Supporting Information

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SI Materials and Methods

Morphology-Based Character State Reconstruction of Floral Symmetry. We used maximum likelihood (ML) character state reconstruction as implemented in Mesquite version 2.6 (1) to infer the evolution of floral symmetry in Malpighiaceae and its closest relatives, Elatinaceae and Centroplacaceae. Floral morphology for 353 species was scored as either zygomorphic or actinomorphic and treated as unordered and optimized onto the rate-smoothed topology for the family (2, 3). Absolute divergence time estimates were ascertained using the methods described by Davis et al. (4, 5) and included nearly one quarter of all species of Malpighiaceae, plus numerous outgroup species of Elantinaceace and Centroplacaceae. These data were analyzed using the Mk1 model (6) with rate parameters estimated directly from the data.

Isolation of CYCLOIDEA Homologues in Malpighiales–Oxalidaceae–Celastrales. We used 3′ RACE to obtain all *CYC* homologues, including *CYC1*, *CYC2*, and *CYC3*, across the closely related clades Malpighiales, Oxalidales, and Celastrales (7). Fresh floral tissue was collected over a broad range of developmental stages (i.e., buds from multiple developmental stages from <1 mm to mature size, and open flowers) from four species of Malpighiaceae (*Byrsonima lucida* DC., *Janusia guaranitica* A. Juss., *Malpighia coccigera* L., and *Tristellateia australasiae* A. Rich.), two additional species of Malpighiales [*Hypericum perforatum* L. (Hypericaceae) and *Euphorbia milii* Des Moul. (Euphorbiaceae)], one species of Oxalidales [*Oxalis herrerae* R. Knuth (Oxalidaceae)], and one species of Celastrales [*Euonymus alatus* (Thunb.) Siebold (Celastraceae)].

Total RNAs were purified using the Concert Plant RNA Reagent (Invitrogen). Single-stranded cDNA was synthesized from 5 μg of total RNA using SuperScript II reverse transcriptase (Invitrogen) by priming with the oligonucleotide 5′-CCGGATC CTCTAGAGCGGCCGC$(T)_{17}$. This poly-T primer was then used with primer 5′-AARGAYMGICAYAGYAARAT (modified from ref. 8) for PCR. PCR products were cleaned with the QIAquick PCR purification kit (Qiagen) and used as the template in a secondary PCR with the degenerate forward primer, 5′-GCIAGRAARTTYTTYGAYYTICARGAYATG, and the poly-T primer described earlier. PCRs were performed in 100 μL of buffer [60 mM Tris-SO_4, pH 8.9; 18 mM $(NH_4)_2SO_4$; 2.5 mM $MgSO_4$] containing 50 pmol 5′ primer, 10 pmol poly-T primer, 25 pmol of dNTPs, and 3.75 units of PlatinumTaq polymerase (Invitrogen). The PCR amplification began with a 12-min activation step at 95 °C, followed by 37 cycles of a 1-min incubation step at 95 °C, a 30-s annealing step at temperatures ranging from 40 °C to 60 °C, and a 1-min extension at 72 °C. The reaction was terminated with a 10-min incubation at 72 °C. Gel-purified secondary PCR products were cloned using the pGEM-T easy vector system (Promega) and XL1-Blue competent cells (Stratagene). Fifty to 200 colonies for each taxon were screened by restriction site analysis and sequencing using the protocols established at Functional Biosciences. The identity of *CYC*-like genes was determined by the presence of the highly conserved TCP domain. Phylogenetic analyses including numerous TCP genes from previously published studies confirmed the identity of *CYC* homologues.

Isolation of CYC2-Like Genes from Malpighiaceae–Elatinaceae–Centroplacaceae–Oxalidaceae. Based on the *CYC*-like gene alignment, we designed degenerate primers to amplify the *CYC2*-like genes from genomic DNA. Our broad taxonomic sampling ensured that these degenerate primers covered a broad range of sequence variation across a diverse set of taxa. One set of nested degenerate primers (i.e., the forward primer located in the TCP domain, 5′-GCIMGIAARTTYTTYGAYYTKCAA, and the reverse primer in the R domain, 5′-GCYCKYGCYCTIGCY-YTHKCYCTWGA), was selected to amplify a 350- to 400-bp fragment of the *CYC2*-like genes from Malpighiaceae and its sister clades. Gel-purified PCR products were cleaned and cloned. Fifty to 200 colonies were initially screened by restriction site analysis, and at least five clones were sequenced for each variant identified using this initial screen. Our criteria to further distinguish these variants included the degree of nucleotide variability and the presence of unique indels. Sequence variants containing no indels and differing by less than 5% sequence similarity were treated as alleles. The coding regions of *CYC2A* and *CYC2B* that fall between the TCP and R domains are 176 ± 14 and 184 ± 5 nucleotides long, respectively. *CYC1*- and *CYC3*-like genes, which were rarely amplified with *CYC2*, were distinguished by using phylogenetic analysis and excluded from subsequent consideration.

