Supporting information

Genetic architecture of floral traits in bee- and hummingbird-pollinated sister species of *Aquilegia* (columbine)

This file includes:

Figs. S1-S10
Tables S1-S2 (Tables S3-S5 are in separate .xlsx files)
Supplementary bibliography

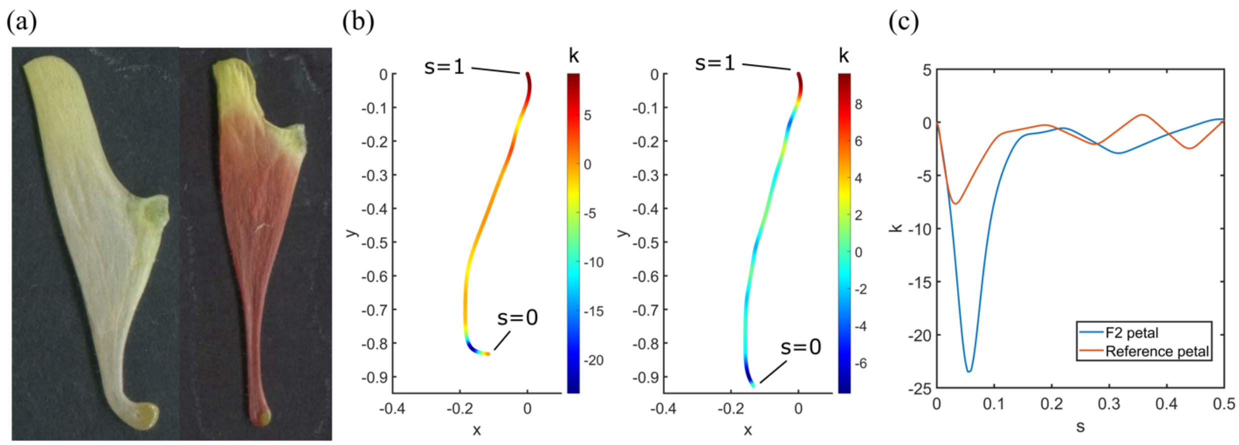
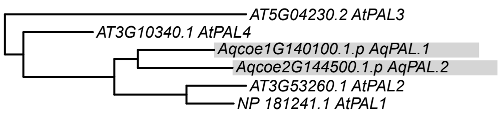


Figure S1. Spur curvature quantification method. **(a)** An F2 petal and the straight-spurred reference petal from an *A. canadensis* flower (not to scale). **(b)** For the spur curvature quantification, the curve segment between the spur tip and the attachment point for each petal was rescaled to be with unit length. The signed curvature k(s) was then computed, with the first zero of the curvature near the spur tip and the attachment point parameterized as s = 0 and s = 1 respectively. **(c)** The spur curvature was quantified by the L^2-norm of the difference between the signed curvature, evaluated from s = 0 to s = 0.5 (i.e. the lower half of the spur)

Figure S2A

PAL



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4CL

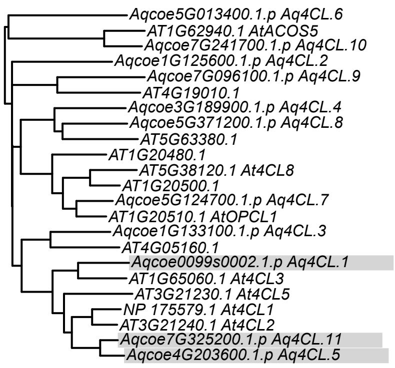

Figure S2B

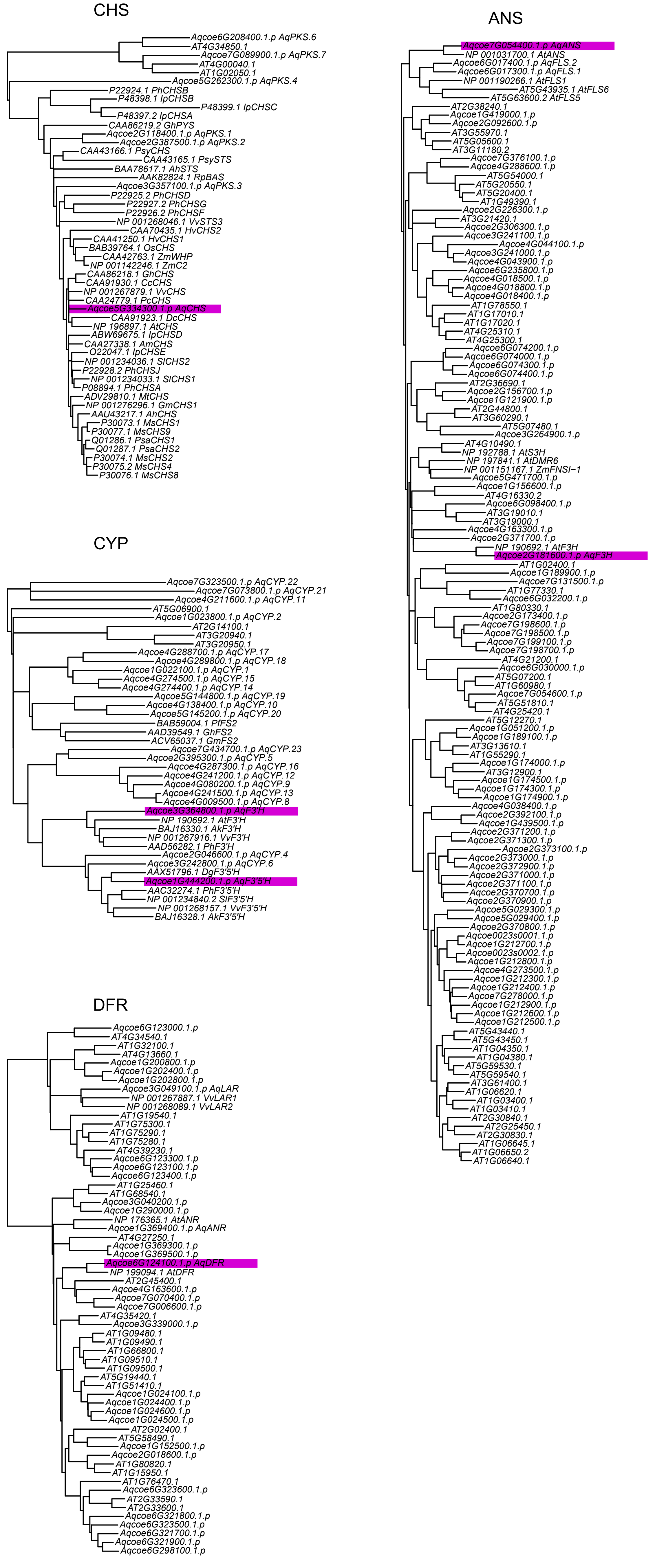
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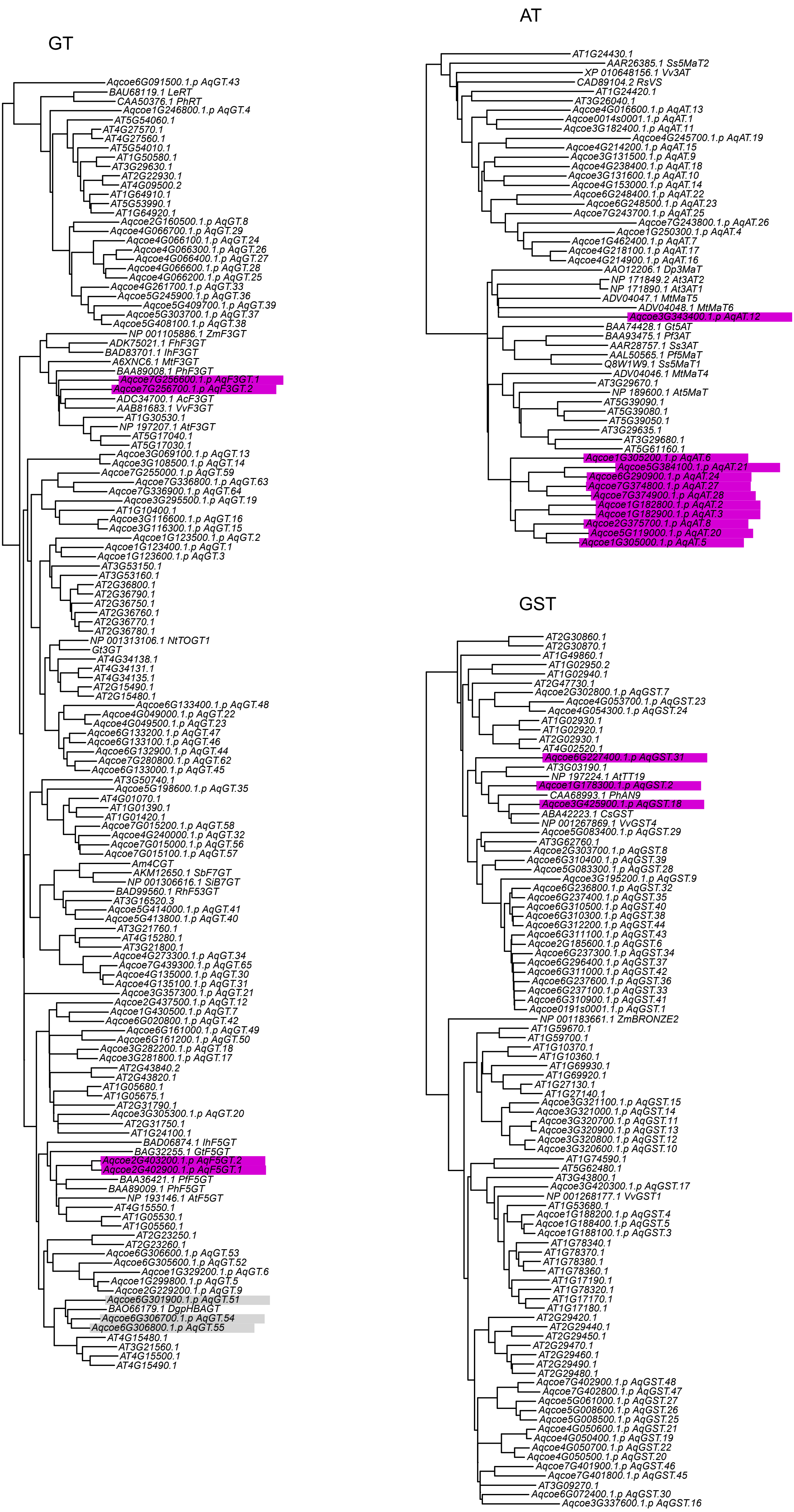
Figure S2C



