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RESEARCH ARTICLE

How to build distylous flowers: comparative floral development and evolution of distylous species across the angiosperms

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PREMISE: Distyly, a plant breeding system characterized by two floral morphs that have reciprocal positioning of anthers and stigmas, is known from at least 27 angiosperm families, making it an excellent example of convergent evolution. The various manners in which patterns of floral development produce the distinct anther and stigma heights in each morph remain largely unexplored from developmental and evolutionary perspectives.

METHODS: In 15 species representing at least 12 origins of distyly, heights and lengths of floral organs in each morph throughout development were examined using light microscopy. Patterns of floral organ development were determined and compared among species. Family-level phylogenies of distylous species and relatives were reconstructed, and patterns of ancestral herkogamy were resolved.

RESULTS: Differences in floral development between morphs resulted in 12 patterns leading to the anther and stigma positions characterizing distyly. Distylous species evolved from ancestors with different types of herkogamy, with approach herkogamy and lack of herkogamy resolved most frequently.

CONCLUSIONS: Seven of the 12 patterns of floral development are known from only one species, with three other patterns described among pairs of close relatives. The most common pattern of floral development, described from at least seven genera, involves for anther heights, distinct intermorph growth rates and for stigma heights, growth rates that differ between morphs only during later development. This pattern is common among subclass Lamiidae, suggesting canalized development within the taxon. Among distylous species, the same type of ancestral herkogamy can give rise to different patterns of floral development.

KEY WORDS breeding system; convergent evolution; distyly; herkogamy; heterostyly; phylogenetics.

Heterostyly is a plant breeding system characterized by two or three floral morphs that occur in the same population of a species, with sexual organs at distinct, fixed heights (Barrett and Shore, 2008; Cohen, 2010). The simplest form, distyly, includes two floral morphs: a long-style (LS) morph that produces flowers with stigmas elevated over the anthers, and a short-style (SS) morph that bears flowers with anthers positioned above the stigmas. The height of the anthers in one morph is the same as that of the stigmas in the other morph, a condition known as reciprocal herkogamy. Along with this morphological aspect of distyly, a self- and intramorph-incompatibility mechanism frequently co-occurs (Barrett and Shore, 2008; Cohen, 2010). Consequently, only pollinations between sexual organs at the same level lead to production of offspring. Micromorphological differences in size or length of various features are also often observed between morphs, such as in pollen or stylar cells (Dulberger, 1992).

Distyly has arisen independently in at least 27 families of angiosperms (Ganders, 1979a; Barrett and Shore, 2008), with multiple origins in some families. For example, at least two, 12, and 20 origins have been resolved in Linaceae, Boraginaceae s.s., and Rubiaceae, respectively (McDill et al., 2009; Ferrero et al., 2012; Jones, 2012; Cohen,

2014), and it is likely that other families, such as Polygonaceae, also include multiple origins of the breeding system (cf. Schuster et al., 2011). Despite the multiple origins of distyly across the angiosperms, the evolution of the breeding system has not been examined, in a phylogenetic context, in multiple families that include distylous species, such as Iridaceae, Lamiacae, and Schoepfiaceae, which has resulted in only a nascent understanding of the evolutionary development of the breeding system in these and other groups.

