD* is gradually down-regulated during later developmental stages (Fig. 9).

Discussion

Our developmental data indicate that convergent evolution of corolla radial symmetry at maturity is produced by distinct developmental trajectories in *Callicarpa cathayana*,

Mentha longifolia and *Lycopus americanus* (Fig. 10). The sepals of all species initiate in a bilaterally symmetrical pattern in early development. While petal initiation in *Mentha longifolia* and *Lycopus americanus* is also bilaterally symmetrical, it is radially symmetrical in *Callicarpa* and this radial symmetry is retained throughout development (Fig. 5d; Fig. 10c). Thus *Callicarpa* achieves radial symmetry at an earlier developmental stage than the other two species. In *Mentha longifolia* and *Lycopus americanus* the four-lobed corolla is both caused by fusion of the two adaxial petals. However, the timing of fusion occurs at different developmental stages, with early fusion in *Mentha longifolia* and late fusion in *Lycopus americanus* (Fig. 4, c versus Fig. 3, d). Therefore, corolla radial symmetry at maturity is structurally and developmentally different among these three sampled species, which in turn indicates that divergent genetic mechanisms may underlie superficial similarity of corolla symmetry at maturity.

Expression patterns of *CYC2A* and *CYC2B* appear to be broadly conserved in higher core Lamiales (Zhong and Kellogg, unpublished data), including *Lycopus americanus* as shown here. *CYC2*-like genes share a common duplication event that occurred predating the diversification of higher core Lamiales and expression study show that *CYC2A* is expressed asymmetrically in adaxial/lateral petals in *Mimulus ringens* (Phrymaceae) and *Schaueria calicotricha* (Acanthaceae), whereas *CYC2B* is broadly detected in all petals (Zhong and Kellogg, unpublished data). Our *in situ* hybridization data show the same pattern for *LaCYC2A* and *LaCYC2B*. Though the flowers of *Lycopus americanus* appear to be almost radially symmetrical at maturity, our developmental data show that early developmental processes of *Lycopus americanus* resemble the normal development of typical bilaterally symmetrical lipped flowers (Endress, 1999). Therefore, early asymmetrical expression of *LaCYC2A* may account for the early adaxial-abaxial asymmetrical development of flowers.

The shift to floral radially symmetrical flowers from bilaterally symmetrical ancestors may or may not correlate with altered expression of *CYC2*-like genes. In contrast to early asymmetrical expression of *CYC2A* in *Lycopus* and other species with conspicuously bilaterally symmetrical flowers in higher core Lamiales, we showed that the derived radially symmetrical corolla in *Callicarpa cathayana* correlates with the expansion of the expression domain of *CYC2A* gene to the abaxial part of the flowers. Though the relative

roles of *CYC2A* and *CYC2B* in patterning the floral symmetry in higher core Lamiales clade remain functionally unknown, *Mimulus ringens CYC2A* is detected exclusively in adaxial/lateral petals in a pattern similar to *Antirrhinum CYC/DICH* and *Primulina GCYC1C*/*GCYC1D* (Zhong and Kellogg, unpublished data) (Luo *et al.*, 1996; Yang *et al.*, 2012), which thus suggests a conserved role of *CYC2A* in shaping floral bilateral symmetry. Our examination of developmental processes shows that the corolla of the species *Callicarpa cathayana* is radially symmetrical throughout flower development. Therefore, we infer that the expanded expression of *CYC2A* gene to abaxial region during the development of flowers may account for the derived corolla radial symmetry in *Callicarpa cathayana*.

The ectopic expression of *CcCYC2A* resembles in part the *CYC* expression pattern in snapdragon *back-petals* mutants (Luo *et al.*, 1999) and in *Cadia* (Fabaceae) with radially symmetrical flowers at maturity (Citerne *et al.*, 2006). However, the *back-petals* mutant in *Antirrhinum majus* has expanded expression of *CYC2*-like gene in the lateral and abaxial petals at late but not early stages of flower development (Luo *et al.*, 1999). The developmental trajectory of *Cadia* flowers is different from that of *Callicarpa*, and seem to start with a bilaterally symmetrical corolla that becomes radially symmetrical flowers at maturity. The expanded expression in snapdragon *back-petals* mutant is caused by a transposon insertion in *cis*-acting region (ca. 4.2kb upstream of start codon) that normally down-regulates *CYC* transcription during the later developmental stages in wild-type *Antirrhinum* flower (Luo *et al.*, 1999).

Flowers of *Mentha longifolia* are bilaterally symmetrical early in development, however, no asymmetrical expression of *CYC2*-like genes is detected that is correlated with the asymmetrical growth along adaxial-abaxial plane. Two *CYC2*-like genes in *Mentha longifolia* (*MlCYC2B1* and *MlCYC2B2*) were derived from a much more recent duplication and show different expression patterns with one being expressed broadly early in the development of flowers and the other being expressed probably in the development of vascular tissues. *CYC2A* appears to be absent in this species.

Taken together, the three sampled species with reversions to radially symmetrical corollas at maturity show distinct developmental trajectories, different *CYC2*-like gene

number and expression of both *CYC2*-like and *RAD*-like genes. Specifically, development and expression pattern of *CYC2*-like genes in *Lycopus americanus* are similar to those of its relatives with bilaterally symmetrical corollas. Loss of *CYC2A* and altered expression of *CYC2Bs* may account for the early bilateral symmetry but late radial symmetry in *Mentha longifolia*. Expanded expression of *CcCYC2A* correlates with radially symmetrical corolla in *Callicarpa cathayana*. Furthermore, expression of *RAD*-like genes, the downstream target of *CYC2*-like genes, is detected neither in *Lycopus americanus* or *Mentha longifolia*, which may further explain the late radial symmetry in these two species. In contrast, *CcRAD* in *Callicarpa cathayana* is broadly expressed like that *CYC2*-like genes.

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Figure Captions

Fig. 1. Character evolution of corolla symmetry using simplified phylogeny of Lamiaceae. Species in bold and italic are three focal species for this study with radially symmetrical corolla at maturity.

Fig. 2. Phylogeny of *CYC2*-like genes in Lamiaceae. Numbers above the branch show maximum likelihood bootstrap support. Sequences in red are *CYC2*-like genes from three focal species in this study. *Lycopus americanus* (LycopusAME) and *Callicarpa cathayana* (CallicarpaCAT) both have CYC2A and CYC2B that derived from a relatively ancient duplication event predating the diversification of higher core Lamiales, whereas *Mentha Longifolia* (MenthaLON) has two copies of *CYC2*-like genes that were all group with CYC2B sequences.

Fig. 3. Flower development of *Lycopus americanus* with radially symmetrical flowers at maturity (a)-(f); the sepals in (d) were removed. Abbreviations: adaxial, ad; abaxial, ab; lateral, l; bracts, b; sepal, s; petal, p; stamen, st; carpel, c; g, gynoecium adaxial sepal, ads; abaxial sepal, abs; lateral sepal, ls; adaxial petal; abaxial petal, abp; lateral petal, lp; lateral stamen, lst; abaxial stamen, abst.

Fig. 4. Flower development of *Mentha longifolia* with radially symmetrical flowers at maturity (a)-(f); Abbreviations: adaxial, ad; abaxial, ab; lateral, l; bracts, b; sepal, s; petal, p; stamen, st; carpel, c; g, gynoecium adaxial sepal, ads; abaxial sepal, abs; lateral sepal, ls; adaxial petal; abaxial petal, abp; lateral petal, lp; lateral stamen, lst; abaxial stamen, abst.