Nucleotide Sequence Analyses. Our analyses in the main text focused on amino acid sequence analyses. Analyses using nucleotide sequence data with third codon positions excluded, yielded a topology nearly identical to the amino acid sequence data. The parameters of the best-fit model for our nucleotide data were estimated using MODELTEST 3.06 (9). The Akaike Information Criterion (10) recommended a general time reversible model with added parameters for invariable sites and a Γ distribution ("GTR + I + Γ"). One hundred ML bootstrap replicates were conducted with the optimal model of sequence evolution. Bayesian analyses were also conducted using this model.

Southern Hybridization. Ten micrograms of genomic DNA was digested from *Bergia texana*, *Byrsonima crassifolia, J. guaranitica,* and *T. australasiae* with restriction enzymes (i.e., HindIII, EcoRI, and HindIII plus EcoRI), fractionated on 0.8% agarose gels, and blotted onto a positively charged nylon membrane (GE Healthcare BioSciences). In addition, for *Janusia*, we ran lanes containing *CYC1*, *CYC2*, and *CYC3* plasmid DNA as controls to test probe efficiency and specificity.

A fragment containing the 3′ end of the TCP domain and the variable region between the TCP and R domains was used as a template to synthesize probes for detecting *CYC2*-like genes (Fig. S3*B*). For *J. guaranitica*, a mixture of *JgCYC2A* (*CYC2A*) and *JgCYC2B-3* (*CYC2B*) sequences in equal molar concentration was used as a template to synthesize probes with ^{32}P-dCTP (Perkin-Elmer) using the Prime-It II kit (Stratagene). This gene region exhibits no more than 31% pair-wise sequence similarity among the *CYC1/2/3* paralogues, which ensures probe specificity. The hybridization was carried out in hybridization solution [900 mM NaCl; 60 mM $NaH_2PO_4 \bullet H_2O$; 6 mM EDTA; 5× Denhart solution (from 50×; Amresco); 1% SDS; 10 μg/mL sheared salmon sperm DNA; pH 7.4] at 65 °C for 18 h. The membranes were then washed at low stringency (900 mM NaCl; 60 mM $NaH_2PO_4 \bullet H_2O$; 6 mM EDTA; 1% SDS; pH 7.4) at 65 °C and exposed for 90 h to phosphor imaging (GE Healthcare Bio-Sciences) to detect for the presence of all *CYC2* homologues.

There are four bands for *J. guaranitica* in the EcoRI digest of the *CYC2* probe (Fig. S3*A*). This result is identical to our cloning experiments, which also identified four copies of *CYC2*. The *CYC1* and *CYC3* plasmid controls gave very faint signals experimentally, demonstrating that the *CYC2* probes are lineage specific. The number of bands in the EcoRI digest therefore

reflects the approximate *CYC2* copy number. In the HindIII and double digests, we expected more than four bands as a result of the presence of a restriction site within the probed region.

Given the ability of our probe to detect lineage specific *CYC2* gene copies, we conducted Southern hybridizations only for *CYC2* on the remaining taxa (i.e., *B. texana*, *B. crassifolia,* and *T. australasiae*). *BtCYC2-1* and *BtCYC2-2* of *B. texana*, *BcCYC2A* and *BcCYC2B* from *B. crassifolia*, and *TaCYC2A* of *T. australasiae* were mixed in equal molar concentrations and used as a template to synthesize our ^{32}P-labeled probes. Our *CYC2*-specific probes revealed two bands in *B. crassifolia*, one band in *T. australasiae*, and six bands in *B. texana* (see EcoRI digest in Fig. S4), which was identical to the *CYC2* copy number inferred by PCR and cloning.

Floral Organ Arrangement in New and Old World Malpighiaceae. New World Malpighiaceae, e.g., *B. crassifolia* and *J. guaranitica*, possess a dorsal flag/banner petal that is always innermost in bud. The remaining four petals can be arranged in one of two ways that form mirror images of each other (Fig. S5*A*). These enantiomorphic flowers can be found on the same inflorescence within a single species. The Old World species *T. australasiae* has a similar petal aestivation (Fig. S5*B*; also see main text). The innermost petal of *T. australasiae* is homologous to the New World dorsal flag/banner petal and can also initiate on the left or right side of the dorsoventral plane of symmetry (Fig. S5*B*). We used the relative positions of these floral organs to sample homologous tissue types from these New and Old World species, and took great care to sample only those *Tristellateia* flowers for RT-PCR in which the innermost petal was located on the left side of the dorsoventral plane of symmetry.