Patterns of floral development in the two morphs differ among the independent origins of distyly (Richards and Barrett, 1992; Cohen, 2010). The morph-specific anther and stigma heights at anthesis can result from various developmental mechanisms, with differences between morphs observed at the genomic (Nowak et al., 2015), transcriptomic (McCubbin et al., 2006; Cohen, 2016), micromorphological (Cohen et al., 2012; Huang et al., 2014), and morphological levels (Richards and Barrett, 1992; Richards and Koptur, 1993; Faivre, 2000; Armbruster et al., 2006; Cohen et al., 2012; Huang et al., 2014), and examinations at all levels aid in the understanding of differences among independent origins of the breeding system. Investigations at the most macroscopic of these levels—morphological—provide insight into the multiple manners in which the same organs can attain different lengths and heights in the flowers of each morph, including different (1) growth rates throughout development; (2) growth rates during particular times of development; (3) organ, structure, and meristem sizes during early development; (4) times of organ initiation and cessation; (5) duration of organ growth or combinations of these possible patterns (Fig. 1) (Richards and Barrett, 1992; Cohen et al., 2012; Bull-Hereñu et al., 2016). Stamens (and corollas, if stamens are adnate) and carpels can each follow distinct, morph-specific patterns of growth that elevate the anthers and stigmas, respectively, to their particular positions. Therefore, the potential for growth in the various androecial and gynoecial structures allows for a large number of potential combinations of morph- and species-specific patterns of growth for these sexual organs. Additionally, given that stamens are sometimes adnate to the corolla and that carpels comprise stigmas, styles, and ovaries, various components of the corolla and androecium and of the gynoecium can differentially contribute, throughout development, to the ultimate heights of the anthers and stigmas. Therefore, even if two species have similar overall developmental patterns for the organs that elevate the anthers and stigmas, different parts of the corolla and androecium and of the gynoecium can be involved in producing the morph-specific heights in each species (e.g., at anthesis, species with stamens adnate to the corolla can have the height of the anthers modified either by changing the length of the filaments or the point of filament attachment to the corolla or a combination of the two) (e.g., Riveros et al., 1987; Richards and Koptur, 1993; Faivre, 2000; Sampson and Krebs, 2013).

Patterns of floral development between the morphs of distylous species differ across the angiosperms (Richards and Barrett, 1992; Richards and Koptur, 1993; Faivre, 2000), and even among members of a medium-sized genus, *Lithospermum* (Boraginaceae), divergent patterns of morph-specific floral development have arisen (Cohen, Litt, and Davis, 2012). To date, however, floral development has only been examined in a limited number of distylous species (Riveros et al., 1987; Richards and Barrett, 1992; Richards and Koptur, 1993; Faivre, 2000; Webster and Gilmartin, 2006; Li and Johnston, 2010; Cohen et al., 2012; Sampson and Krebs, 2013; Huang et al., 2014; Bull-Hereñu et al., 2016), and only rarely have multiple species been included in a single study. The present study is an examination of comparative floral development of the two distylous morphs for 15 species from across the angiosperms. This broad sampling allows for a comprehensive understanding of not only the diverse manners in which distyly can develop, but also possible constraints on evolution and development, which cannot be fully explored by examinations of individual species. When coupled with phylogenetic data, investigations of morph-specific patterns of development allow for a greater understanding of the evolution of the breeding system, including the origin of distyly from a homostylous ancestor, the evolutionary signatures of independent origins of the breeding system (i.e., various approaches to developing distylous flowers in unrelated taxa), and modifications of floral developmental patterns among related distylous species, such as those that may involve refinement of floral organ height for adaptation to pollinators.