Fig. 5. Flower development of *Callicarpa cathayana* with radially symmetrical flowers at maturity (a)-(f); Abbreviations: adaxial, ad; abaxial, ab; lateral, l; bracts, b; sepal, s; petal, p; stamen, st; carpel, c; g, gynoecium adaxial sepal, ads; abaxial sepal, abs; lateral sepal, ls; adaxial petal; abaxial petal, abp; lateral petal, lp; lateral stamen, lst; abaxial stamen, abst.

Fig. 6. RNA *In situ* hybridization expression of *CYC2*-like genes during *Lycopus americanus* (Lamiaceae) flower development; (a)-(e) anti-sense probe of *LaCYC2A*; (f)-(g) sense probe of *LaCYC2A*; (h)-(k) anti-sense probe of *LaCYC2B*; (l)-(m) sense probe of *LaCYC2B*. Abbreviations: adaxial, ad; abaxial, ab; lateral, l; bracts, b; sepal, s; petal, p; stamen, st; carpel, c; g, gynoecium adaxial sepal, ads; abaxial sepal, abs; lateral sepal, ls; adaxial petal; abaxial petal, abp; lateral petal, lp; lateral stamen, lst; abaxial stamen, abst.

Fig. 7. RNA *In situ* hybridization expression of *CYC2*-like genes during *Mentha longifolia* (Lamiaceae) flower development; (a)-(b) anti-sense probe of *MlCYC2B1*; (c)-(d) sense probe of *MlCYC2B1*; (e)-(g) anti-sense probe of *MlCYC2B2*; (h) sense probe of *MlCYC2B2*. Abbreviations: adaxial, ad; abaxial, ab; lateral, l; bracts, b; sepal, s; petal, p; stamen, st; carpel, c; g, gynoecium adaxial sepal, ads; abaxial sepal, abs; lateral sepal, ls; adaxial petal; abaxial petal, abp; lateral petal, lp; lateral stamen, lst; abaxial stamen, abst.

Fig. 8. RNA *In situ* hybridization expression of *CYC2*-like genes during *Callicarpa cathayana* (Lamiaceae) flower development; (a)-(e) anti-sense probe of *CcCYC2A*; (f)-(i) sense probe of *CcCYC2A*; (j)-(k) anti-sense probe of *CcCYC2*B (l)-(m) sense probe of *CcCYC2B*. Abbreviations: adaxial, ad; abaxial, ab; lateral, l; bracts, b; sepal, s; petal, p; stamen, st; carpel, c; g, gynoecium adaxial sepal, ads; abaxial sepal, abs; lateral sepal, ls; adaxial petal; abaxial petal, abp; lateral petal, lp; lateral stamen, lst; abaxial stamen, abst.

Fig. 9. RNA *In situ* hybridization expression of *RAD*-like genes during *Callicarpa cathayana* (Lamiaceae) flower development; (a)-(d) anti-sense probe of *CcRAD*; (e)-(f) sense probe of *CcRAD*. Abbreviations: adaxial, ad; abaxial, ab; lateral, l; bracts, b; sepal, s; petal, p; stamen, st; carpel, c; g, gynoecium adaxial sepal, ads; abaxial sepal, abs; lateral sepal, ls; adaxial petal; abaxial petal, abp; lateral petal, lp; lateral stamen, lst; abaxial stamen, abst.

Fig. 10. Summary of floral development trajectories and gene expression pattern of three focal species in this study. * indicate the early expression in all sepals but not in any petals.

Figures

Fig. 1.

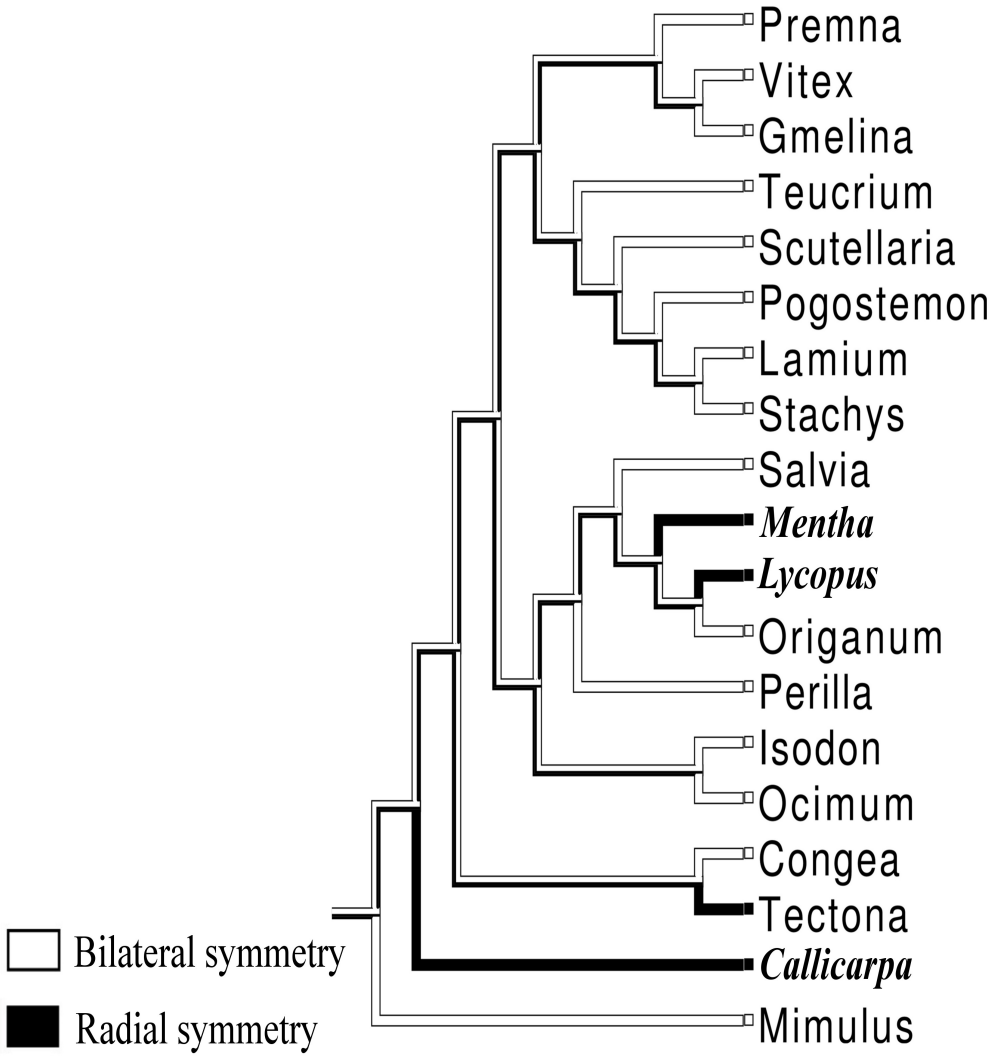
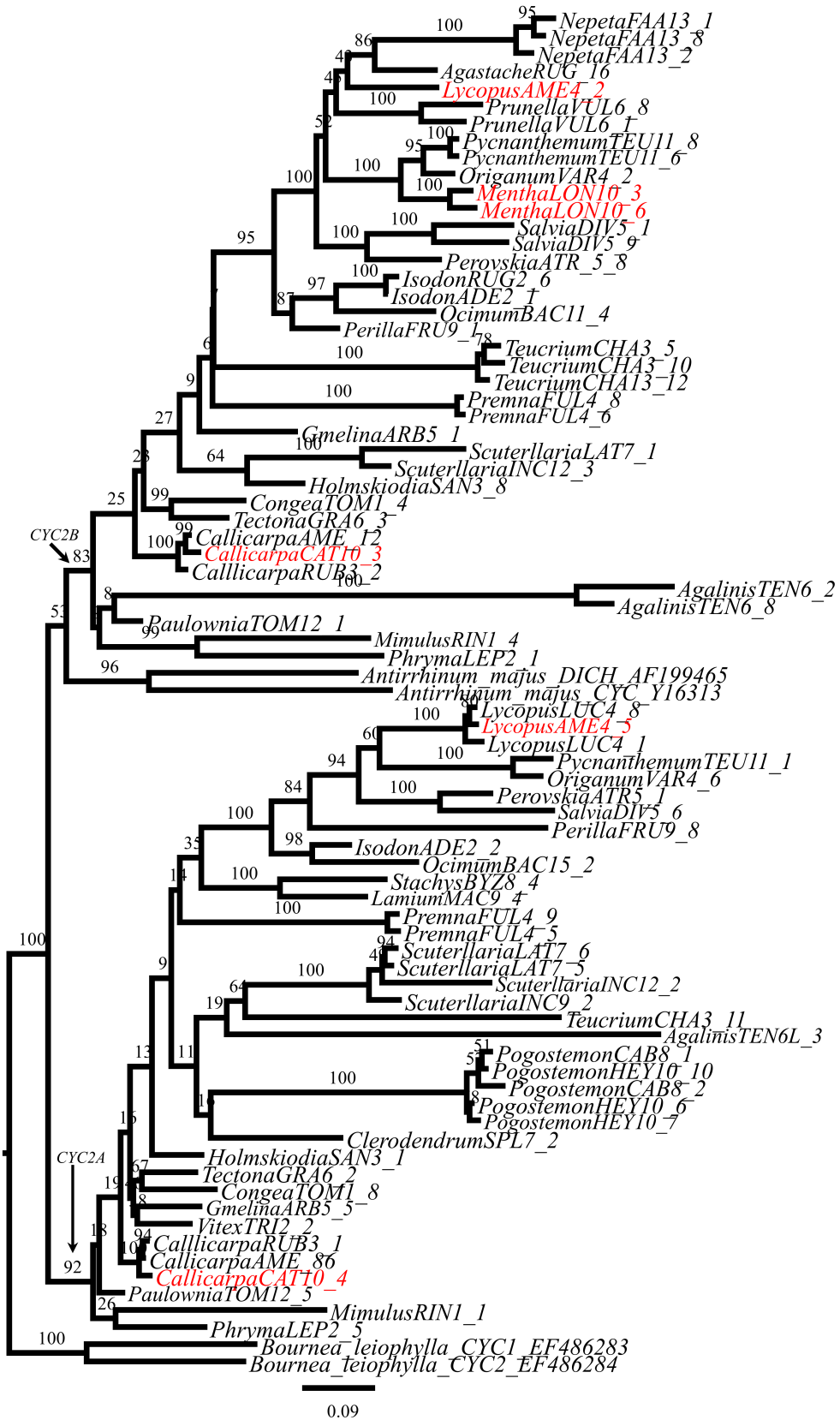