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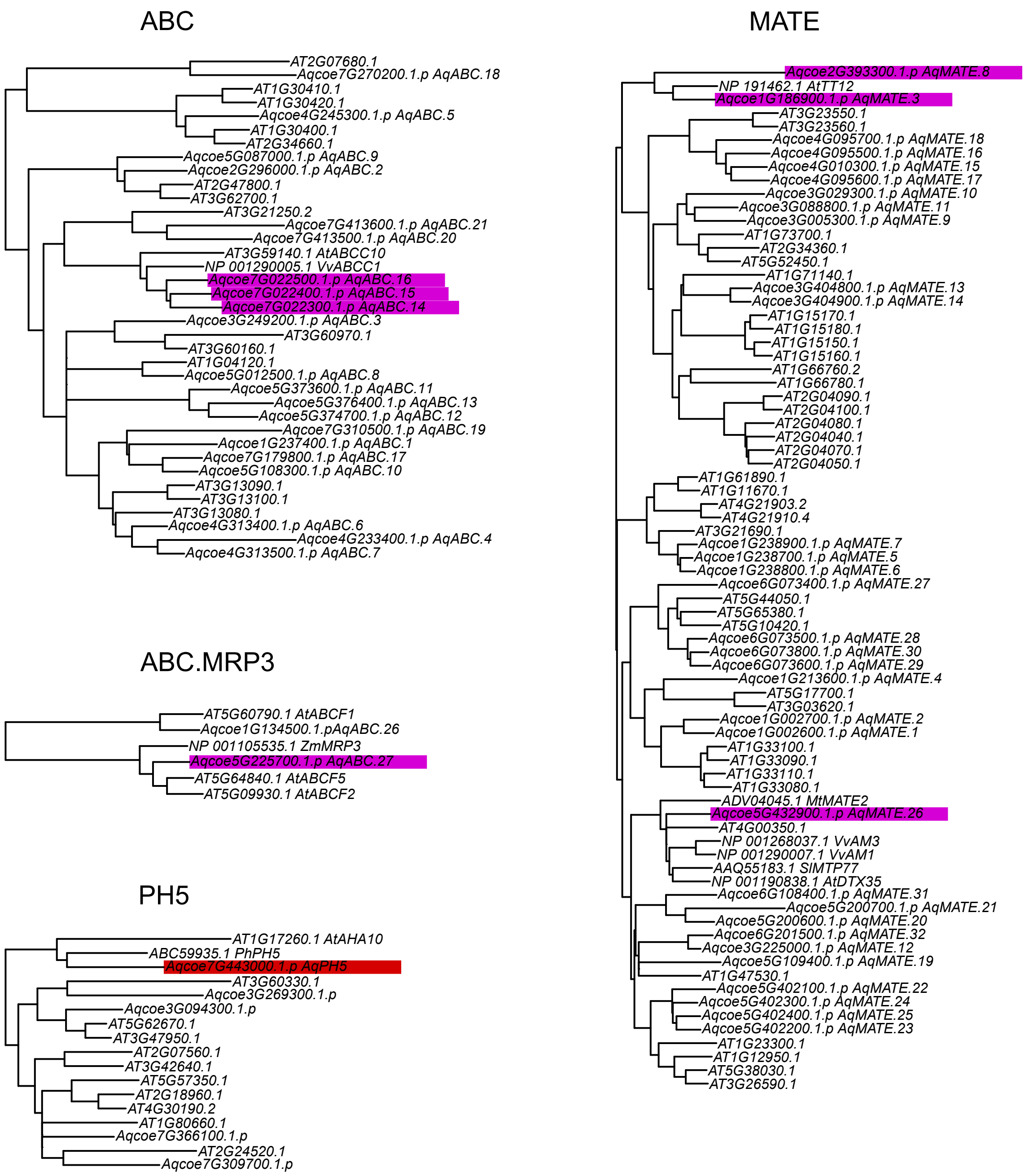
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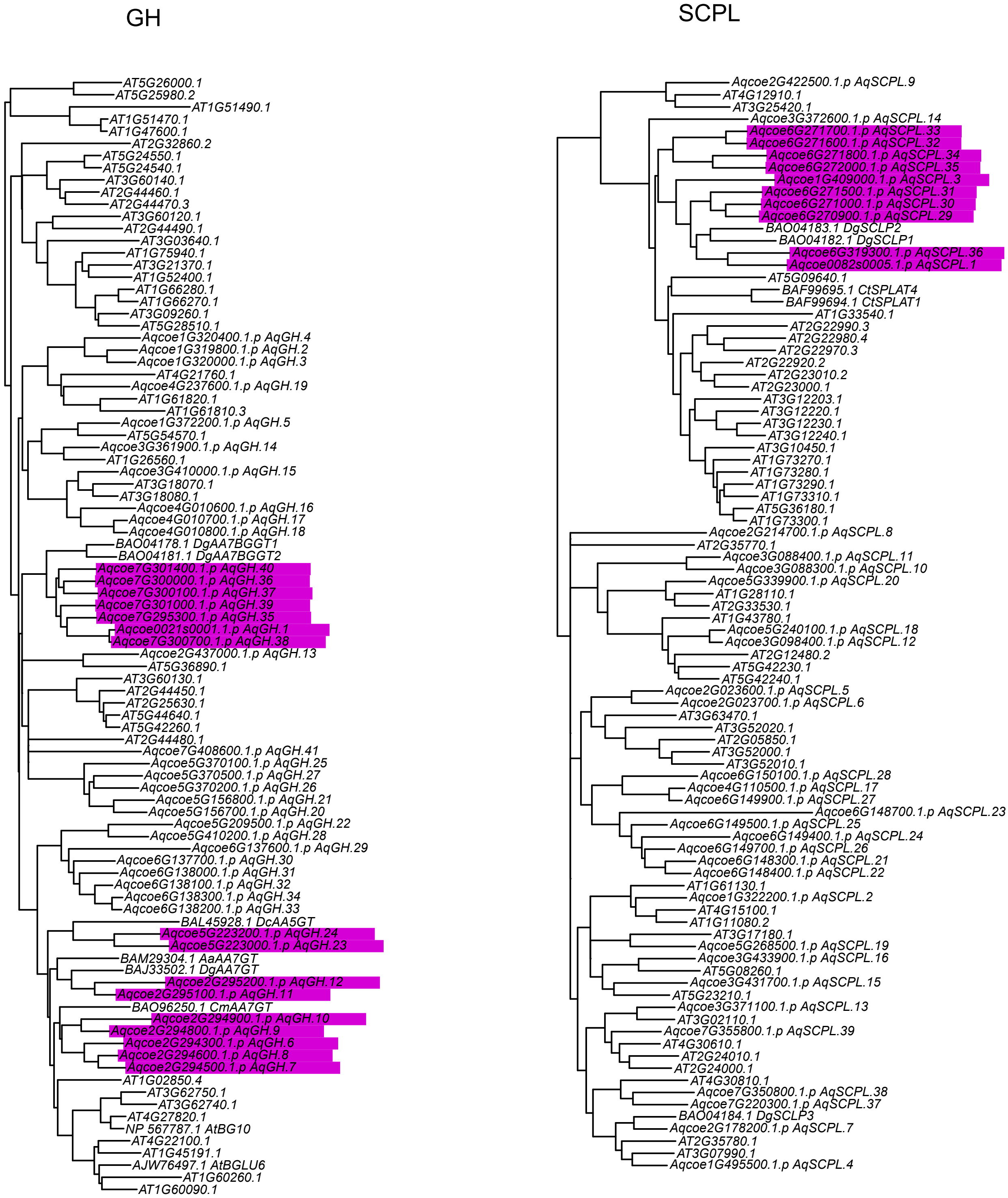
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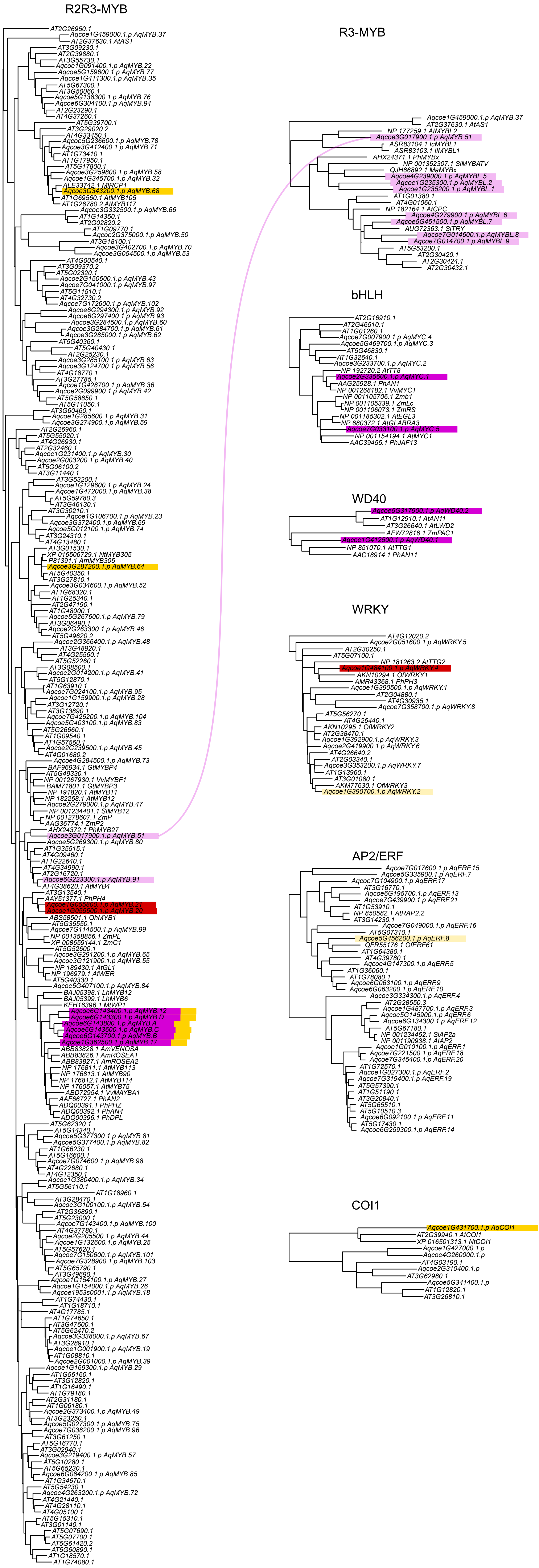
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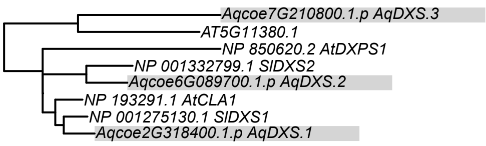
Figure S2G