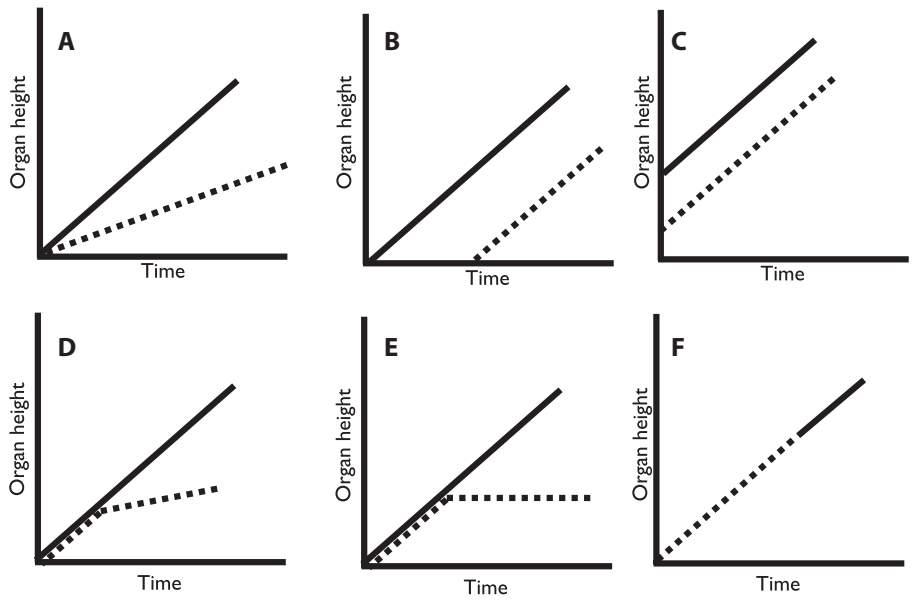

FIGURE 1. Possible patterns of development for anther and stigma height in long-style and short-style morphs of distylous species, including (A) different growth rates, (B) different times of organ initiation, (C), different organ sizes during early development, (D) different growth rates later in development, (E) growth cessation of shorter organ, and (F) increased duration of growth for longer organ. Shorter organ: dashed line; longer organ: solid line. Adapted from Richards and Barrett (1992).

MATERIALS AND METHODS

Plant material

Fifteen distylous species from across the angiosperms representing at least 12 independent origins of the breeding system were selected for the present study (Table 1). The species include *Aliciella*

TABLE 1. Distylous species, collection number or citation, collection location, number of long-style and short-style morph flowers examined in present study and pattern of anther and stigma height development. USDA is the U. S. Department of Agriculture.

Species	Voucher/Citation	Collection location	Long-style flowers	Short-style flowers	Anther height pattern	Stigma height pattern
Aliciella heterostyla	Cohen 400	Nevada, USA	32	32	Different growth rates	Different growth rates later
Averrhoa carambola	WA1 1803, HAVE 1, HAVE 22, B17 Maha	Puerto Rico (USDA)	110	52	Growth ceases later	Different growth rates later
Cordia boissieri	Cohen 419	Texas, USA	282	264	Growth ceases later	Different growth rates
Fagopyrum esculentum	S. Sherman-Broyles, Cohen 476	New York, USA	37	44	Different growth rates later	Different growth rates and length of growth period
Houstonia acerosa	Cohen 381	Texas, USA	51	52	Different growth rates	Different growth rates later
Houstonia nigricans	Cohen 384	Texas, USA	49	55	Different growth rates and length of growth period	Different growth rates later
Houstonia wrightii	Cohen 414	Arizona, USA	49	49	Different growth rates and length of growth period	Different growth rates later
Linum perenne	Cohen 397	Arizona, USA	67	63	Growth ceases later	Growth ceases later
Nivenia parviflora	M. Grantham, Cohen 477	California, USA (cultivated)	19	31	Different growth rates later	Different growth rates
Nivenia stenosiphon	M. Grantham, Cohen 478	California, USA (cultivated)	29	36	Different growth rates and length of growth period	Different growth rates later
Oreocaraya paysonii	Cohen 386	New Mexico, USA	53	56	Different growth rates	Different growth rates later
Plumbago auriculata	TARS 17935	Puerto Rico (USDA)	46	48	Different growth rates later	Growth ceases later
Pulmonaria mollis	V. Kolarčik, Cohen 479	Slovakia (cultivated)	52	49	Different growth rates	Growth ceases later
Salvia brandegeei	D. Rodriguez, Cohen 480	California, USA	49	49	Different growth rates	Different growth rates later
Turnera diffusa	Cohen 379, 380	Texas, USA	49	42	Different growth rates later	Different growth rates later
Lithospermum canescens	Cohen et al. (2012)				Different growth rates	Growth ceases later
Psychotria chiapensis	Faivre (2000)				Different growth rates	Early differences in development
Psychotria poeppigiana	Faivre (2000)				Different growth rates	Early differences in development
Bouvardia ternifolia	Faivre (2000)				Different growth rates	Different growth rates
Guettarda scabra	Richards and Koptur (1993)				Different growth rates	Different growth rates later
Amsinckia	Li and Johnston (2010)				Different growth rates	Different growth rates later
Houstonia caerulea	Sampson and Krebs (2013)				Different growth rates and length of growth period	Different growth rates later
Quinchamalium chilense	Riveros et al. (1987)				Different growth rates	Different growth rates later
Oreocarya crassipes	Cohen et al. (in review)				Different growth rates	Different growth rates later
Polygonum jucundum	Huang et al. (2014)				Different growth rates later	Different growth rates and length of growth period
Primula vulgaris	c.f. Webster and Gilmartin (2006)				Different growth rates	Different growth later, either slowing or ceasing