Fig. 2.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OriganumVAR4_2
MenthaLON10_3
MenthaLON10_6
SalviaDIV5_1
SalviaDIV5_9
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OcimumBAC11_4
PerillaFRU9_1
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TeucriumCHA3_10
TeucriumCHA13_12
PremnaFUL4_8
PremnaFUL4_6
GmelinaARB5_1
ScuterllariaLAT7_1
ScuterllariaINC12_3
HolmskiodiaSAN3_8
CongeaTOM1_4
TectonaGRA6_3
CallicarpaAME_12
CallicarpaCAT10_3
CalllicarpaRUB3_2
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PaulowniaTOM12_1
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CYC2B
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Fig. 3.

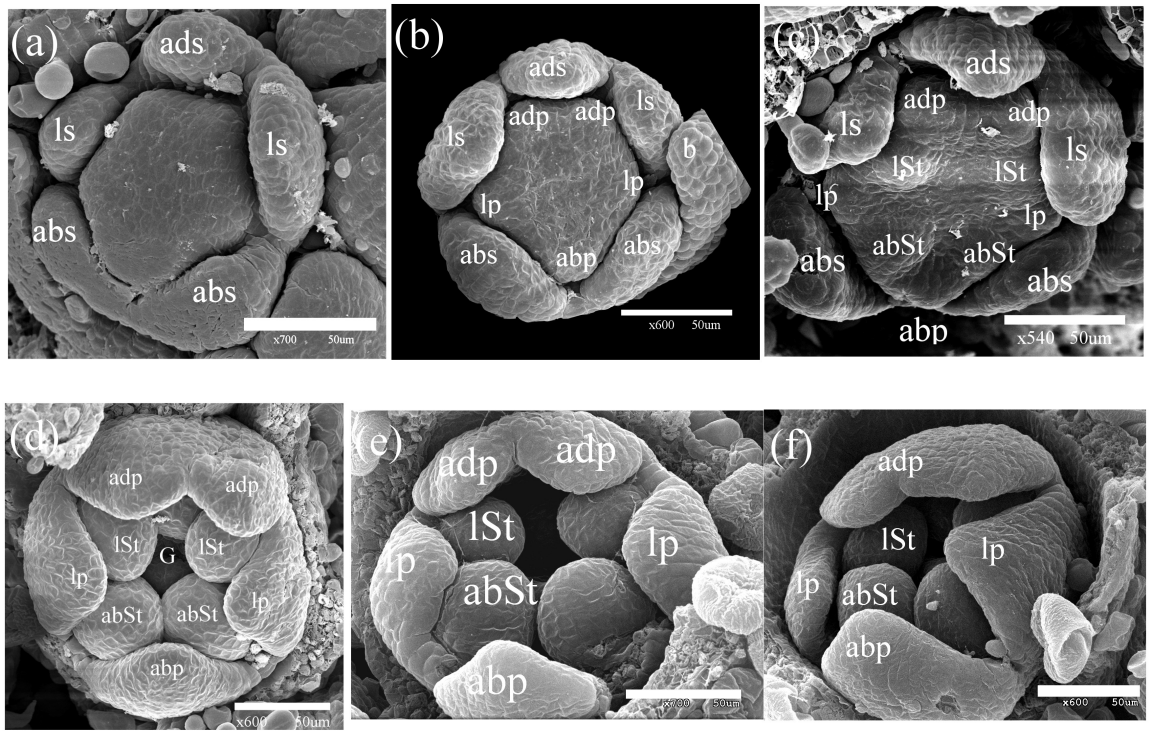
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Fig. 4.

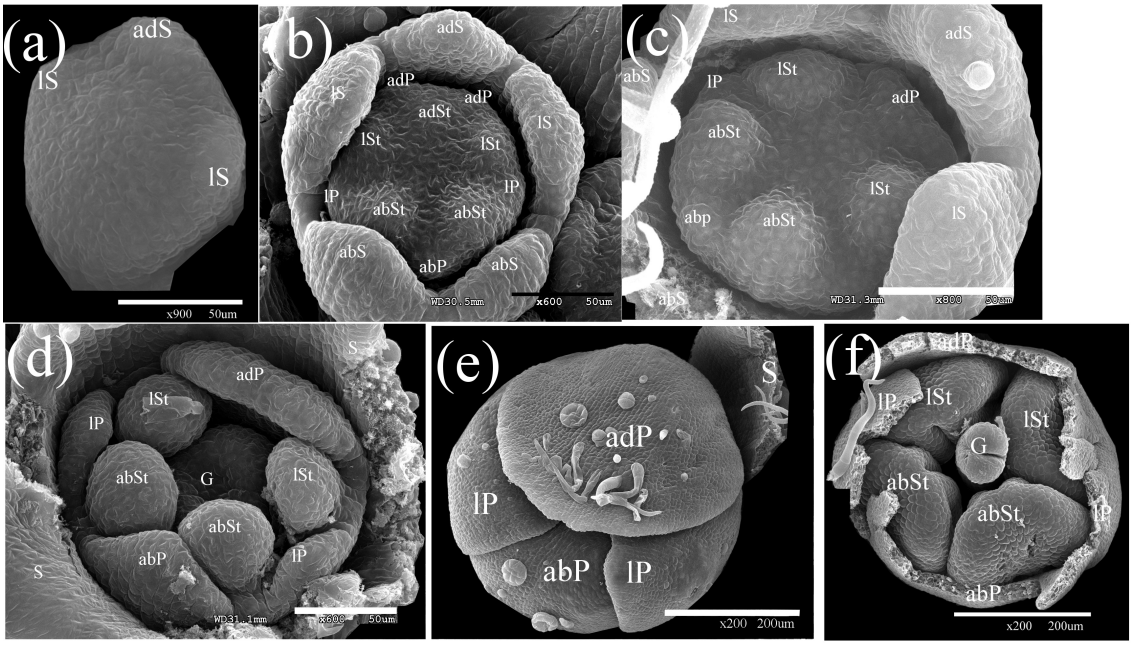
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Fig. 5.