DXS



GGPPS

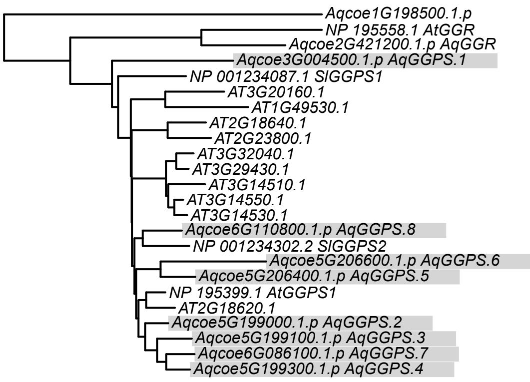
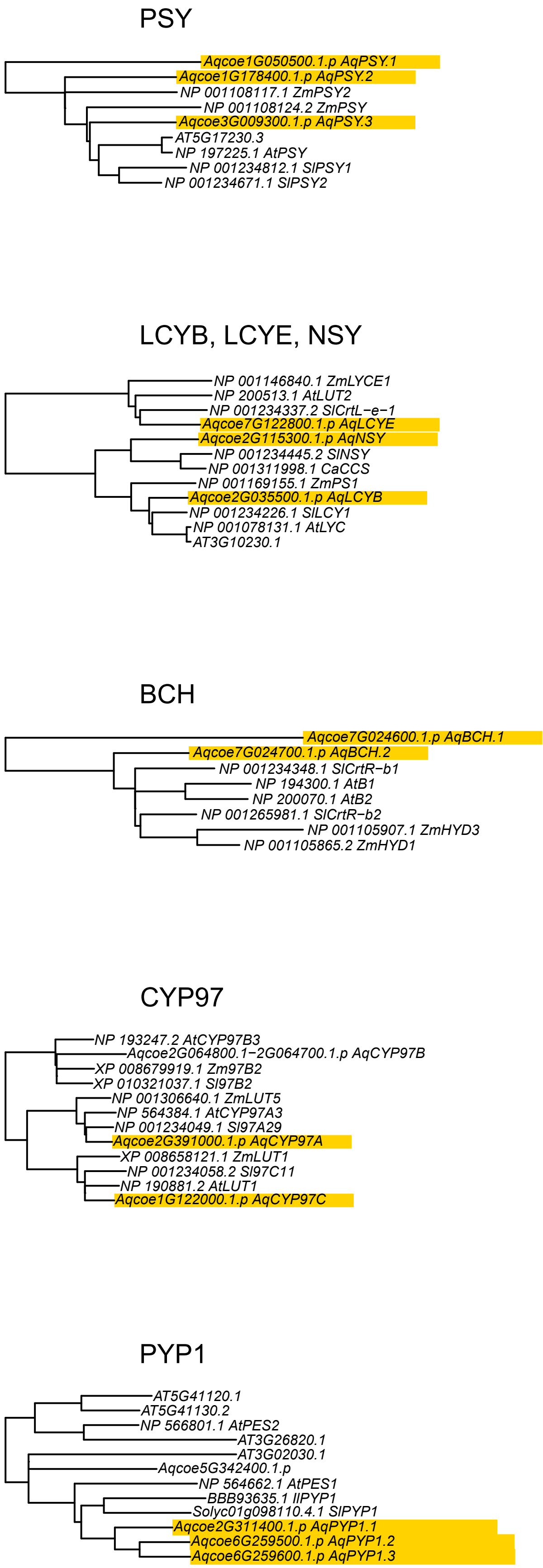