heterostyla (S.Cochrane & A.G.Day) J.M.Porter (Polemoniaceae), *Averrhoa carambola* L. (Oxalidaceae), *Cordia boissieri* A.DC. (Cordiaceae), *Fagopyrum esculentum* Moench (Polygonaceae), *Houstonia acerosa* (A.Gray) Benth. & Hook.f. (Rubiaceae), *H. nigricans* (Lam.) Fernald, *H. wrightii* A.Gray, *Linum perenne* L. (Linaceae), *Nivenia parviflora* Goldblatt (Iridaceae), *N. stenosiphon* Goldblatt, *Oreocarya paysonii* J.F.Macbr. (Boraginaceae), *Plumbago auriculata* Lam. (Plumbaginaceae), *Pulmonaria mollis* Wolff ex F.Heller (Boraginaceae), *Salvia brandegeei* Munz (Lamiaceae), and *Turnera diffusa* Willd. (Turneraceae). Multiple inflorescences of each morph of each species were collected from wild populations or obtained from cultivated material. Inflorescences were fixed in formalin–acetic acid–alcohol (FAA) (Ruzin, 1999) or 70% ethanol and subsequently preserved in either 70% ethanol or a mixture of 70% ethanol and 10% glycerol. For each species, inflorescences included flowers at various stages of development that were used to examine the patterns of floral development of each morph. Herbarium vouchers of collections were deposited in the herbarium at the University of Michigan, Ann Arbor (MICH) (Table 1).

Microscopy

Flowers at various stages of development were dissected and examined with light microscopy (LM) using either a Zeiss Stemi 2000c (Carl Zeiss AG, Oberkochen, Germany) or a Nikon SMZ 1500 stereomicroscope (Nikon, Melville, NY, USA). Images of the dissected flowers were captured using either a Jenoptik ProgRes C3 digital camera and ProgRes CapturePro software (Jenpotik AG, Jena, Thuringia, Germany) or a Nikon DMX 1200f digital camera and Nikon NIS-Elements software. For each morph of each species, at least 30 flowers at various stages of development (early sexual organ development through anthesis) were dissected, except for the LS morphs of the two species of *Nivenia* (Table 1).

Anther and stigma heights throughout development were measured for all species as, ultimately, it is the height of these two organs that help characterize the presence of distyly and the influence of pollinators. Because the flowers of the species included in the study vary in structure, the heights of the anthers were obtained by measuring the lengths of the filaments, if free, or the lengths of the free part of the filaments and the corolla below the point of attachment, if filaments were adnate to the corolla (Fig. 2A, B). The height of the stigmas was determined by measuring the length of the style, and if the stigma was not of negligible length and the ovary was not inferior or gynobasic, the lengths of these organs were measured as well (Fig. 2A, B). Corolla length was measured for all flowers, and corolla length serves as a proxy for flower age (i.e., time) (Cohen et al., 2012; Huang et al., 2014). All measurements were taken from the base of the corolla, point of filament attachment, or base of the organ measured (Fig. 2). Lengths and heights of floral organs were measured using the ProgRes or Nikon software or, if too large to fit under the microscope, with a ruler. Raw data for all species is available in Appendix S1, and species-specific patterns of development are described in Appendix S2.