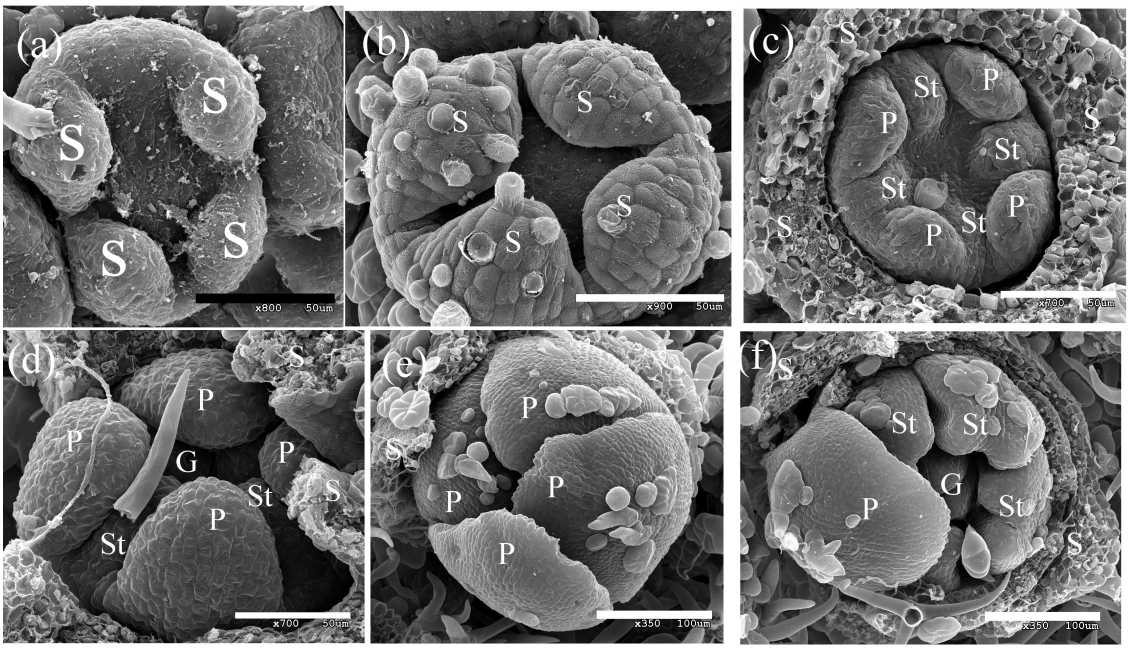
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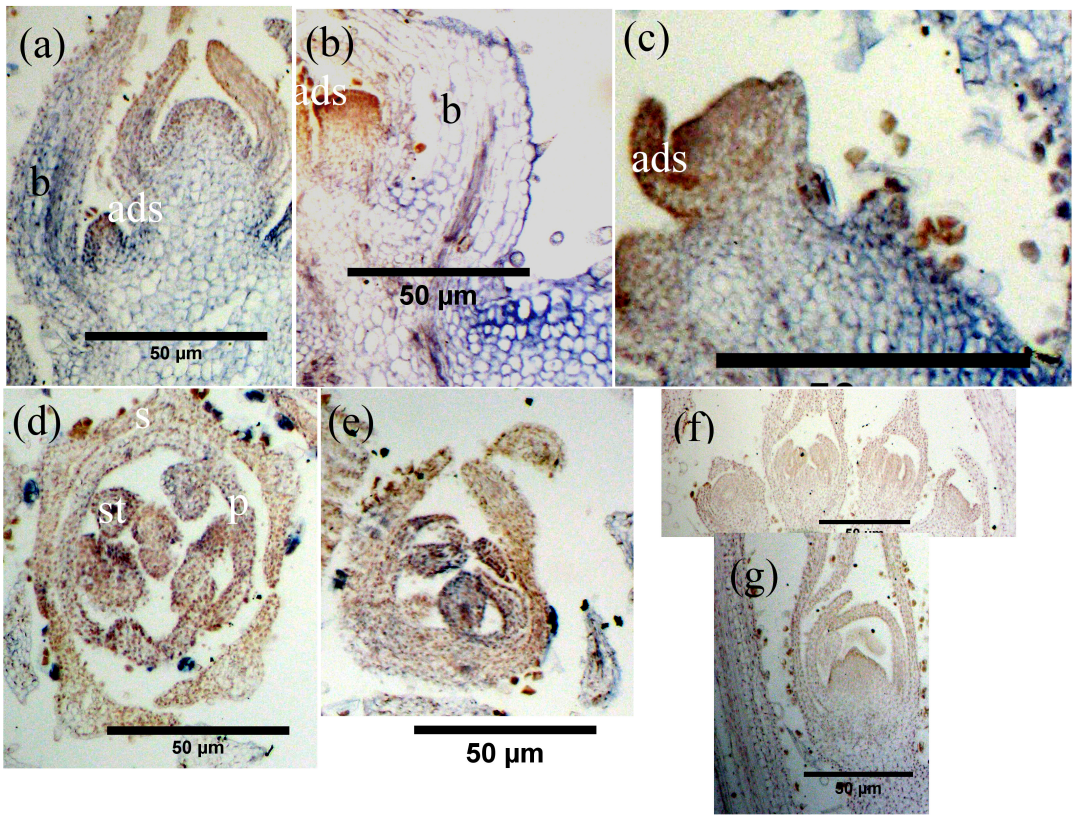
Fig. 6.