Figure S2H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Aqcoe3G009300.1.p AqPSY.3
AT5G17230.3
NP 197225.1 AtPSY
NP 001234812.1 SlPSY1
NP 001234671.1 SlPSY2
LCYB, LCYE, NSY
NP 001146840.1 ZmLYCE1
NP 200513.1 AtLUT2
NP 001234337.2 SlCrtL−e−1
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NP 001234445.2 SlNSY
NP 001311998.1 CaCCS
NP 001169155.1 ZmPS1
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NP 001234226.1 SlLCY1
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AT3G10230.1
BCH
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Aqcoe7G024700.1.p AqBCH.2
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NP 194300.1 AtB1
NP 200070.1 AtB2
NP 001265981.1 SlCrtR−b2
NP 001105907.1 ZmHYD3
NP 001105865.2 ZmHYD1
CYP97
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NP 001306640.1 ZmLUT5
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XP 008658121.1 ZmLUT1
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NP 564662.1 AtPES1
BBB93635.1 IlPYP1
Solyc01g098110.4.1 SlPYP1
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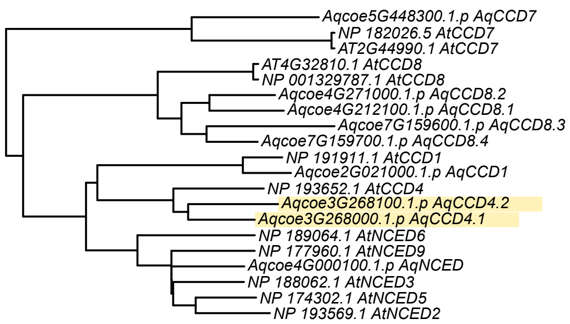
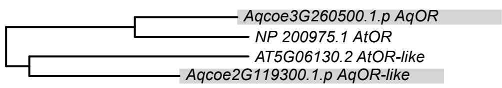
Figure S2I

CCD/NCED



OR



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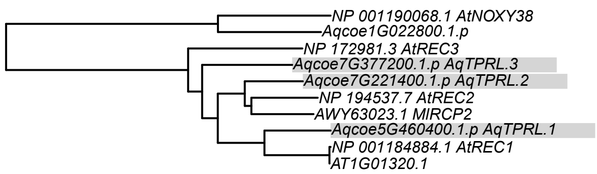

Figure S2. Neighbor-joining trees of genes involved in anthocyanin and carotenoid pigmentation

A. Neighbor-joining trees of anthocyanin precursor biosynthetic genes. Query group names are abbreviated as: phenylalanine ammonia-lyase (PAL); cinnamate-4-hydroxylase (C4H); and 4-coumaroyl:CoA-ligase (4CL). Likely *Aquilegia* homologs are marked with a gray background.

B. Neighbor-joining trees of biosynthetic genes producing the core anthocyanidins pelargonidin, cyanidin, and delphinidin**.** Query group names are abbreviated as: chalcone synthase (CHS); cytochromes P450 (CYP); dihydroflavonol-4-reductase (DFR); and anthocyanidin synthase (ANS). The CYP group included flavonoid 3'-hydroxylases (F3'H) and flavonoid 3',5'-hydroxylases (F3'5'H). The ANS group included ANS and flavanone 3-hydroxylase (F3H). Likely *Aquilegia* homologs are marked with a purple background.

C. Neighbor-joining trees of anthocyanidin modification genes**.** Query group names are abbreviated as: glycosyltransferase (GT); acyltransferase (AT); and glutathione S-transferases (GST). GSTs do not appear to modify anthocyanins, but are important for transport into the vacuole (Zhao 2015). The GT group includes anthocyanidin 3-O-glucosyltransferases (F3GT) and anthocyanidin 5-O-glucosyltransferases (F5GT), as well a UDP-glucose dependent p-hydroxybenzoic acid glucosyltransferase (pHBAGT) identified in *Delphinium*. DgpHBAGT plays an indirect role in producing flower color, as its product (pHBG) is a donor molecule for both glycosylation and acylation of anthocyanins in the vacuole (Nishizaki et al 2013). Likely *Aquilegia* homologs of genes directly involved in anthocyanin biosynthesis or transport are marked with a purple background, and homologs of DgpHBAGT are marked with gray.

D. Neighbor-joining trees of vacuolar transporter genes. Query group names are abbreviated as: dultidrug resistance-associated protein transporters (ABC, ABC.MRP3); multidrug and toxic compound extrusion transporters (MATE); and H+-APTase (PH5). Likely *Aquilegia* homologs for anthocyanin transporters are marked with a purple background, and the homolog for proton transporter PH5 is marked with red.

E. Neighbor-joining trees of anthocyanin modification genes active in the vacuole. Query group names are abbreviated as: glycosyl hydrolase (GH); and serine carboxypeptidase-like proteins (SCPL). Likely *Aquilegia* homologs for anthocyanin transporters are marked with a purple background.

F. Neighbor-joining trees of transcriptional regulatory genes control pigment-related genes. Query group names are abbreviated as: myeloblastosis viral oncogene transcription factor (R2R3-MYB, R3-MYB); basic helix-loop-helix transcription factor (bHLH); WD40 repeat containing domain transcription factor (WD40); WRKY transcription factor (WRKY); APETALA2 and ethylene response factor transcription factors (AP2/ERF); and CORONATINE INSENSITIVE1 (COI1). R3-MYBs are found as two separate clades nested within R2R3-MYBs (Gates et al 2018); we pruned the R3-MYB tree to include only those clades plus an outgroup (AtAS1 and Aq.MYB.37). AqMYB5.1 falls within the AtMYBL2 clade, but retains an R2 motif so is classified here as an R2R3-MYB. Transcriptional regulation of floral carotenoid production is poorly characterized. Co-option of an anthocyanin-type R2R3-MYB for control of carotenoid biosynthetic genes has to date only been seen in *Medicago truncatula* (Meng et al 2019), and conserved function of the R2R3-MYB RCP1 from *Erythranthe lewisii* has not yet been demonstrated (Sagawa et al 2016). Similarly, positive regulation of CCD4 by WRKY and ERF transcrption factors is only known from one species, *Osmanthus fragrans* (Han et al 2016, Han et al 2019). Likely *Aquilegia* homologs for transcription factors directly controlling expression anthocyanin biosynthetic genes are marked with a purple background, with light purple indicating negative regulation. *Aquilegia* homologs for transcription factors controlling vacuolar pH are marked with red. Homologs for potential transcriptional regulators of carotenoid biosynthetic genes are marked with yellow, with light yellow indicating positive regulators of CCD4, an enzyme that degrades carotenoids.

G. Neighbor-joining trees of carotenoid precursor biosynthetic genes. Query group names are abbreviated as: 1-deoxy-D-xylulose 5-phosphate synthase (DXS); and geranylgeranyl diphosphate synthase (GGPPS). Likely *Aquilegia* homologs are marked with a gray background.

H. Neighbor-joining trees of carotenoid biosynthetic and modification genes. Query group names are abbreviated as: phytoene synthase (PSY); lycopene beta-cyclase (LCYB); lycopene epsilon-cyclase (LCYE); neoxanthin synthase (NSY); carotenoid beta-hydroxylase (BCH); and PALE YELLOW PETAL1 (PYP1). Likely *Aquilegia* homologs are marked with a yellow background.

I. Neighbor-joining trees of genes indirectly regulating carotenoid biosynthesis and accumulation. Query group names are abbreviated as: carotenoid cleavage dioxygenase (CCD); nine-cis-epoxycarotenoid dioxygenase (NCED); ORANGE protein (OR); and tetratricopeptide repeat-like superfamily protein (TPRL). Likely *Aquilegia* homologs for CCD4 are marked with a pale yellow background, and homolgs for OR and a TPRL from *Erythranthe lewisii* (RCP2) are marked with grey.