Notes on the Floral Orientation of *Bergia texana* and *Bhesa paniculata*. *B. texana* has a single dorsal petal with respect to the stem, which is uncommon relative to most angiosperms, which have one ventral petal (11, 12). Two dorsal and one ventral sepal are glandular, and two lateral sepals are eglandular (Fig. 3 in the main text). Moreover, the two dorsal sepals tightly clasp the stem, so it is relatively easy to determine that there is a single dorsal petal in *B. texana*. In Centroplacaceae, the orientation is more difficult to interpret. The flowers of *B. paniculata* are sessile, and three tightly congested flowers are commonly borne in an inflorescence. More than one kind of floral orientation was observed, in which case these flowers may twist during development.

Character State Reconstruction of CYC2-Like Gene Expression. We used ML character state optimization as implemented in Mesquite 2.6 (1) to reconstruct the evolution of *CYC2*-like gene expression. We used the single ML topology inferred from *CYC2* nucleotide sequences to reconstruct the pattern of *CYC2* gene expression under the general Mk1 model (6) with the rate parameter estimated from the data. The character states of gene expression were coded as follows: none, uniform, broad differential, and narrow differential. Ancestral character states were reconstructed assuming that all transition states are unordered. Although we used genetic distance as an approximate measure of the "opportunity for selection" (13), we also conducted our analysis with the topology calibrated for absolute divergence time estimates (4, 5, 14). Those results are very similar to those presented here and do not affect our conclusions.

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Fig. S1. Ancestral character state reconstruction of floral symmetry. ML analysis indicates the relative likelihood of floral symmetry at the nodes of Centroplacaceae–Elatinaceae–Malpighiaceae (actinomorphy, 0.77; zygomorphy, 0.23), Elatinaceae–Malpighaceae (actinomorphy, 0.75; zygomorphy, 0.25), and crown group Malpighiaceae (actinomorphy, 0.0; zygomorphy, 1.0). These results indicate that the common ancestor of Centroplacaceae–Elatinaceae–Malpighiaceae and Elatinaceae–Malpighiaceae are likely actinomorphic, and that zygomorphy evolved in the common ancestor of all Malpighiaceae.

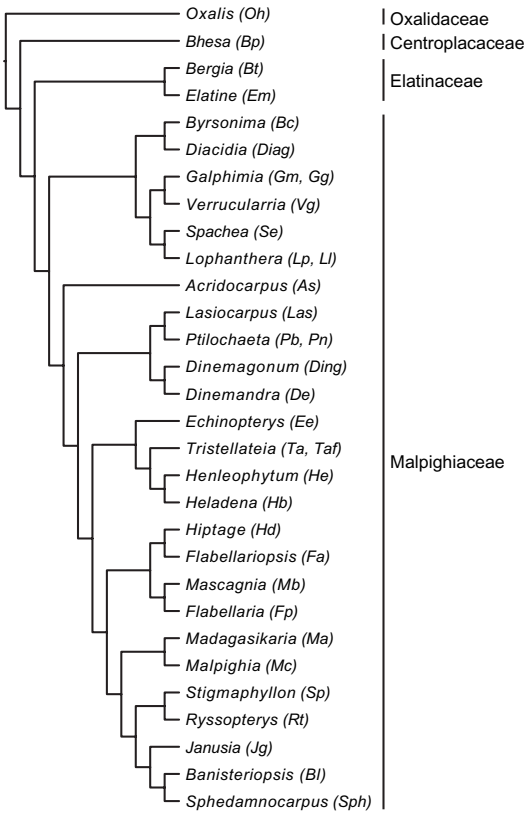

Fig. S2. Phylogeny showing accepted species relationships (derived from refs. 2, 3, 15–17).