Analyses

The growth rates for organ heights were examined in JMP v12-14 (SAS Institute, Cary, NC, USA). For each species, a standard least-squares regression line was fit to each group of data for each morph (Tables 2, 3). To examine patterns of development for the same organ, four comparisons were undertaken for each species: (1) between morphs throughout entire development, (2) between morphs during only early development, (3) between morphs during only later development, and (4) within one morph between early

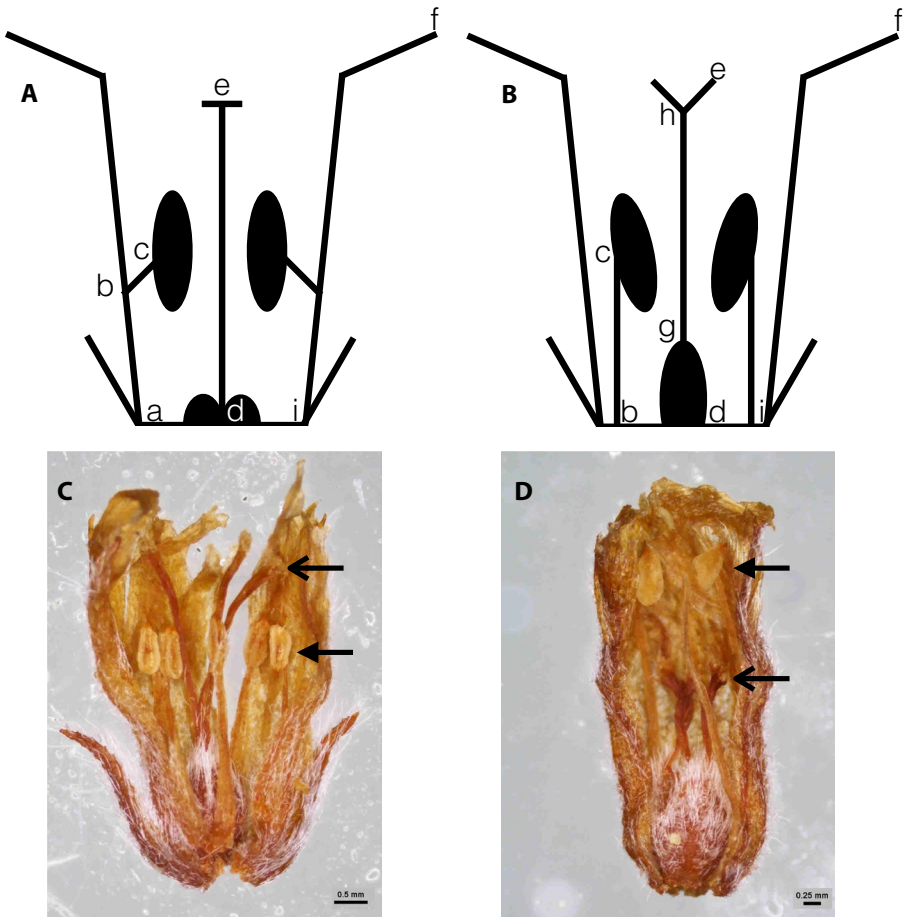

FIGURE 2. Flowers and measurements of floral organs for (A) a flower with filaments adnate to corolla and a gynobasic style and (B) a flower with free filaments and a gynoecium with well-developed ovary, style, and stigmas. Letters on flowers indicate sites of measurements for at least some species; a→b: corolla length below point of filament attachment; b→c, filament length; a→b + b→c, anther height; d→e, stigma height; d→g. ovary length; g→h, style length; h→e, stigma length; g→e, style and stigma length; i→f, corolla length. Dissected flowers of long-style (C) and short-style (D) morphs of *Turnera diffusa* (Cohen 379); closed arrows point to anthers, and open arrows point to stigmas. Scale bars: 0.5 mm (C), 0.25 mm (D).