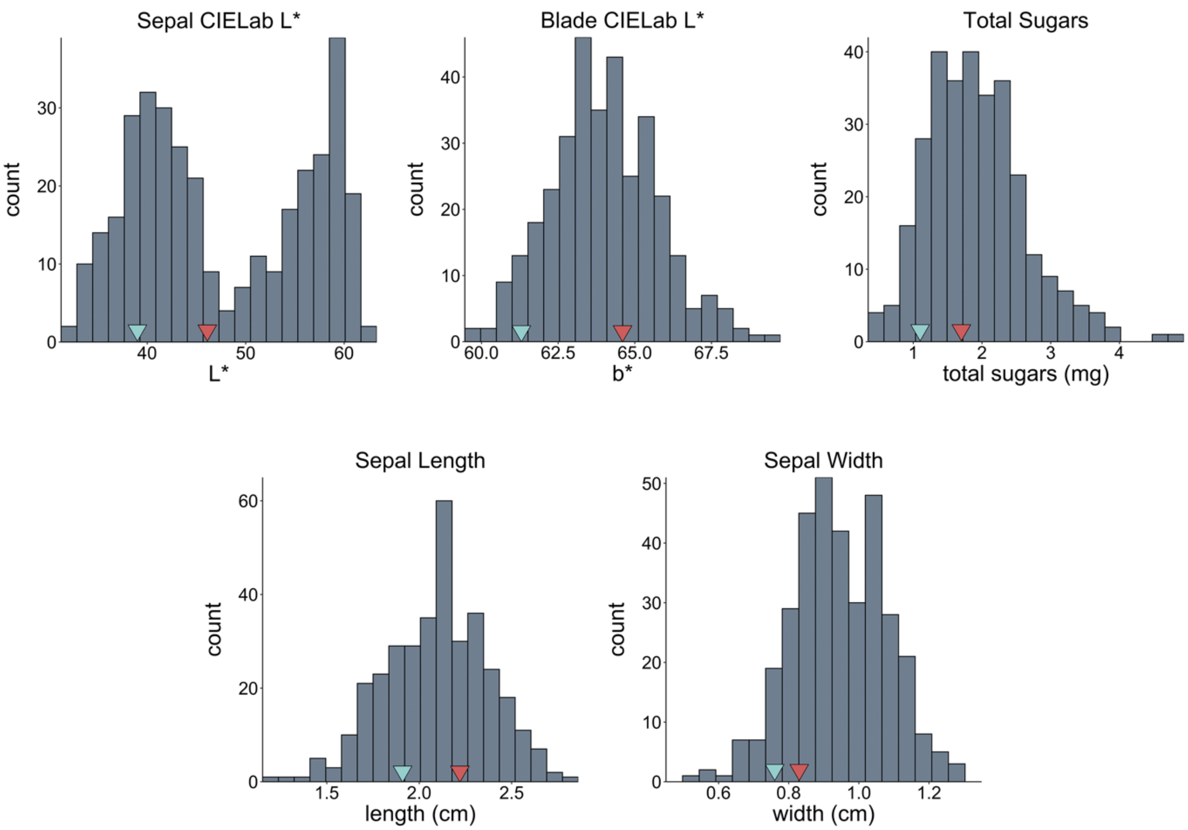

Figure S3. F2 population floral trait histograms (continued from Fig. 2). Blue and red arrows mark the phenotypic means of *A. brevistyla* (brev mean) and *A. canadensis* (can mean) plants that were closely related to the parents.

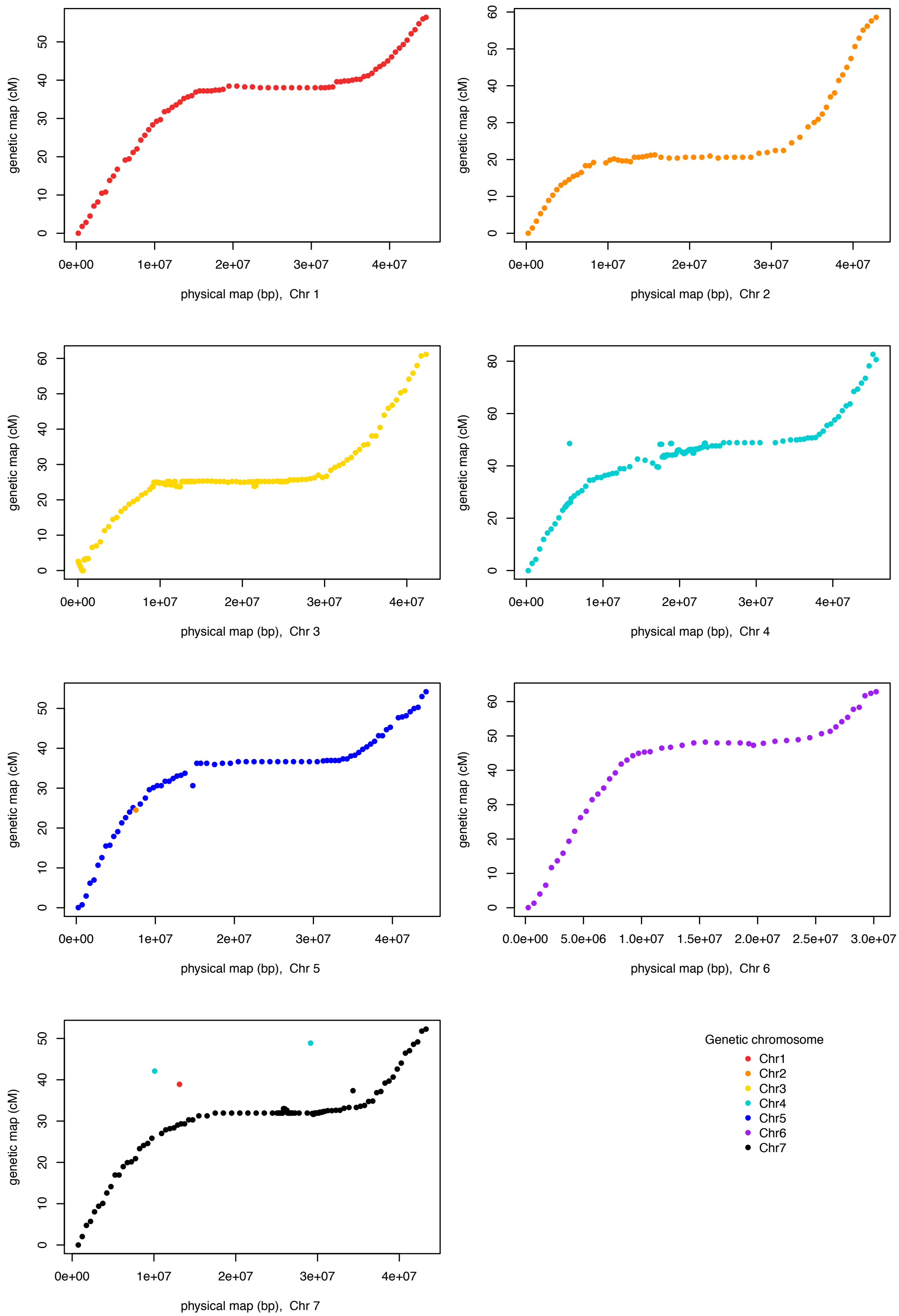
genetic map (cM)
physical map (bp), Chr 1
physical map (bp), Chr 2
physical map (bp), Chr 3
physical map (bp), Chr 4
physical map (bp), Chr 5
physical map (bp), Chr 6
physical map (bp), Chr 7
0e+00
1e+07
2e+07
3e+07
4e+07
0.0e+00
5.0e+06
1.0e+07
1.5e+07
2.0e+07
2.5e+07
3.0e+07
Genetic chromosome
Chr1
Chr2
Chr3
Chr4
Chr5
Chr6
Chr7

Figure S4. Genetic map position versus physical map position of QTL mapping markers. Each box represents the different *Aquilegia* chromosomes (1-7) as assembled in the reference genome (v3.1; e.g., the physical map). The physical map assembly was broken into bins (500 kb or 1 Mb depending on recombination frequency) and bins were genotyped as markers for genetic and QTL mapping. The chromosome that these markers map to in the genetic map are color coded. For the most part, the physical and genetic maps are consistent in marker order and chromosome, however there are several physical map markers that map to different genetic map positions (physical map markers that do not map contiguously in the genetic map) or chromosomes (e.g., several makers on the chromosome 7 physical map to the chromosome 1 or chromosome 4 genetic map). All chromosomes have extended regions of low recombination in the center.