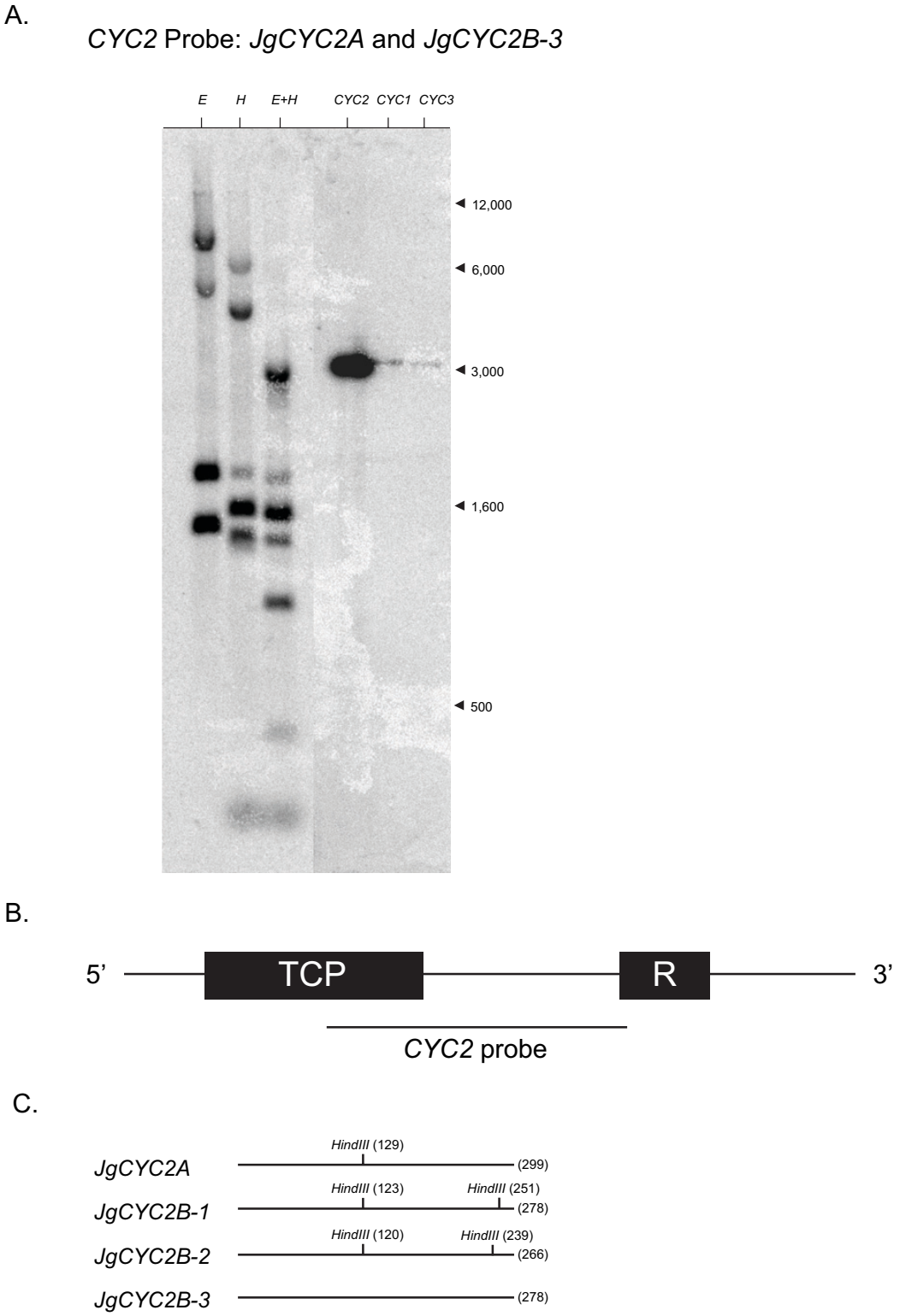

Fig. S3. *CYC2* Southern hybridization results for *J. guaranitica*. (*A*) To assess gene copy number, we developed a *CYC2*-specific probe and tested it against *J. guaranitica*. That probe preferentially identified only *CYC2* and not other *CYCLOIDEA* homologues in *Janusia*, i.e., *CYC1* and *CYC3*. The result of this test of the probe's specificity is shown on the right side of the *Janusia* blot. Restriction digests using EcoRI (E), HindIII (H), and EcoRI + HindIII (E+H) are shown for *Janusia*. (*B*) The position of the probe region within *CYC*-like genes. (*C*) Restriction cut sites were inferred from sequence analysis and are indicated on the *CYC2* gene copies shown at bottom. Arrows and numbers indicate molecular size markers (in base pairs).