and later development. In each species, apparent points of divergence in development of the same organ in the two morphs were used as a boundary between early and later development (Table 3, Figs. 3–5; Appendices S1 and S2) (e.g., when growth slowed or stopped in the shorter organ compared to the longer organ continuing to develop). The comparison between early and later development within morphs allows for a statistical test of growth rates that may change. Additionally, in some species, multiple floral structures contribute to anther and stigma heights at anthesis (i.e., height of the point of filament attachment to the corolla and filament length for anthers; ovary, style, and stigma lengths for stigmas), and these were compared throughout development. Because increasing floral length throughout development accompanies increasing height and length of the various floral organs, measurements taken from the organs are interconnected. To account for the interaction between organs, the "cross" function was used in the standard least-squares model, with the morph or developmental stage (i.e., early or later) "crossed" with corolla length (SAS Institute, 2009; Cohen et al., 2012). For each organ, structure, or time period, R^2 and the slope and intercept of the regression lines were recorded because these data represent the linear developmental patterns of the floral organs (Figs. 3–5).

Morph-specific differences in developmental patterns were observed to identify underlying causes for differences in heights of anthers and stigmas of the two morphs. Results from previous studies also were compared to results from the present study (Table 1). Previous studies collected data in a comparable manner to the present study: examining flowers from early sexual organ development through anthesis and employing similar measurements and analytical methods.

Phylogenetics of distyly

Phylogenetic analyses were conducted to resolve the evolutionary relationships and identify the ancestral type of herkogamy of distylous species and their homostylous relatives. To reconstruct phylogenetic trees, I included all species of the family available in GenBank (based on the Plant List 2013), with two exceptions: Misodendraceae and Loranthaceae were included along with Schoepfiaceae because this last family has a small number of species (ca. 50 species), and only species of the genus *Salvia* L. were used because the genus is large (ca. 1000 species) and part of a much more speciose family (Lamiaceae) that only includes one distylous species, *S. brandegeei* (Drew et al., 2017). The list of species was input into the OneTwoTree pipeline (Drori et al., 2018), which was used to gather sequence data from GenBank based on matched names in the National Center for Biotechnology Information (NCBI; Bethesda, MD, USA), cluster the resulting sequences into homologous groups using OrthoMCL (Li et al., 2003), and align homologous sequences with MAFFT (Katoh and Standley, 2013). Based on these alignments, phylogenies for each family were inferred using maximum parsimony (MP) and maximum likelihood (ML) methods. For MP, the program TNT (Goloboff et al., 2008) was employed using the following parameters: 100,000 trees held in memory, 1000 iterations of the parsimony ratchet (Nixon, 1999) with 4–10% upweighting and downweighting, 1000 iterations of tree drifting, 100 rounds of tree fusing, and random sectorial searches (Goloboff, 1999). Jackknife support was calculated using 1000 replicates. For ML, RAxML (Stamatakis, 2014) was utilized to search for the tree with the greatest likelihood and to conduct 1000 bootstrap replicates. For these analyses, two searches were undertaken, each with a different model of sequence evolution (GTR+Γ and GTR+Γ+I). RAxML analyses were conducted using the Kettering University High-Performance Cluster, with the exception of analyses of Rubiaceae. Because of the large size of the matrix, RAxML analyses of this family were undertaken at CIPRES (Miller et al., 2010) and, due to run times, with only 100 or 200 bootstrap replicates, depending on the model.

The type of herkogamy of distylous species and close relatives was determined via examination of herbarium specimens and from the literature (Standley, 1934; Terrell, 1996, 2001a, b; Porter, 1998; Selvi et al., 2006; Tomlinson, 2016; eFloras, 2019; Jepson eFlora, 2019), and these data were used for the ancestral character reconstructions in both MP and ML frameworks. For each family, ancestral characters were reconstructed using Fitch optimization (Fitch, 1971) with MP trees in WinClada (Nixon, 2002), with acctran and deltran employed if the reconstruction was ambiguous (Farris, 1970; Swofford and Maddison, 1987, 1992). Ancestral characters were resolved, for each ML tree, using the rayDISC function in corHMM (https://github.com/thej022214/corHMM) and three models—all rates different (ARD), equal rates (ER), and symmetrical rates (SYM)—and

TABLE 2. *P*-values of intermorph comparisons, for distylous species, for various organs and organ combinations. Not all measurements are appropriate for each species, and blank entries represent no measurement taken.