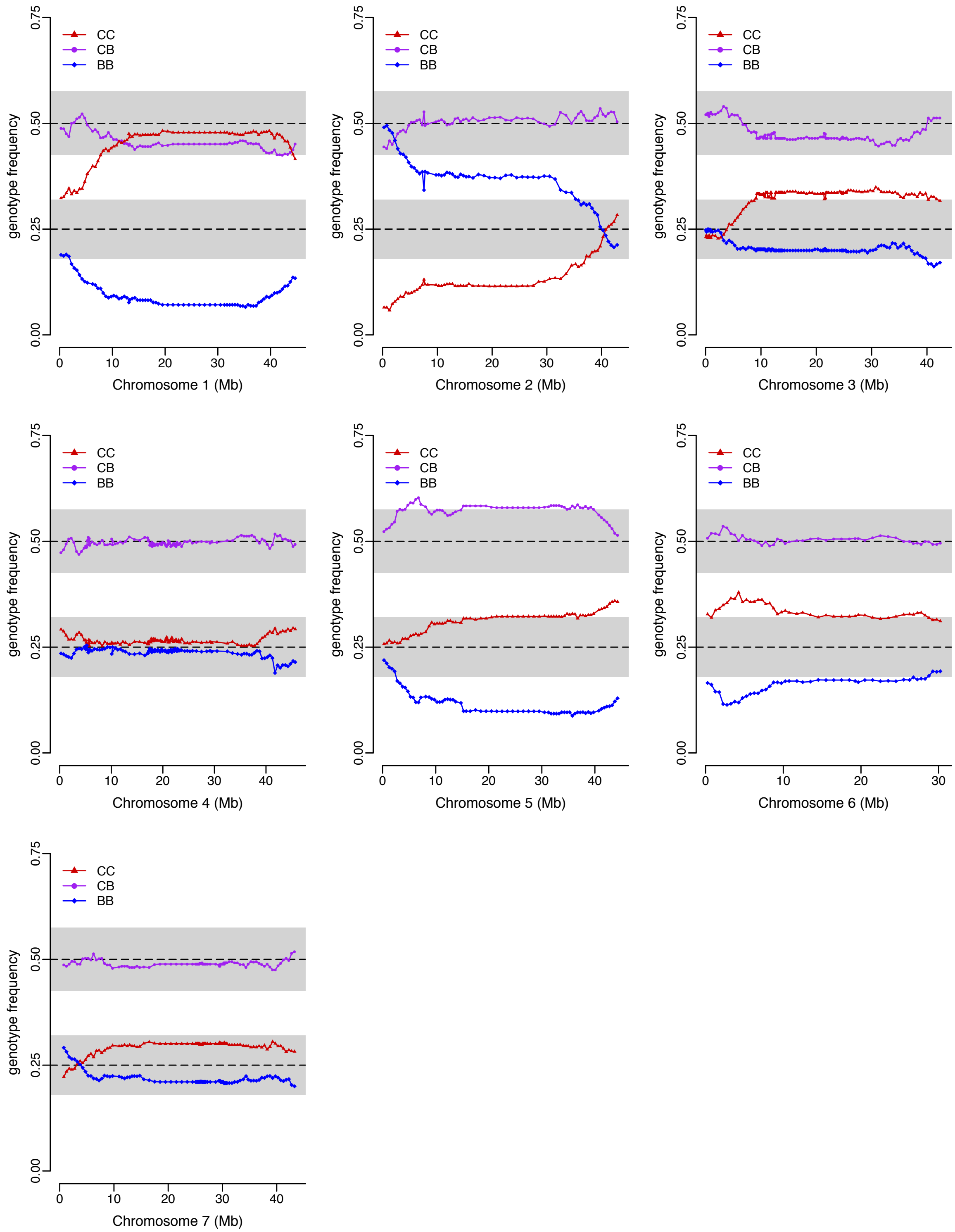

Figure S5. F2 population genotype frequency across each chromosome. Loci homozygous for *A. canadensis* alleles are overrepresented across much of chromosomes 1, 3, 5, and 6. Loci homozygous for *A. brevistyla* alleles are overrepresented across much of chromosome 2. CC – homozygous *A. canadensis*, CB – heterozygous, BB – homozygous *A. brevistyla*.

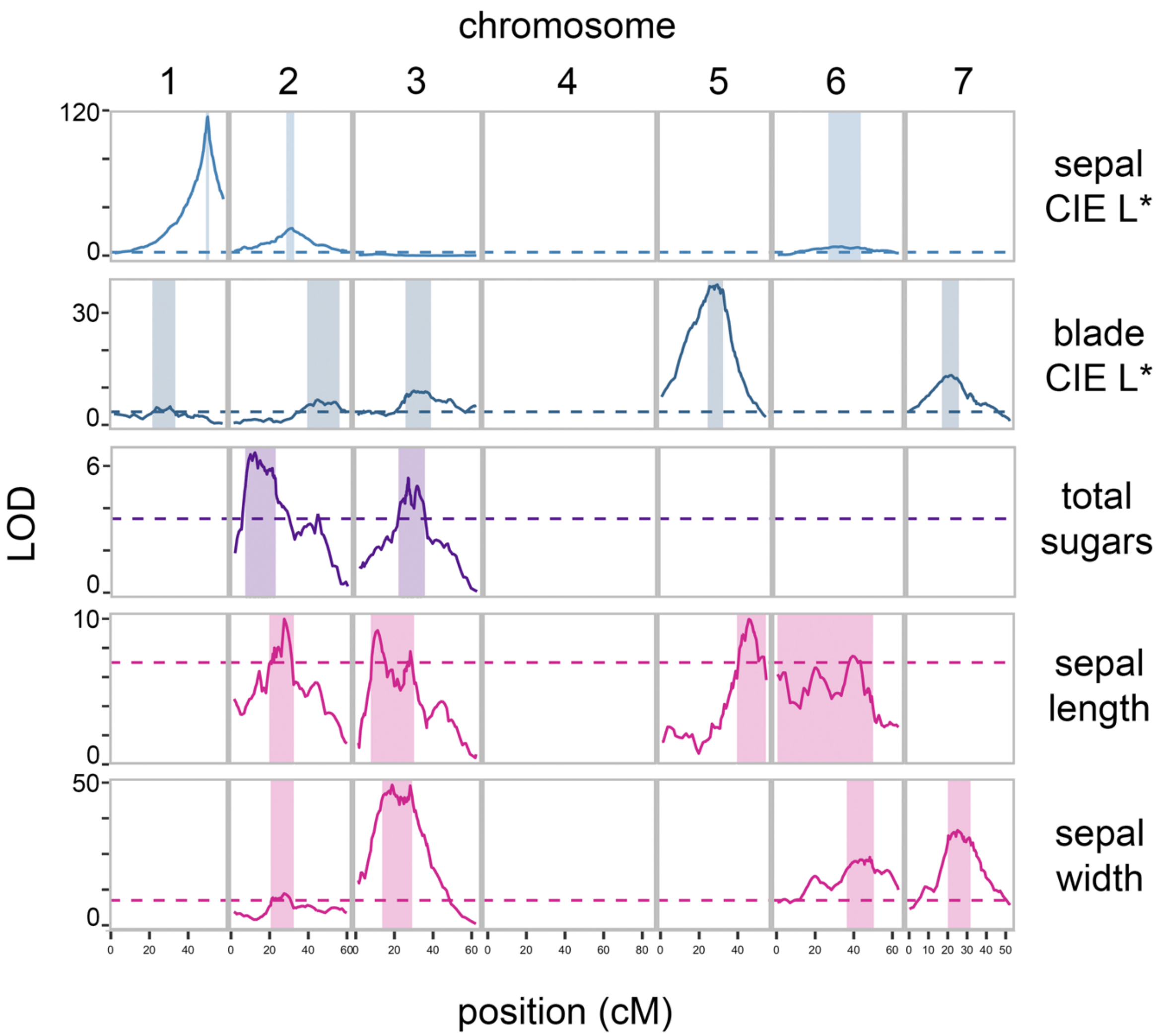

Figure S6. Floral trait QTL maps (continued from Fig. 4). Dashed line represents the significant LOD cutoff of 3.5, shaded areas represent the 1.5 LOD interval for each peak. Blue, color traits; purple, nectar traits; pink, morphological traits.