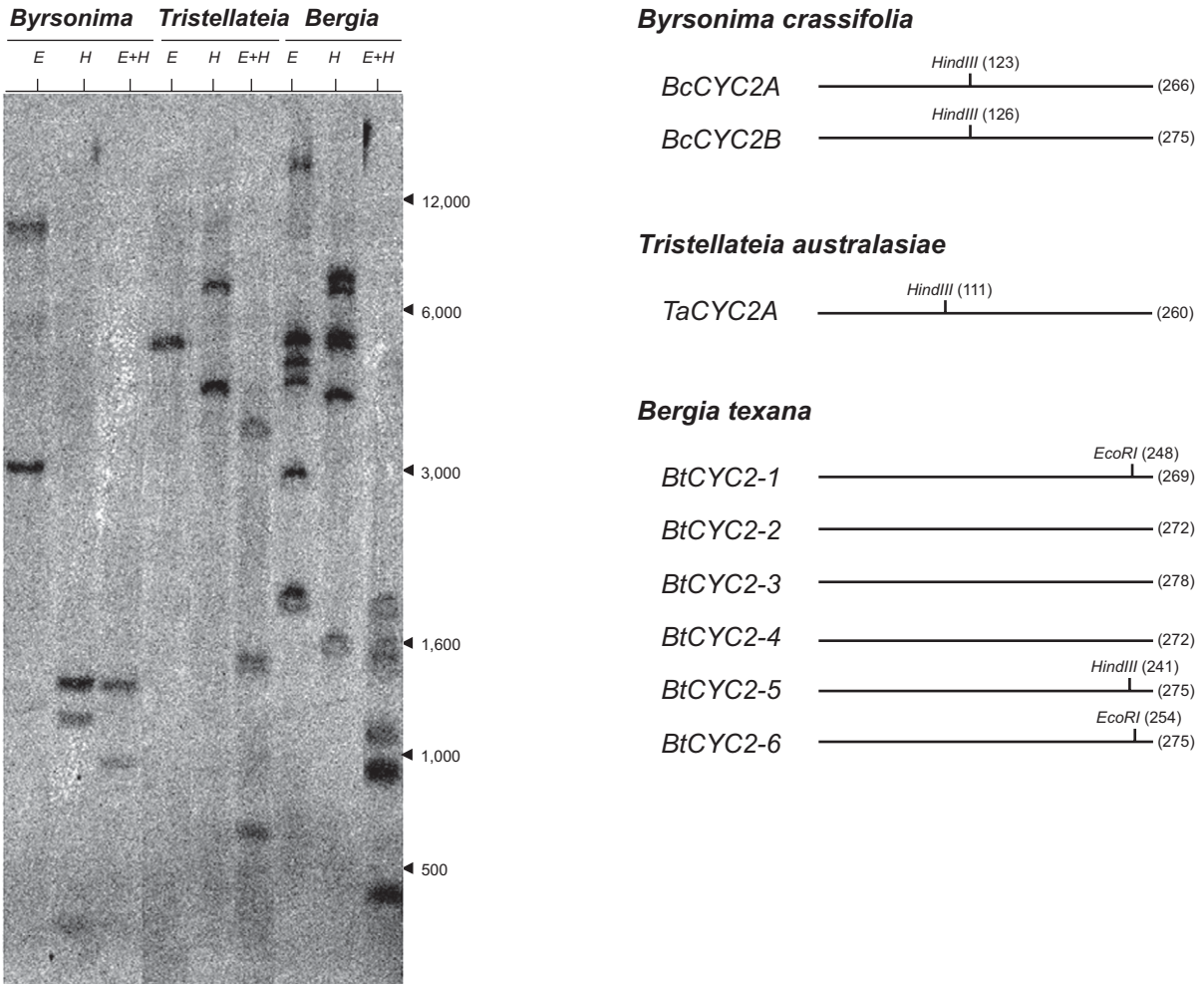

Fig. S4. *CYC2*-like gene Southern hybridization results for *Byrsonima crassifolia*, *T. australasiae*, and *Bergia texana*. (*A*) Restriction digests using EcoRI (E), HindIII (H), and EcoRI + HindIII (E+H) are shown for *B. crassifolia*, *T. australasiae*, and *B. texana*. (*B*) Restriction cut sites were determined from sequence analysis and are indicated on the *CYC2* gene copies shown at bottom. Arrows and numbers indicate molecular size markers (in base pairs).