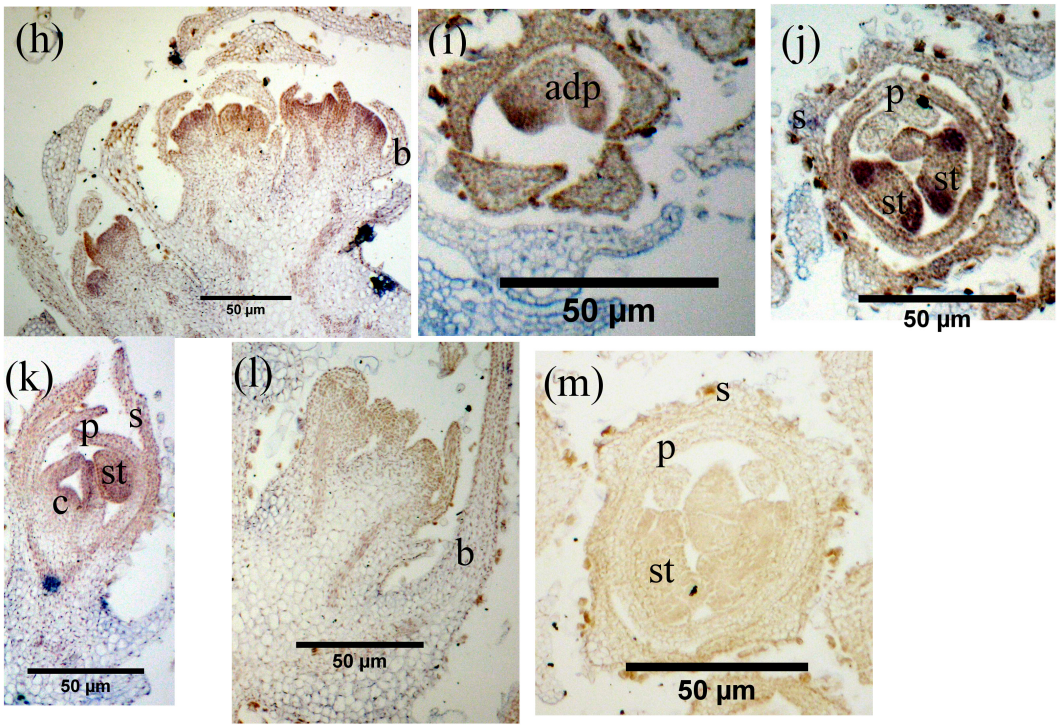

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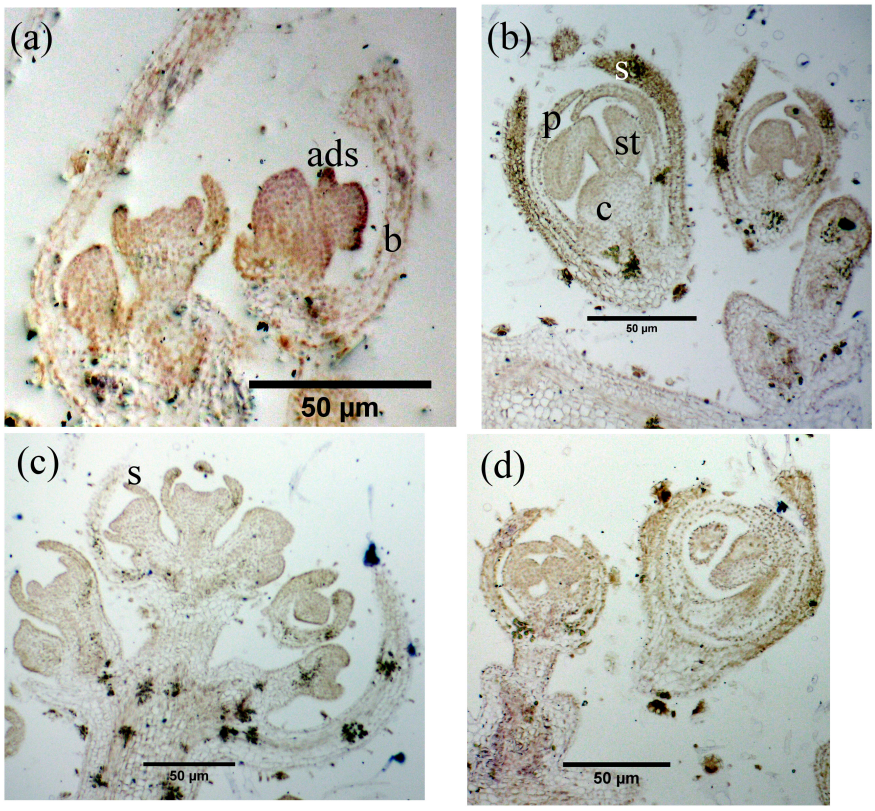
Fig. 7.