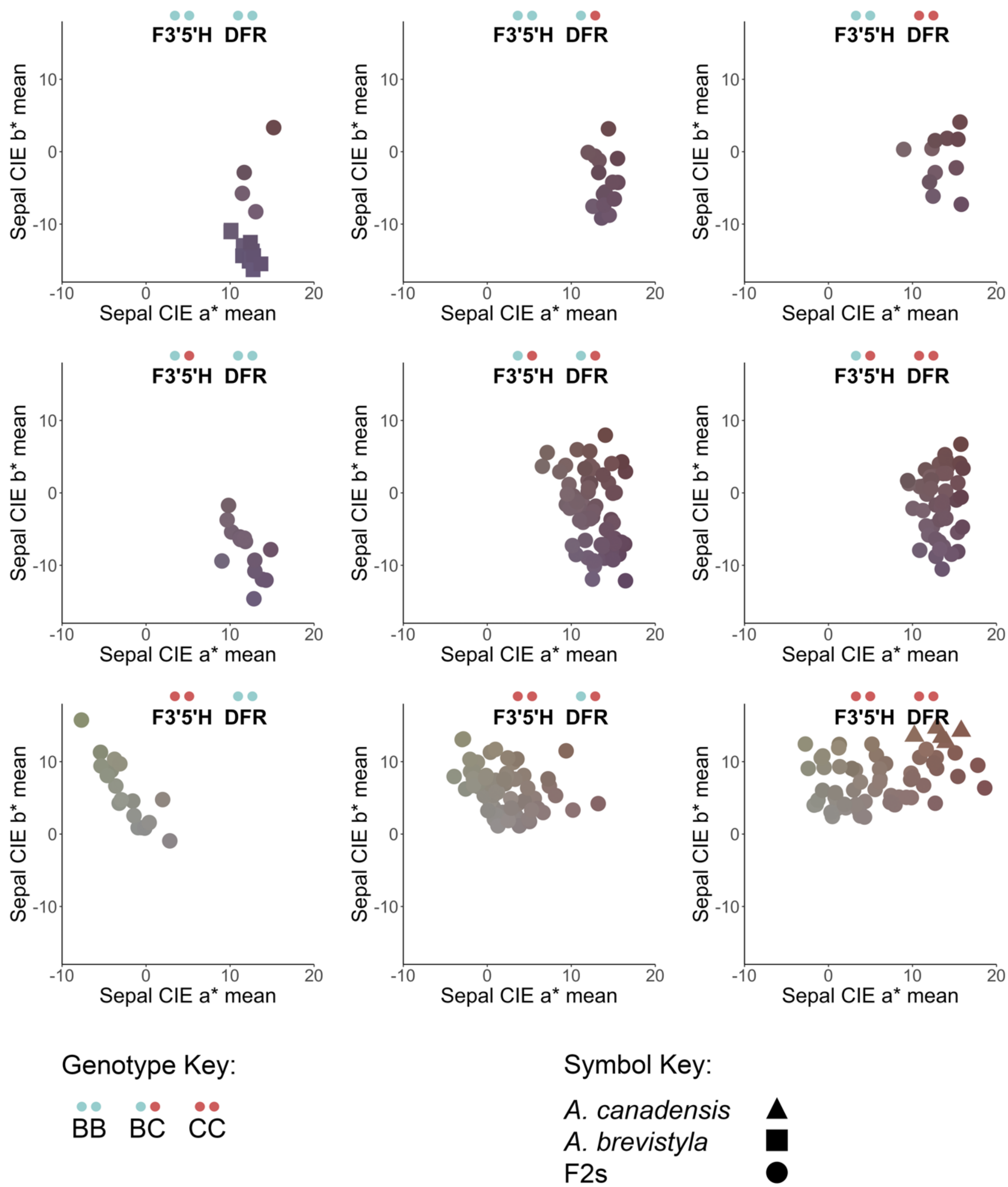

Figure S7. Sepal color phenotypes by genotype at *AqF3′5′H* and *AqDFR*. Allelic genotypes of the two loci are represented by pairs of blue (*A. brevistyla* allele) and red (*A. canadensis* allele) dots. Each of the nine panels represents a different allelic genotype combination. Points are color-coded based on the mean RGB values of the sepal for that individual F2 or parent plant. F3′5′H, flavonoid 3′,5′-hydroxylase; DFR, dihydroflavonol reductase.

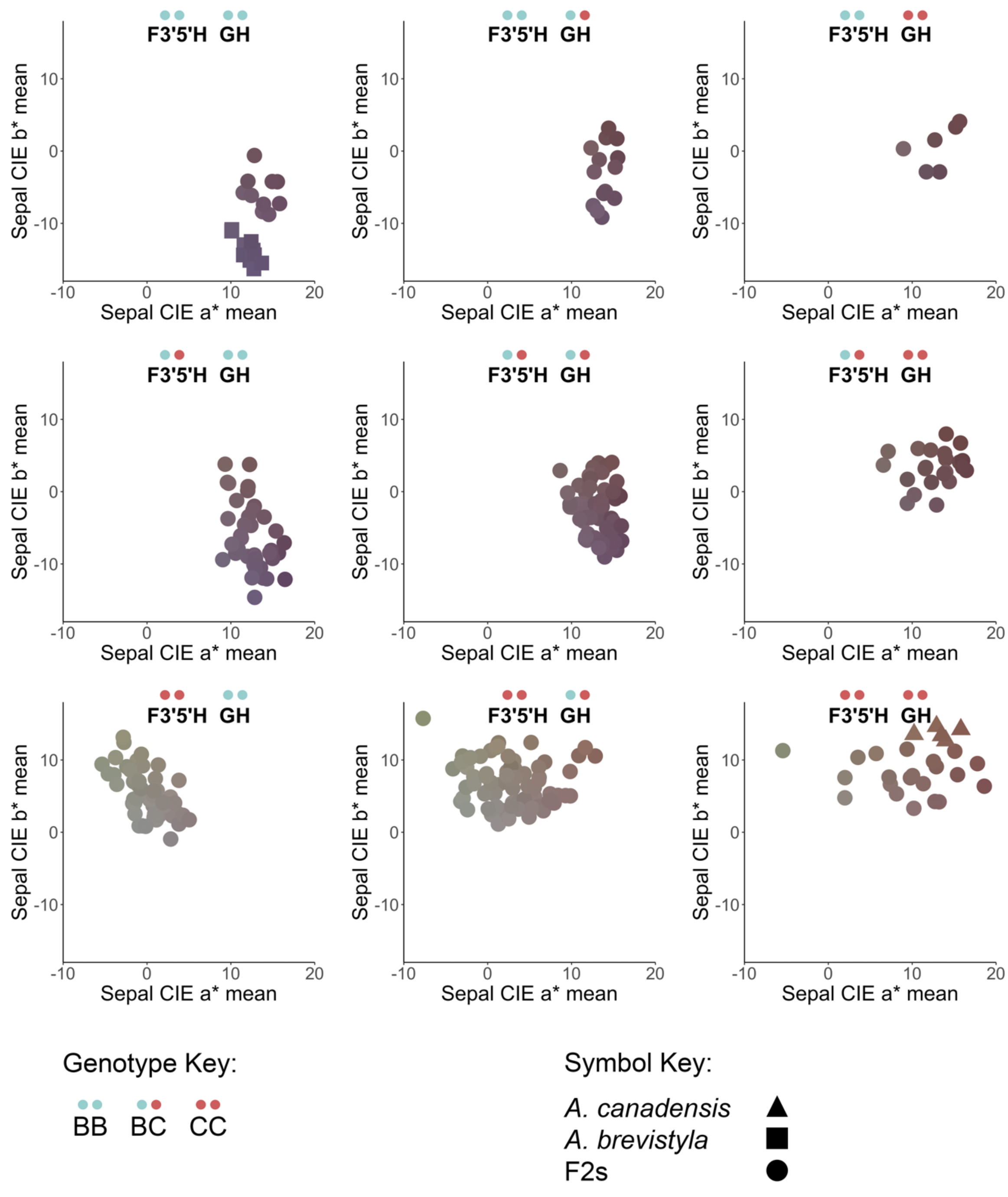

Figure S8. Sepal color phenotypes by genotype at *AqF3′5′H* and *AqGH*. Allelic genotypes of each locus are represented by pairs of blue (*A. brevistyla* allele) and red (*A. canadensis* allele) dots. Each of the nine panels represents a different allelic genotype combination. Points are color-coded based on the mean RGB values of the sepal for that individual F2 or parent plant. F3′5′H, flavonoid 3′,5′-hydroxylase; GH, glycosyl hydrolase.