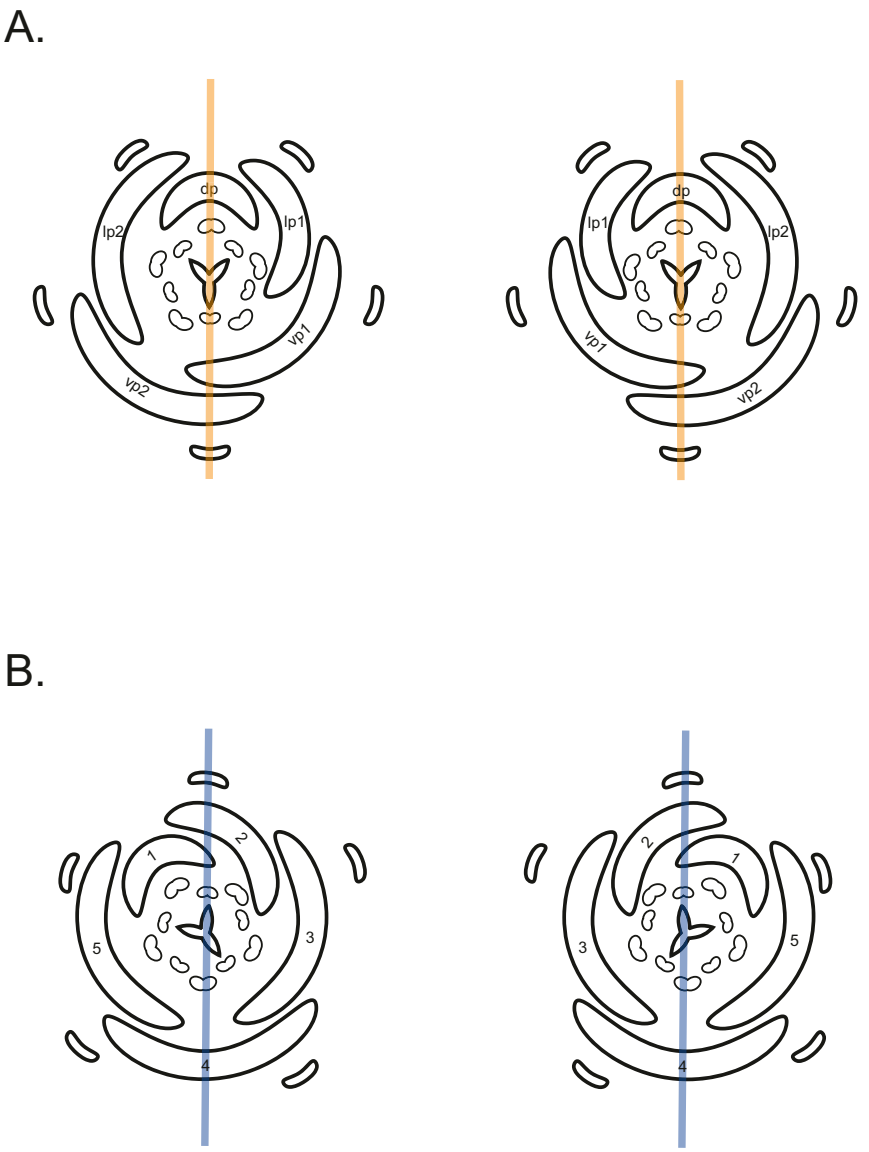

Fig. S5. Floral aestivation and enantiomorphy of Malpighiaceae. (*A*) New World Malpighiaceae. Petal identities are indicated as follows: dp, dorsal petal; lp1-2, lateral petals; vp1-2, ventral petals. (*B*) *T. australasiae*, an Old World Malpighiaceae species. The dorsoventral planes of floral symmetry are indicated with a colored vertical line. Petal identities are indicated as follows: 1 and 2, dorsal petals; 3 and 5, lateral petals; 4, ventral petal. The petals are not intended to be drawn proportional to scale in *Tristellateia*.

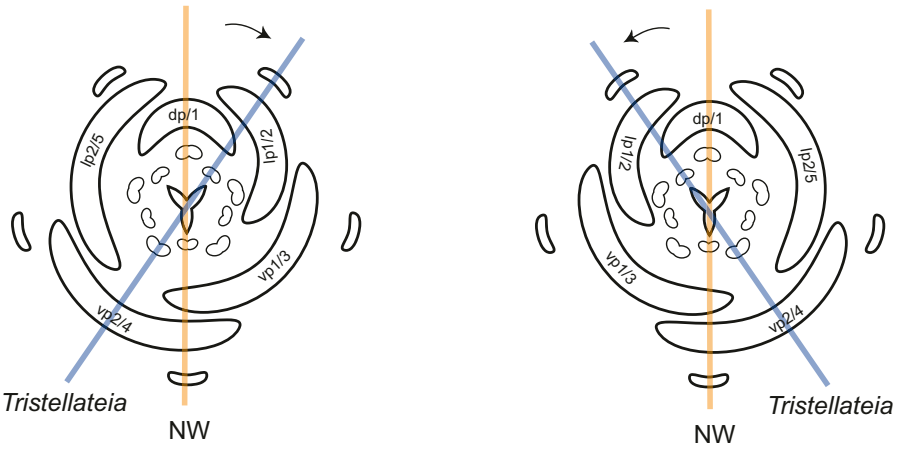

Fig. S6. Hypothesized shift in the dorsoventral plane of symmetry in the Old World species *T. australasiae*. Floral arrangement of New World Malpighiaceae shown for comparison. The dorsoventral planes of floral symmetry of New World Malpighiaceae and *Tristellateia* are indicated in orange and blue, respectively. The arrow illustrates the hypothesized 36° rotation in *Tristellateia* relative to their New World ancestors. For the New World arrangement abbreviations are as follows: dp, dorsal petal; lp1-2, lateral petals; vp1-2, ventral petals. For the Old World arrangement, abbreviations are as follows: 1 and 2, dorsal petals; 3 and 5, lateral petals; 4, ventral petal.