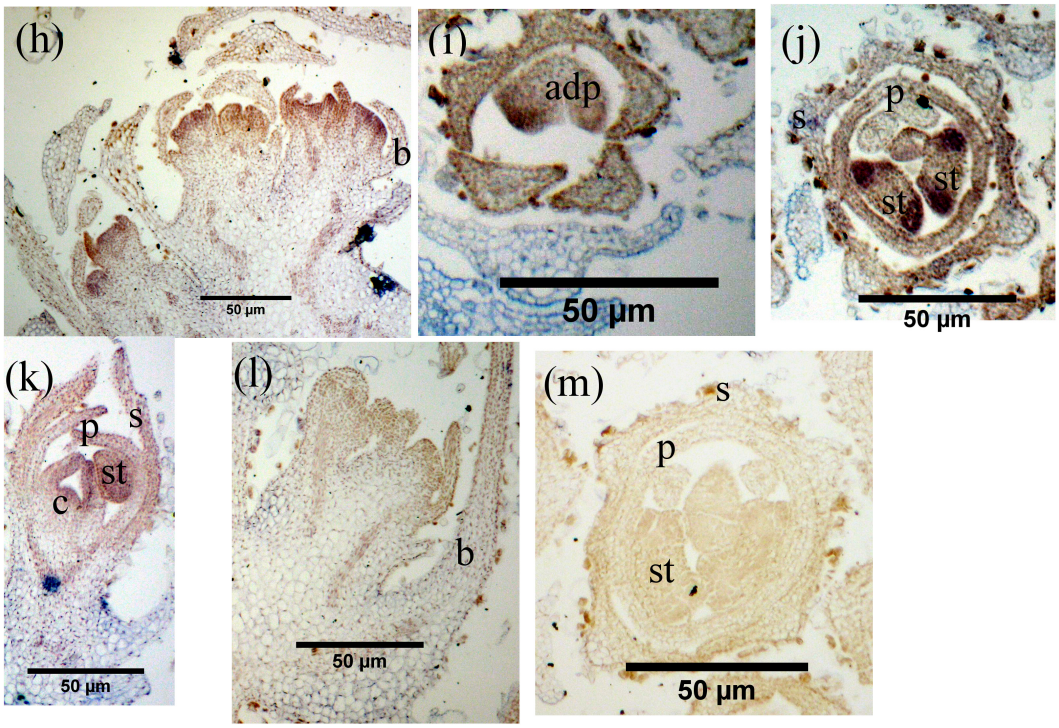

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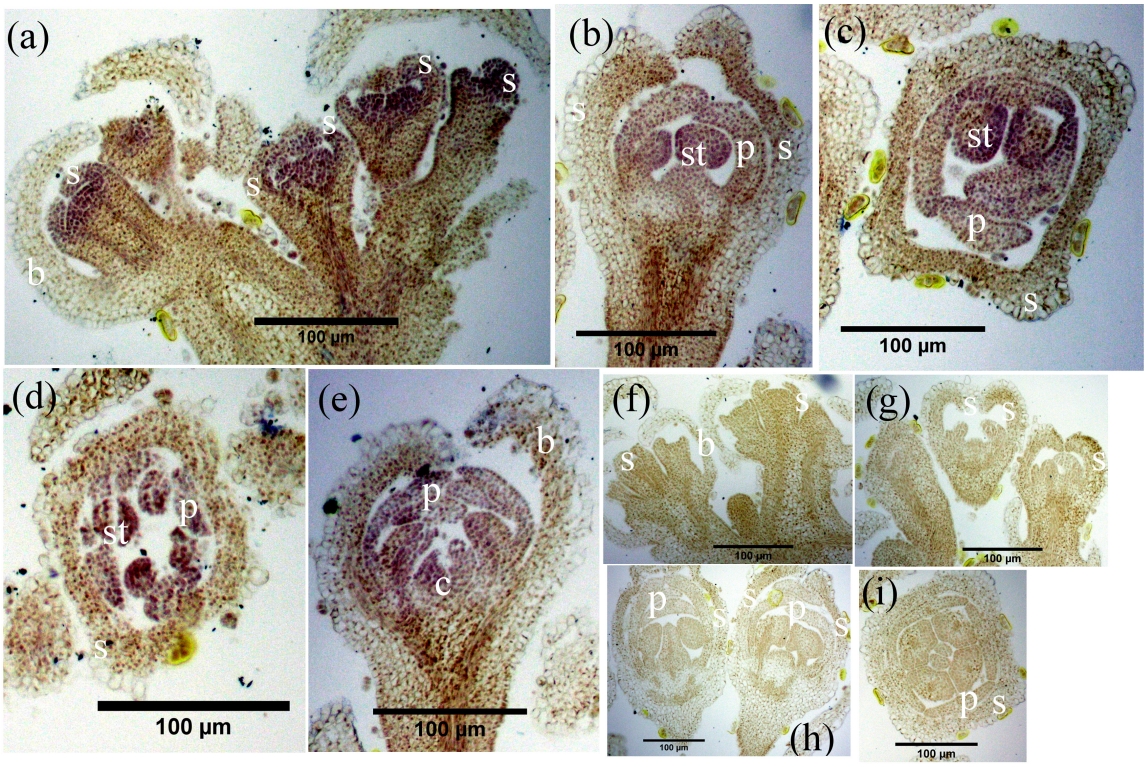
Fig. 8.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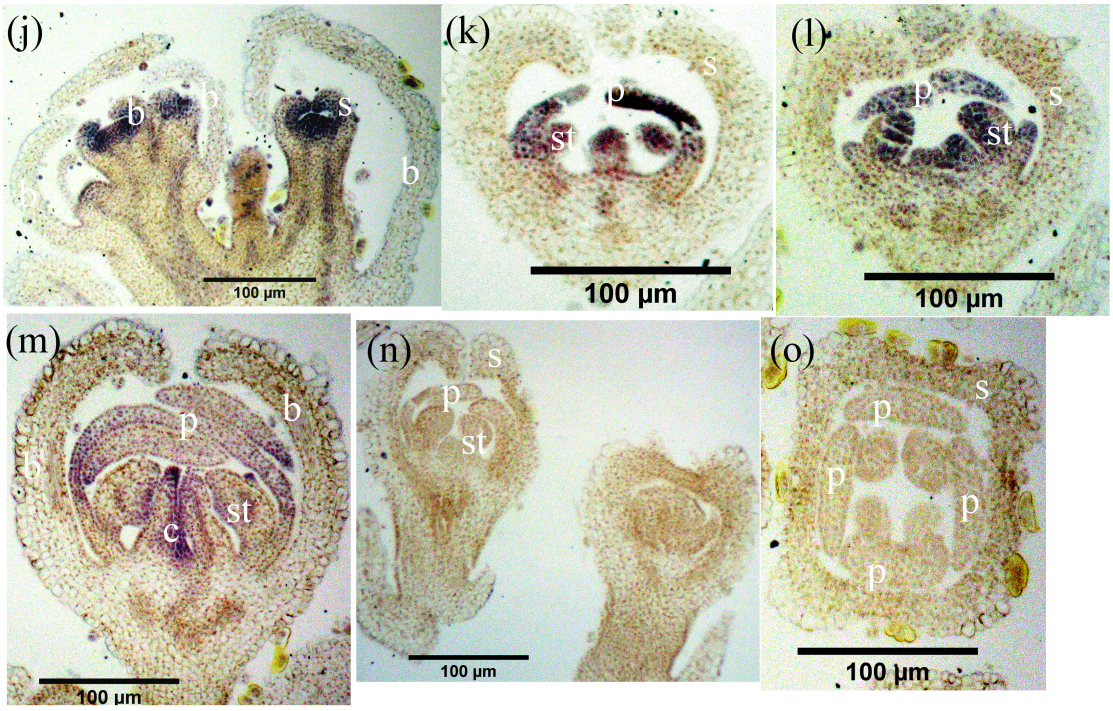
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Fig. 9.

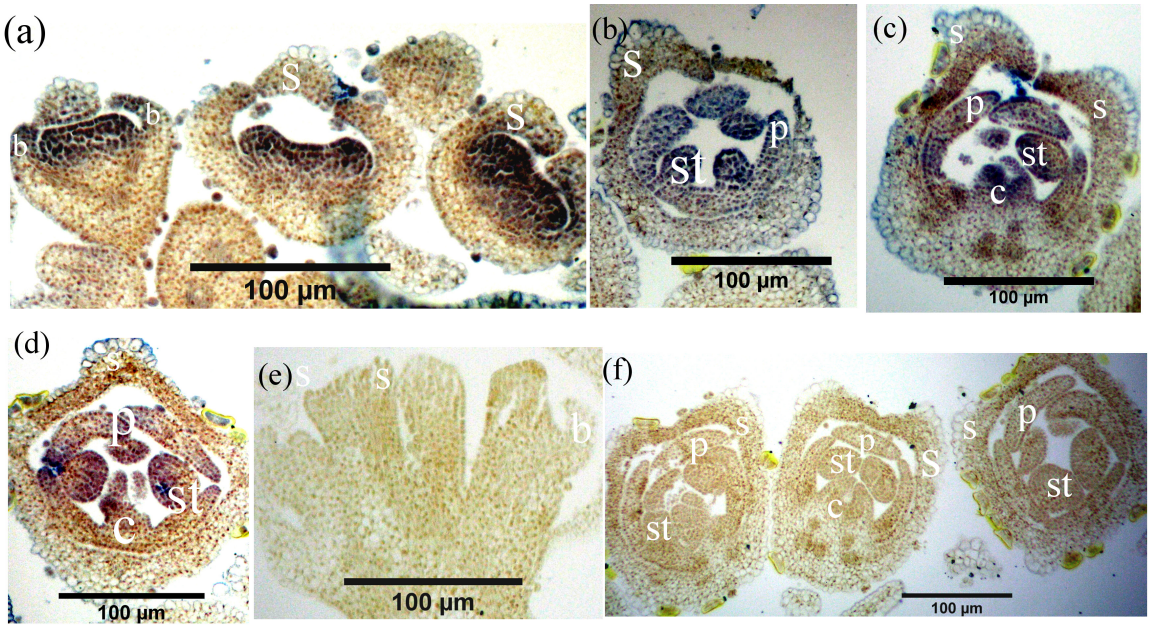
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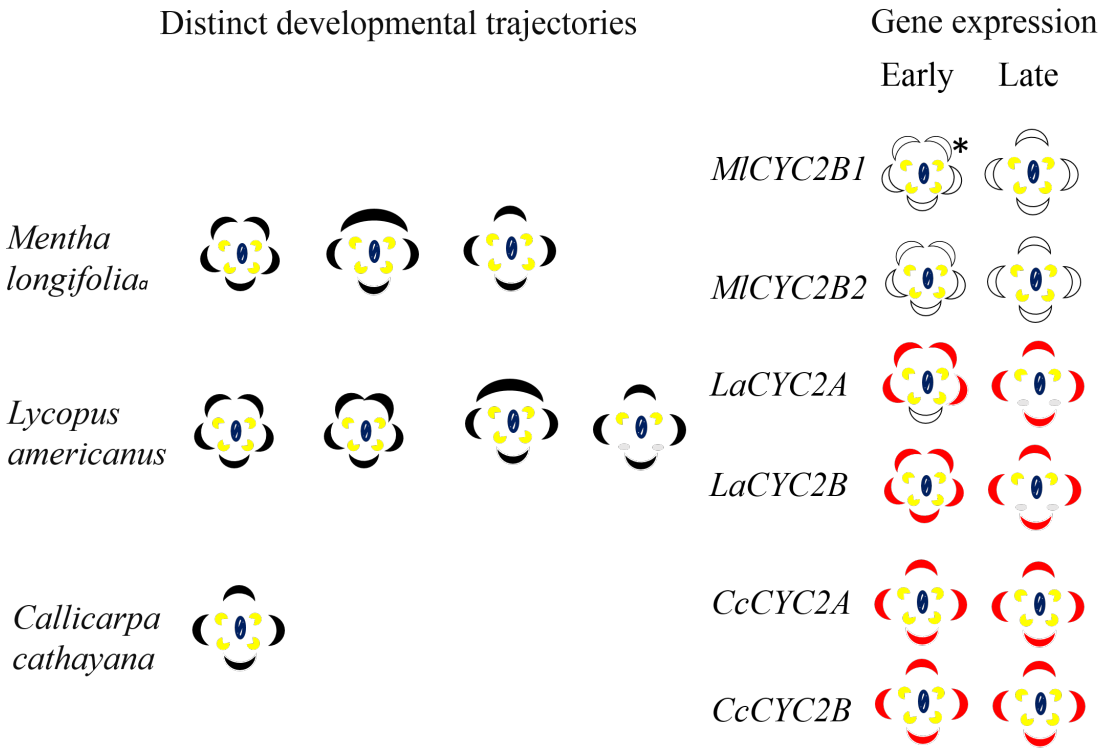
Fig. 10.