Species	Corolla length below point of filament attachment	Filament length	Anther height	Ovary length	Style length	Stigma length	Style and stigma length	Stigma height
Aliciella heterostyla	0.2442	0.0031	0.0054	0.655	—	—	<0.0001	<0.0001
Averrhoa carambola	—	<0.0001	<0.0001	0.048	—	—	<0.0001	<0.0001
Cordia boissieri	<0.0001	<0.0001	<0.0001	0.8206	—	—	<0.0001	<0.0001
Fagopyrum esculentum	—	—	<0.0001	0.2061	—	—	<0.0001	<0.0001
Houstonia acerosa	<0.0001	<0.0001	<0.0001	—	<0.0001	0.4417	—	<0.0001
Houstonia nigricans	<0.0001	<0.0001	<0.0001	—	<0.0001	0.037	—	<0.0001
Houstonia wrightii	<0.0001	<0.0001	<0.0001	—	<0.0001	0.5843	—	<0.0001
Linum perenne	—	<0.0001	<0.0001	<0.0001	<0.0001	0.0103	—	<0.0001
Nivenia parviflora	0.1719	<0.0001	<0.0001	—	—	—	<0.0001	<0.0001
Nivenia stenosiphon	0.8948	<0.0001	<0.0001	—	—	—	<0.0001	<0.0001
Oreocarya paysonii	<0.0001	0.4572	<0.0001	—	—	—	<0.0001	<0.0001
Plumbago auriculata	—	—	<0.0001	<0.0001	<0.0001	0.2395	—	<0.0001
Pulmonaria mollis	—	—	<0.0001	—	—	—	<0.0001	<0.0001
Salvia brandegeei	0.0035	<0.0001	<0.0001	—	—	—	<0.0001	<0.0001
Turnera diffusa	—	—	<0.0001	—	—	—	—	<0.0001

TABLE 3. *P*-values of intra- and intermorph comparisons of early and of later development for anther and stigma height in distylous species. Corolla length of boundary between early and later development included for each species. For statistically significant differences, time period (early [E] or late [L]) or morph (long-style [LS] and short-style [SS]) with organs contributing to faster and slower rates of growth are identified.