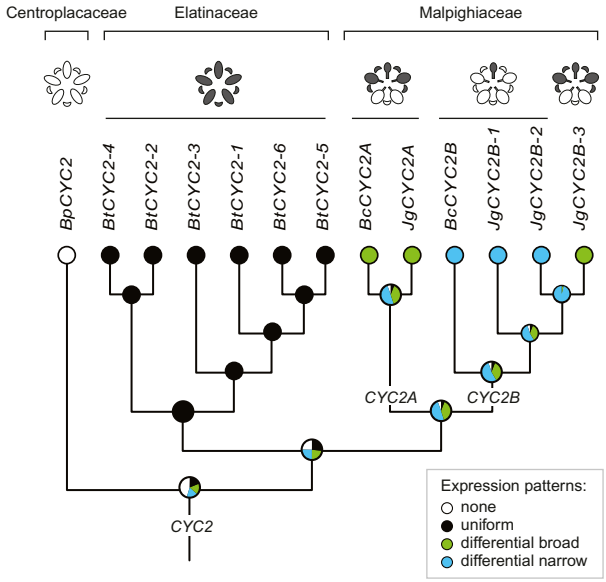

Fig. S7. Character state reconstruction of *CYC2* gene expression. Expression patterns are treated as character states shown in different colors. Areas of pies indicate the relative degree of support for alternative ancestral character states. Gray highlighting in flower diagram indicates the spatial pattern of *CYC2* expression. The most recent common ancestor of Centroplacaceae–Elatinaceae–Malpighiaceae likely exhibited the pattern of gene expression observed in Centroplacaceae (*Bhesa paniculata*). *CYC2* expression in the most recent common ancestor of Malpighiaceae–Elatinaceae is equivocal. The most recent common ancestor of Malpighiaceae likely exhibited differential expression of *CYC2* before the *CYC2A/B* duplication. In addition, our results support the independent evolution of a broader pattern of gene expression of *JgCYC2B-3*, one of the *CYC2B* copies in *J. guaranitica*, from a narrow patterned *CYC2B* ancestor.

Table S1. Species sampled, with collection locations, voucher information, and *CYC2* identities

Species	Location	Voucher	Identity of obtained *CYC2* copies		
			2	2A	2B
Acridocarpus smeathmanni Guill. and Perr.	Ghana	Davis 99–13 (A)	–	*AsCYC2A*	*AsCYC2B*
Banisteriopsis latifolia (A.Juss.) B. Gates	Distrito Federal, Brazil	Azeuedo 698 (MICH)	–	*BlCYC2A*	*BlCYC2B-1*, *BlCYC2B-2*
Bergia texana Seub. ex Walp.	Butte County, California, US	Zhang, Ahart, and Bartholomew 84 (A)	*BtCYC2-1* ~ *BtCYC2-6*	–	–
Bhesa paniculata Arn.	Negeri Sembilan, Malaysia	Zhang and Boufford 160 (A)	*BpCYC2*	–	–
Byrsonima crassifolia Kunth	Cult. OEB, Harvard U.	Matamoros and Cerda 301	–	*BcCYC2A*	*BcCYC2B*
Diacidia galphimioides Griseb.	Amazonas, Venezuela	Berry et al., 5275 (MICH)	–	*DiagCYC2A*	-
Dinemagonum gayanum A.Juss.	Chile	Simpson 83–10-23–2c (MICH)	–	*DingCYC2A*	*DingCYC2B*
Dinemandra ericoides A.Juss.	Chile	Dillon and Teillier 5103 (MICH)	–	*DeCYC2A*	*DeCYC2B*
Echinopterys eglandulosa (A. Juss.) Small	Sonora, Mexico	Van Devender 98–178 (MICH)	–	*EeCYC2A*	*EeCYC2B*
Elatine minima (Nutt.) Fisch. & C. A. Meyer	Gemini lake, North Michigan, US	Voss 11739 (MICH)	*EmCYC2-1*, *EmCYC2-2*	–	–
Flabellaria paniculata Cav.	Tanzania	Congdon 414 (K)	–	–	*FpCYC2B*
Flabellariopsis acuminata (Engl.) R. Wilczek	Tanzania	Faulkner 783 (K)	–	*FaCYC2A*	*FaCYC2B-1*, *FaCYC2B-2*
Galphimia gracilis Bartl.	Fairchild T.G., Florida, US	FTG 79–235 (FTG)	–	*GgCYC2A-1*, *GgCYC2A-2*	*GgCYC2B*
Galphimia mexiae C.E. Anderson	Jalisco, Mexico	Anderson and Anderson 6122 (MICH)	–	*GmCYC2A*	*GmCYC2B*
Heladena bunchosioides A. Juss.	Espírito Santo, Brazil	Folli 4653 (MICH)	–	*HbCYC2A*	*HbCYC2B*
Henleophytum echinatum (Griseb.) Small	nr. Havana, Cuba	Curtiss 688 (K, NY)	–	*HeCYC2A*	*HeCYC2B*
Hiptage detergens Craib	Thailand	Middleton et al., 2095 (A, MICH)	–	*HdCYC2A-1*, *HdCYC2A-2*	*HdCYC2B-1*, *HdCYC2B-2*
J. guaranitica A. Juss.	Cult. OEB, Harvard U.	Zhang 165 (A)	–	*JgCYC2A*	*JgCYC2B-1*, *JgCYC2B-2*, *JgCYC2B-3*
Lasiocarpus sp.	Mexico	Anderson 13828 (MICH)	–	*LasCYC2A*	*LasCYC2B*
Lophanthera longifolia Griseb.	Amazonas, Venezuela	Zimmerman 27 (MICH)	–	*LlCYC2A*	*LlCYC2B*
Lophanthera pendula Ducke	Amazonas, Brazil	Lima and Lima 3185 (MICH)	–	*LpCYC2A*	*LpCYC2B*
Madagasikaria andersonii C. Davis	Madagascar	Davis 20–01 (A)	–	*MaCYC2A*	–
Malpighia coccigera L.	UMBG	UMBG 20626 (MICH)	–	*McCYC2A*	*McCYC2B*
Mascagnia bracteosa Griseb.	Manaus, Brazil	Anderson 13777 (MICH)	–	*MbCYC2A*	*MbCYC2B*
Oxalis herrerae R.Knuth	Cult. OEB, Harvard U.	Zhang 20 (A)	*OhCYC2-1*, *OhCYC2-2*	–	–
Ptilochaeta bahiensis Turcz.	Bahia, Brazil	Anderson 13725 (MICH)	–	–	*PbCYC2B-1*, *PbCYC2B-2*, *PbCYC2B-3*
Ptilochaeta nudipes Griseb.	Jujuy, Argentina	Anderson 13588 (MICH)	–	*PnCYC2A*	*PnCYC2B-1*, *PnCYC2B-2*
Ryssopterys timoriensis (DC.) Blume ex A. Juss.	Cult. Bogor	XVIII.F.172 (BO)	–	*RtCYC2A*	*RtCYC2B-1*, *RtCYC2B-2*
Spachea elegans A. Juss.	Guyana	Janson-Jacobs et al., 3907 (MICH)	–	*SeCYC2A*	*SeCYC2B-1*, *SeCYC2B-2*
Sphedamnocarpus sp.	Madagascar	Phillipson et al., 4104 (MICH, MO, P)	–	–	*SphCYC2B*
Stigmaphyllon paralias A. Juss.	Bahia, Brazil	Anderson 13693 (MICH)	–	–	*SpCYC2B*
Tristellateia africana S. Moore	Dar es Salaam, Tanzania	Davis 99–25 (A)	–	*TafCYC2A-1*, *TafCYC2A-2*, *TafCYC2A-3*	–
T. australasiae A. Rich.	Cult. OEB, Harvard U.	Zhang 163 (A)	–	*TaCYC2A*	–
Verrucularia glaucophylla A. Juss.	Bahia, Brazil	Amorim 3662 (CEPEC, MICH)	–	*VgCYC2A-1*, *VgCYC2A-2*	*VgCYC2B*