Distinct developmental trajectories
Gene expression
Early
Late
Mentha longifolia
Lycopus americanus
Callicarpa cathayana
MlCYC2B1
MlCYC2B2
LaCYC2A
LaCYC2B
CcCYC2A
CcCYC2B
*

Supporting Information

Table S1. Newly sampled Lamiaceae species for the amplification of *CYC2*-like genes for this study on the convergent evolution of radially symmetrical corolla.

VOUCHER	TAXON	SOURCE
2009002	*Nepeta faassenii*	Missouri Botanical Garden
2009020	*Callicarpa dichotoma* (Lour.) K. Koch	Missouri Botanical Garden
2009025	*Salvia divinorum* Epling & Jativa	Missouri Botanical Garden
2010003	*Vitex trifolia* L.	Xishuangbanna, CHINA
2010004	*Callicarpa rubella* var. *crenata* C. P'ei	Xishuangbanna, CHINA
2010016	*Isodon rugosiform (*Hand.-Mazz) H.Hara	Kunming, Xishan, CHINA
2010018	*Isodon adenanthus* (Diels) Kudo	Kunming, Xishan, CHINA
2010020	*Lycopus lucidus* Turcz.	Kunming, KIB, CHINA
2010040	*Lycopus americanus* Muhl.	Shaw Nature Reserve
2010049	*Prunella vulgaris* L.	Shaw Nature Reserve

Table S2. Primers used for the preparation of locus-specific probes in this study. Abbreviations: LycAME, *Lycopus americanus*; CalCAT, *Callicarpa cathayana*; MenLON, *Mentha longifolia*.

Name	**Primer sequences: 5' to 3'**
Forward	
***CYC2*-like genes**	
LycAMECYC2A_471F	TGC AGC TGA TGG ATT TCG TG
LycAMECYC2B_377F	AAG CTC AAC GAA ACC AGA AAC
LycAMECYC2B_771F	TTGGGATCACCACAAGTTCA
LycAMECYC2A_731F	ATGTTTTTGGCCTCAACTCG
MenLON619F	CGTCCAAACCCCTTACAATG
MenLON679F	TCGATCACCACAAGTTCATCA
CalCATCYC2A_441F	CAGGAACACGGGTTCTGATT
CalCATCYC2A_811F	TATGCCATTTTGGATCAGCA
CalCATCYC2B_466F	CCCTCGATCCCAACTCAGTA
CalCATCYC2B_689F	TCTTGGGATTCCAGCAAAAC
***RAD*-like genes**	
CalCAT_RAD_183F	CTACAGGACCMCCGGAGG

CalCAT_RAD_171F	ACCCTYTCCCAACTACAGGAC
RAD40F	TGG WCC GCS AAG GAR AAC AAR G
RAD41F	GGW CCG CSA AGG ARA ACA ARG
RAD44FA	CC GCS AAG GAR AAC AAR GMS TTC G
RAD44FB	CC GCS AAG GAR AAC AAR GMS TT
RAD46FA	GCS AAG GAR AAC AAR GMS TTC GA
RAD46FB	GCS AAG GAR AAC AAR GMS TTC G
RAD49F	AAGGARAACAARGMSTTYG
RAD88FA	GACAARGACAYNCCNGANMGKTGG
RAD88FB	GACAARGACAYNCCNGANMG
RAD149F	ARG AAG TKA AGA RRC AYT AYG
RAD152F	AAG ARR CAY TAY GAA RTT CT
RAD154F	GTK AAG ARR CAY TAY GAA RTT C
RAD217F	CCCWTYCCYAAYTACAGGRC
Reverse	
***RAD*-like genes**	
CalCAT_RAD_3UTRL	GTGGAACATTATTGCTTCATTCATAC
CalCAT_RAD_3UTRs	ATTTCRAACAACATKAATTAAAAGG
RAD152R	TCATARTGYCTCTTVACYTCYT
RAD154R	TCR TAR TGY YTC TTN ACY TC
RAD232R	CWC SRG KDG TMM TRT AST TVG G

Supporting information

Table S1. Sampled taxa and GenBank accession number;

VOUCHER	TAXON	SOURCE	FAMILY
G. Yatskievych 11-73	*Polypremum procumbens* L.	United States	Tetrachondraceae
CHW SN9	*Abeliophyllum distichum* Nakai	Cult. MBG, United States	Oleaceae
CHW SN2	*Chionanthus virginicus* L.	Cult. MBG, United States	Oleaceae
CIR 1757	*Comoranthus minor* H. Perrier	Madagarscar	Oleaceae
CHW SN11	*Forsythia suspensa* (Thunb.) Vahl	Cult. MBG, United States	Oleaceae
CHW SN6	*Fraxinus americana* L.	Cult. MBG, United States	Oleaceae
2011020	*Jasminum angustifolium* Willd.	Cult. MBG, United States	Oleaceae
CHW SN7	*Jasminum nudiflorum* Lindl.	Cult. MBG, United States	Oleaceae
2011021	*Jasminum tortuosum* Willd.	Cult. MBG, United States	Oleaceae
CHW SN8	*Ligustrum lucidum* W.T. Aiton	Cult. MBG, United States	Oleaceae
CHW 699	*Noronhia candicans* H. Perrier	Cult. MBG, United States	Oleaceae
CHW SN1	*Olea europaea* L.	Cult. MBG, United States	Oleaceae
CHW SN3	*Osmanthus fragrans* (Thunb.) Lour.	Cult. MBG, United States	Oleaceae
CHW SN5	*Phillyrea angustifolia* L.	Cult. MBG, United States	Oleaceae
CHW SN4	*Syringa pekinensis* Rupr.	Cult. MBG, United States	Oleaceae
	Syringa vulgaris L.	Cult. MBG, United States	Oleaceae
2011015	*Ruellia tweediana* Griseb.	MBG	Acanthaceae
2010058	*Ruellia strepens* L.	Shaw Nature Reserve, United States	Acanthaceae
2010066	*Eranthemum pulchellum* Andrews	Cult. MBG, United States	Acanthaceae
2010070	*Schaueria calicotricha* (Link & Otto) Nees	Cult. MBG, United States	Acanthaceae
2010071	*Hygrophila corymbosa* (Blume) Lindau	Cult. MBG, United States	Acanthaceae
2011006	*Acanthus mollis* L.	Cult. MBG, United States	Acanthaceae
2011019	*Crossandra infundibuliformis* (L.) Nees	Cult. MBG, United States	Acanthaceae