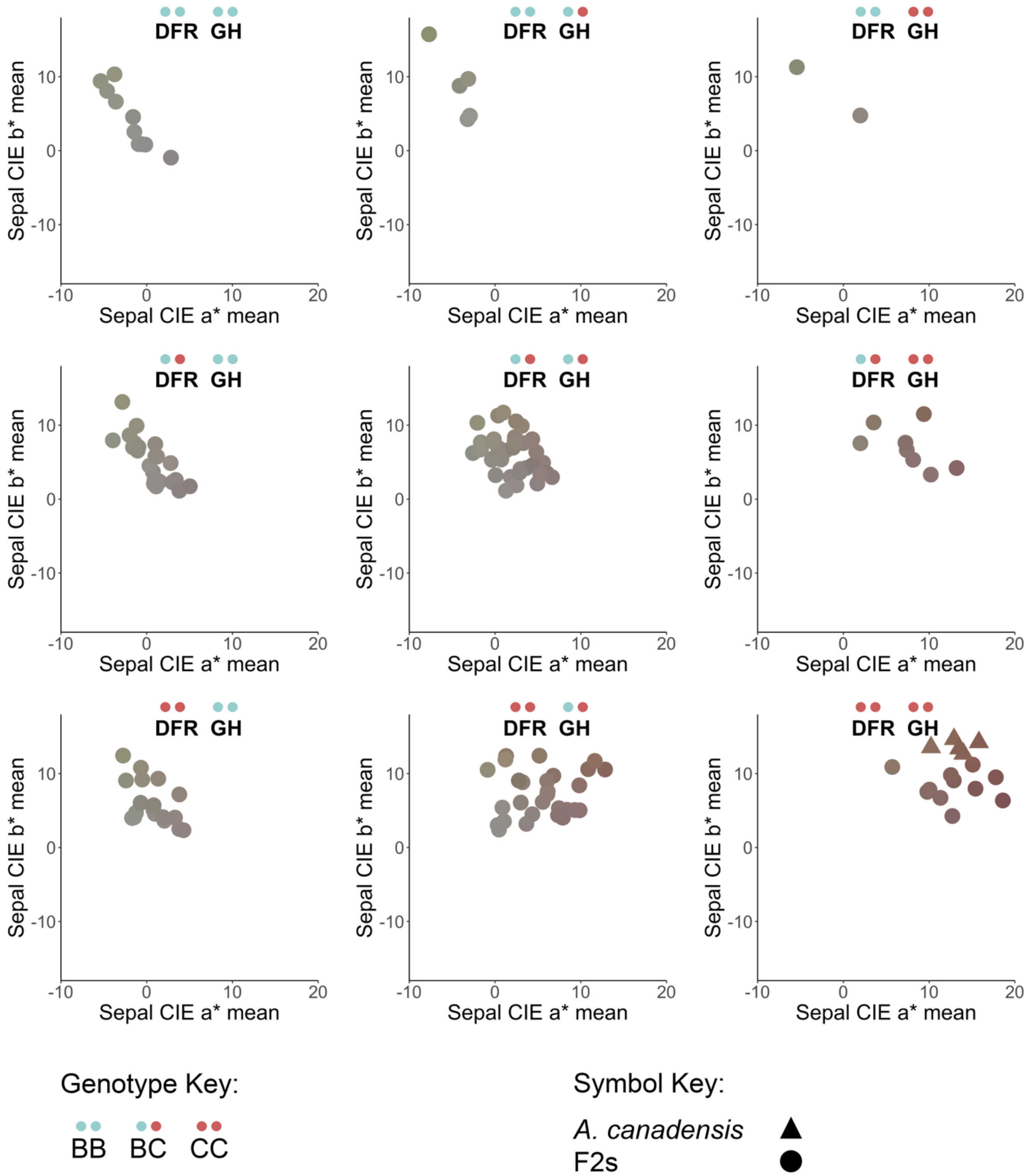

Figure S9. Sepal color phenotypes by genotype at *AqDFR* and *AqGH* in plants homozygous for *A. canadensis* at *F3′5′H*. Allelic genotypes of DFR and GH are represented by pairs of blue (*A. brevistyla* allele) and red (*A. canadensis* allele) dots. Each of the nine panels represents a different allelic genotype combination. Points are color-coded based on the mean RGB values of the sepal for that individual F2 or parent plant. F3′5′H, flavonoid 3′,5′-hydroxylase; DFR, dihydroflavonol reductase; GH, glycosyl hydrolase.

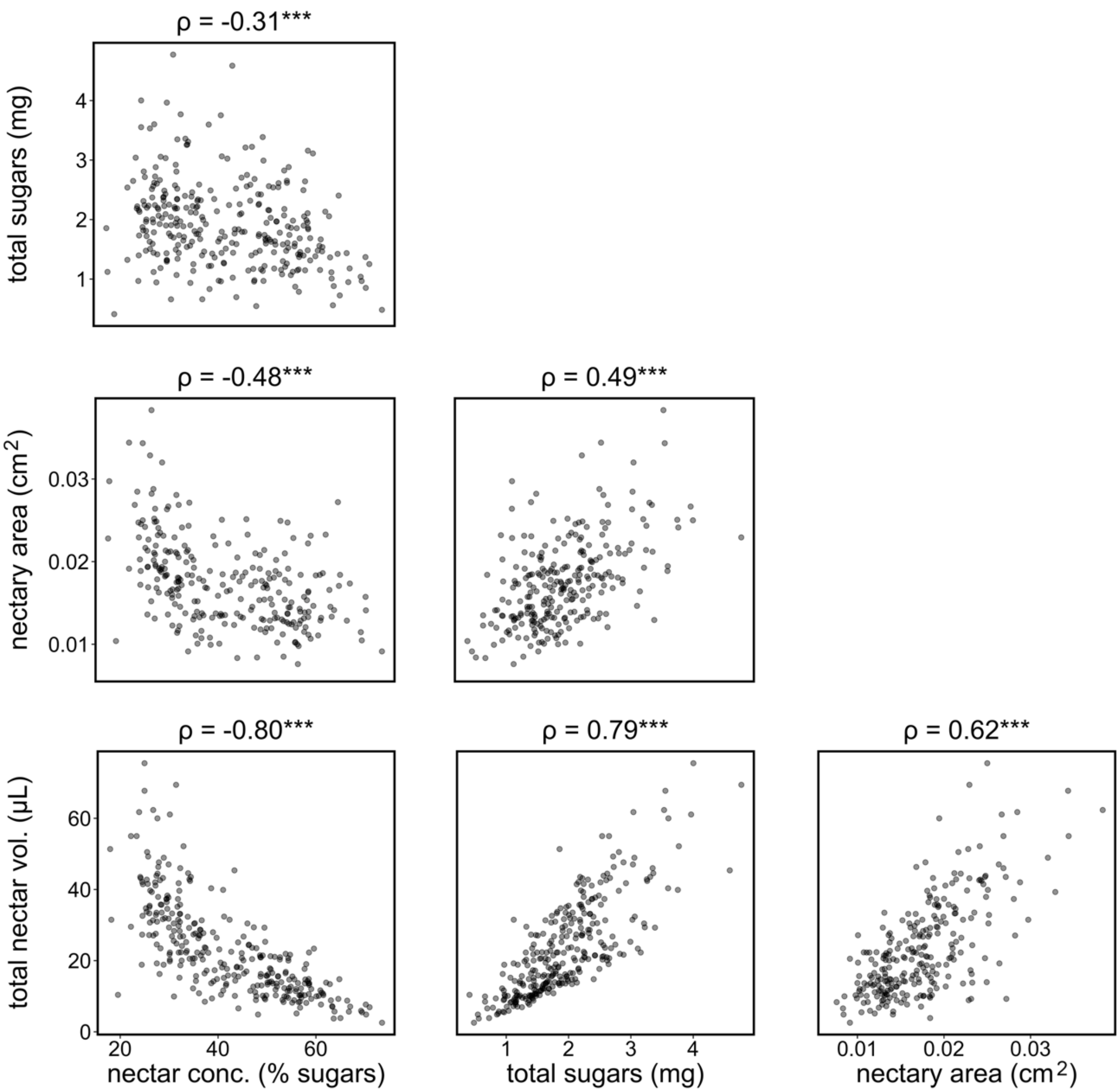

Figure S10. Pairwise correlation scatterplots among nectar and nectary traits. Conc., concentration; vol., volume; ρ, Spearman's correlation coefficient; *** $p < 0.001$

Traits under study
Sepal CIE a*
Sepal CIE b*
Sepal CIE L*
Blade CIE a*
Blade CIE b*
Blade CIE L*
Nectar volume
Nectar concentration
Total sugars
Nectary area
Sepal area
Sepal length
Sepal width
Blade length
Spur length
Spur curvature
Pistil length

Table S1. List of traits under study.

Trait	***A. brevistyla***	***A. canadensis***
Sepal CIE L*	39.03 ± 0.47	46.10 ± 0.94
Sepal CIE a*	12.11 ± 0.29	13.28 ± 0.91
Sepal CIE b*	-13.91 ± 0.46	13.70 ± 0.34
Blade CIE L*	61.30 ± 0.28	64.60 ± 0.69
Blade CIE a*	-5.73 ± 0.23	-11.11 ± 0.69
Blade CIE b*	13.32 ± 0.65	41.02 ± 0.64
Nectar volume (µL)	9.9 ± 1.3	19.8 ± 1.2
Nectar conc. (w/w)	48.6 ± 2.2	36.4 ± 1.3
Total sugars (mg)	1.1 ± 0.08	1.7 ± 0.25
Nectary area (cm^2)	0.010 ± 0.0002	0.013 ± 0.0009
Speal area (cm^2)	0.89 ± 0.04	1.08 ± 0.08
Sepal length (cm)	1.91 ± 0.06	2.22 ± 0.05
Sepal width (cm)	0.76 ± 0.02	0.83 ± 0.04
Spur length (cm)	1.16 ± 0.01	2.83 ± 0.05
Spur curvature	5.48 ± 0.30	1.26 ± 0.04
Blade length (cm)	1.06 ± 0.01	0.69 ± 0.03
Pistil length (cm)	1.00 ± 0.01	2.25 ± 0.03

Table S2. Phenotypic means and standard errors of floral traits in *A. brevistyla* and *A. canadensis* plants that were close relatives of the parents used in the cross. Conc., concentration.

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