Arnold Arboretum (Arn. Arb.) is in Jamaica Plain, MA. A, Arnold Herbarium, Harvard University Herbaria; BO, Herbarium Bogoriense, Bogor, West Java, Indonesia; CEPEC, Herbário Centro de Pesquisas do Cacau, Bahia, Brazil; FTG, Fairchild Tropical Botanic Garden; K, Royal Botanic Gardens, Kew, England; MICH, University of Michigan Herbarium; MO, Missouri Botanical Garden, St. Louis, Missouri; NY, New York Botanical Garden, Bronx, New York; *P*, Muséum National d'Histoire Naturelle, Paris. GenBank numbers are given for each copy found. The GenBank numbers are GU982187–GU982264.

Table S2. RT-PCR primer sequences used in this study

Name	Taxa	Forward (5′ to 3′)	Reverse (5′ to 3′)
BtCYC2-1	*Bergia texana*	GGTCTTACAATTCTACTAGTGAATTACTTG	GAATTCTGGAAGCAAACTTTTGTAAT
BtCYC2 (-2, -4)	*Bergia texana*	CAAGAWACTYTAGGGTTTGATAAAGCAA	CAAATGACTCCATYTTTGAAACTGTCC
BtCYC2 (-3, -5, -6)	*Bergia texana*	CAAGAWACTYTAGGGTTTGATAAAGCAA	GAATCCAMCTTTGAAACTSTTGTCC
BpCYC2	*Bhesa paniculata*	GATCTTCAAGACATTCTAGGGTTTGAC	GACTCCTTTGCAAGAAGTGTACTG
BcCYC2A	*Byrsonima crassifolia*	AAGATTTGTTAGGGTTTGATAGGG	AGGTCTCATTTCACTATAATCAACACA
BcCYC2B	*Byrsonima crassifolia*	AAGACCTTCTAGGGTTTGATAGGG	TCTCCCCTCACTAGAATCAACAGT
JgCYC2A	*J. guaranitica*	ACAATCTCTGGAGCTGAAAAGG	ACTCACATCCTGCCTGAACC
JgCYC2B-1	*J. guaranitica*	TTCCATAYTCAAGATCCGATTTA	CTCAATTGTTCTGATGATGACCT
JgCYC2B-2	*J. guaranitica*	TTCCATAYTCAAGATCCGATTTA	CTTCCCATGATTTGCAGTATACTTATT
JgCYC2B-3	*J. guaranitica*	CTAACAAGCGATCAAATCGAA	GATCTCAATTGTTCTGGTGATCTT
TaCYC2A	*T. australasiae*	TTAGGGTTTGACAGGGCAAG	GCTTAGCAAGAAGTGGGATTT