2012004	*Thunbergia erecta* (Benth.) T. Anderson	Cult. MBG, United States	Acanthaceae
2012035	*Thunbergia mysorensis* (Wight) T. Anderson	Cult. MBG, United States	Acanthaceae
T.P.Prinzie 131	*Avicennia germinans* (L.) L.	United States	Acanthaceae
A.F. Fuentes - 5307	*Mendoncia aspera* Ruiz & Pav.	Bolivia	Acanthaceae
RAZANATSIMA - 297	*Mendoncia flagellaris* (Baker) Benoist	Madagascar	Acanthaceae
James S. Miller - 9268	*Mendoncia hoffmannseggiana* Nees	Suriname	Acanthaceae
2010051	*Campsis radicans* (L.) Bureau	Shaw Nature Reserve, United States	Bignoniaceae
2012020	*Amphitecna apiculata* A.H. Gentry	Cult. MBG, United States	Bignoniaceae
2012021	*Amphitecna tuxtlensis* A.H. Gentry	Cult. MBG, United States	Bignoniaceae
2012022	*Anemopaegma orbiculatum* (Jacq.) A. DC.	Cult. MBG, United States	Bignoniaceae
2012023	*Bignonia capreolata* L.	Cult. MBG, United States	Bignoniaceae
2012024	*Catalpa speciosa* Warder ex Engelm.	Cult. MBG, United States	Bignoniaceae
2012025	*Catalpa bignonioides* Walter	Cult. MBG, United States	Bignoniaceae
2012026	*Crescentia cujete* L.	Cult. MBG, United States	Bignoniaceae
2012027	*Crescentia portoricensis* Britton	Cult. MBG, United States	Bignoniaceae
2012028	*Macfadyena tweediana* Griseb. ex Lorentz	Cult. MBG, United States	Bignoniaceae
2012029	*Markhamia lutea* (Benth.) K. Schum.	Cult. MBG, United States	Bignoniaceae
2012030	*Martinella obovata* (Kunth) Bureau & K. Schum.	Cult. MBG, United States	Bignoniaceae
2012031	*Melloa quadrivalvis* (Jacq.) A.H. Gentry	Cult. MBG, United States	Bignoniaceae
2012032	*Phryganocydia corymbosa* (Vent.) Bureau ex K. Schum.	Cult. MBG, United States	Bignoniaceae
2012033	*Tabebuia impetiginosa* (Mart. ex DC.) Standl.	Cult. MBG, United States	Bignoniaceae
2012034	*Tanaecium crucigerum* Seem.	Cult. MBG, United States	Bignoniaceae
2012036	*Jacaranda cuspidifolia* Mart. ex A. DC.	Cult. MBG, United States	Bignoniaceae
2012037	*Oroxylum indicum* (L.) Kurz	Cult. MBG, United States	Bignoniaceae
W.D. Stevens - 28076	*Paragonia pyramidata (Rich.) Bureau*	Nicaragua	Bignoniaceae
A.F. Fuentes - 12782	*Calceolaria engleriana* Kraenzl.	Bolivia	Calceolariaceae
A.F. Fuentes - 12761	*Calceolaria rivularis* Kraenzl.	Bolivia	Calceolariaceae
2009001	*Teucrium chamaedrys* L.	Cult. MBG, United States	Lamiaceae

2009004	*Stachys byzantina* C. Koch	Cult. MBG, United States	Lamiaceae
2009005	*Mentha longifolia* (L.) L.	Cult. MBG, United States	Lamiaceae
2009006	*Scutellaria incana* Vent.	Cult. MBG, United States	Lamiaceae
2009007	*Scutellaria lateriflora* L.	Cult. MBG, United States	Lamiaceae
2009014	*Lamium maculatum* L.	Cult. MBG, United States	Lamiaceae
2009008	*Ocimum basilicum* L.	Cult. MBG, United States	Lamiaceae
2009019	*Origanum vulgare* L.	Cult. MBG, United States	Lamiaceae
2009021	*Holmskioldia sanguinea* Retz	Cult. MBG, United States	Lamiaceae
2010005	*Premna fulva* Craib	China	Lamiaceae
2009003	*Perovskia atrplicifolia* Benth.	Cult. MBG, United States	Lamiaceae
2009023	*Pogostemon heyneanus* Benth.	Cult. MBG, United States	Lamiaceae
2010002	*Congea tomentosa* Roxb.	China	Lamiaceae
2010006	*Gmelina arborea* Roxb.	China	Lamiaceae
2010007	*Tectona grandis* fo. Abludens Koord. & Valeton	China	Lamiaceae
2010054	*Callicarpa cathayana* H.T. Chang	Cult. MBG, United States	Lamiaceae
ZW19	*Pogostemon cablin* (Blanco) Benth.	China	Lamiaceae
2010042	*Agalinis tenuifolia* (Vahl) Raf.	Shaw Nature Reserve, United States	Orobanchaceae
2010026	*Paulownia tomentosa* (Thunb.) Steud.	Cult. MBG, United States	Paulowniaceae
Neil W. Snow - 6857	*Sesamum triphyllum* Welw. ex Asch.	Botswana	Pedaliaceae
2010041	*Phryma leptostachya* L.	Shaw Nature Reserve, United States	Phrymaceae
2010043	*Mimulus ringens* L.	Shaw Nature Reserve, United States	Phrymaceae
2010062	*Scoparia sp*	Cult. MBG, United States	Plantaginaceae
2012038	*Schlegelia parviflora* (Oerst.) Monach.	Cult. MBG, United States	Schlegeliaceae
2010076	*Buddleja davidii* Franch.	Cult. MBG, United States	Scrophulariaceae
Mary Merello - 2377	*Scrophularia orientalis* L.	Republic of Georgia	Scrophulariaceae
Mary Merello - 2244	*Scrophularia variegata* M. Bieb.	Republic of Georgia	Scrophulariaceae
Zhong - 2011013	*Scrophularia marilandica* L.	Shaw Nature Reserve, United States	Scrophulariaceae

Robert M. King - 13931	*Verbascum thapsus* L.	United States	Scrophulariaceae
2011018	*Verbascum chaixii* Vill.	Cult. MBG, United States	Scrophulariaceae
Adam F. Bradley - 1052	*Thomandersia laurifolia* (T. Anderson ex Benth.) Baill.	Gabon, Haut-Ogooue	Thomandersiaceae
2010045	*Lippia nodiflora* Cham.	Shaw Nature Reserve, United States	Verbenaceae
2010050	*Verbena canadensis* (L.) Britt.	Shaw Nature Reserve, United States	Verbenaceae
2010079	*Glandularia canadensis* (L.) Nutt.	Cult. MBG, United States	Verbenaceae
W.D. Stevens - 30011	*Citharexylum schottii* Greenm.	Nicaragua	Verbenaceae
A. Araujo M. - 2112	*Bouchea fluminensis* (Vell.) Moldenke	Bolivia	Verbenaceae
W.D. Stevens - 27497	*Stachytarpheta calderonii* Moldenke	Nicaragua	Verbenaceae
W.D. Stevens - 29615	*Lantana velutina* M. Martens & Galeotti	Nicaragua	Verbenaceae
W.D. Stevens - 29194	*Lippia myriocephala* Schltdl. & Cham.	Nicaragua	Verbenaceae
Charlotte M. Taylor - 11541	*Junellia seriphioides* (Gillies & Hook.) Moldenke	Chile	Verbenaceae
Charlotte M. Taylor - 11607	*Acantholippia trifida* (Gay) Moldenke	Chile	Verbenaceae
W.D. Stevens - 27257	*Rehdera trinervis* (S.F. Blake) Moldenke	Nicaragua	Verbenaceae