Species	Early-later corolla-length boundary (mm)	Long-style morph		Short-style morph		Early		Later	
		Anther height	Stigma height	Anther height	Stigma height	Anther height	Stigma height	Anther height	Stigma height
Aliciella heterostyla	6	0.0264, L > E	0.0434, E > L	0.0741, L > E	0.0046, E > L	0.0251, SS > LS	0.001, LS > SS	0.6428	0.9351
Averrhoa carambola	5	<0.0001, E > L	0.2111	<0.0001, E > L	0.0014, E > L	0.0533	0.0284, LS > SS	<0.0001, SS > LS	0.0138, LS > SS
Cordia boissieri	27	<0.0001, E > L	<0.0001, E > L	<0.0001, E > L	<0.0001, E > L	<0.0001, SS > LS	<0.0001, LS > SS	<0.0001, SS > LS	<0.0001, LS > SS
Fagopyrum esculentum	3	0.6783	0.8618	0.4362	0.4582	0.0499, SS > LS	0.2191	0.0093, SS > LS	0.267
Houstonia acerosa	5.3	0.3796	<0.0001, E > L	0.014, E > L	<0.0001, E > L	<0.0001, SS > LS	0.7525	0.0063, SS > LS	<0.0001, LS > SS
Houstonia nigricans	4.3	0.016, L > E	0.0003, E > L	0.4263	<0.0001, E > L	<0.0001, SS > LS	0.042, LS > SS	<0.0001, SS > LS	<0.0001, LS > SS
Houstonia wrightii	3.8	0.1424	0.3113	0.8143	<0.0001, E > L	<0.0001, SS > LS	0.0323, LS > SS	<0.0001, SS > LS	<0.0001, LS > SS
Linum perenne	6	<0.0001, E > L	<0.0001, E > L	0.299	<0.0001, E > L	0.6025	<0.0001, LS > SS	0.0095, SS > LS	<0.0001, LS > SS
Nivenia parviflora	8	0.5033	0.6414	0.2678	0.5165	0.0078, SS > LS	0.0131, LS > SS	0.3319	0.2544
Nivenia stenosiphon	20	0.8923	0.5999	0.566	0.0008, E > L	0.1229	0.0023, LS > SS	0.2392	0.005, LS > SS
Oreocarya paysonii	6.7	0.3081	0.0016, E > L	0.4181	0.012, E > L	<0.0001, SS > LS	<0.0001, LS > SS	<0.0007, SS > LS	0.0003, LS > SS
Plumbago auriculata	30	0.6859	<0.0001, E > L	0.0063, E > L	<0.0001, E > L	0.0172, SS > LS	<0.0001, LS > SS	0.0846	0.1134, LS > SS
Pulmonaria mollis	9	0.6471	<0.0001, E > L	<0.0001, E > L	<0.0001, E > L	<0.0001, SS > LS	<0.0001, LS > SS	0.0057, SS > LS	0.8091
Salvia brandegeei	9	0.225	<0.0001, E > L	0.0142, E > L	<0.0001, E > L	0.0003, SS > LS	0.109	0.5604	0.5873
Turnera diffusa	4.3	0.0075, E > L	<0.0001, E > L	0.7188	0.2391, E > L	0.4397	0.0028, LS > SS	0.0219, SS > LS	0.3009

node states calculated via marginal probabilities. For Passifloraceae and Primulaceae, only the presence/absence of distyly (i.e., distyly or homostyly) was recorded as distyly has been resolved as ancestral in these families (Shore et al., 2006; de Vos et al., 2014). *Psychotria chiapensis* Standl. was not included in the phylogeny of Rubiaceae due to lack of available sequence data. The sequence data used in phylogenetic analyses are included in Appendix S3, and data on the type of herkoogamy in the species and results from ancestral character reconstruction are in Appendix S4.

RESULTS

Patterns of development in distylous flowers

In the species, developmental patterns of anther height and of stigma height differed significantly between morphs (Figs. 3–5, Tables 2, 3), but not for all organs and structures examined. Morph-specific patterns for structures composing anther and stigma heights differ among species and genera. Species-specific patterns of floral development are detailed in Appendix S2.

The rate of elongation of filaments between morphs differed significantly in all species except *O. paysonii*, and it was faster in the SS morph of these species. For species with stamens adnate to the corolla, the rate of growth of the corolla below the point of filament attachment differed significantly between morphs in six of nine species, and it was also faster in the SS morph compared to the LS morph (Table 2). The structures contributing to the height of the anthers grew faster in the SS morph than in the LS morph early and later in development in 11 and 10 species, respectively (Table 3). Four species (*A. heterostyla*, *N. parviflora*, *P. auriculata*, and *S. brandegeei*) had faster growth for these structures only during early development, while those of three others (*A. carambola*, *L. perenne*, and *T. diffusa*) only grew faster during later development. The structures grew faster in seven species during early and later development, and only *N. stenosiphon* lacked intermorph differences in early or later development (Table 3).

Multiple measurements were made to understand the growth rate of the gynoecium and, ultimately, the contributors to stigma height. The growth rate for ovary length, stigma length, and collective style and stigma length differed significantly between morphs in three of six species, in two of five species, and in all species, respectively (Table 2), with the LS morph being faster than the SS morph. During early and later development for 12 species and nine species, respectively, the structures contributing to the height of the stigma grew faster in the LS morph than in the SS morph (Table 3). Three species (*A. heterostyla*, *N. parviflora*, and *T. diffusa*) had faster rates of growth for these organs only during early development, while only one species, *H. acerosa*, grew faster only during later development. Eight species grew faster during early and later development,

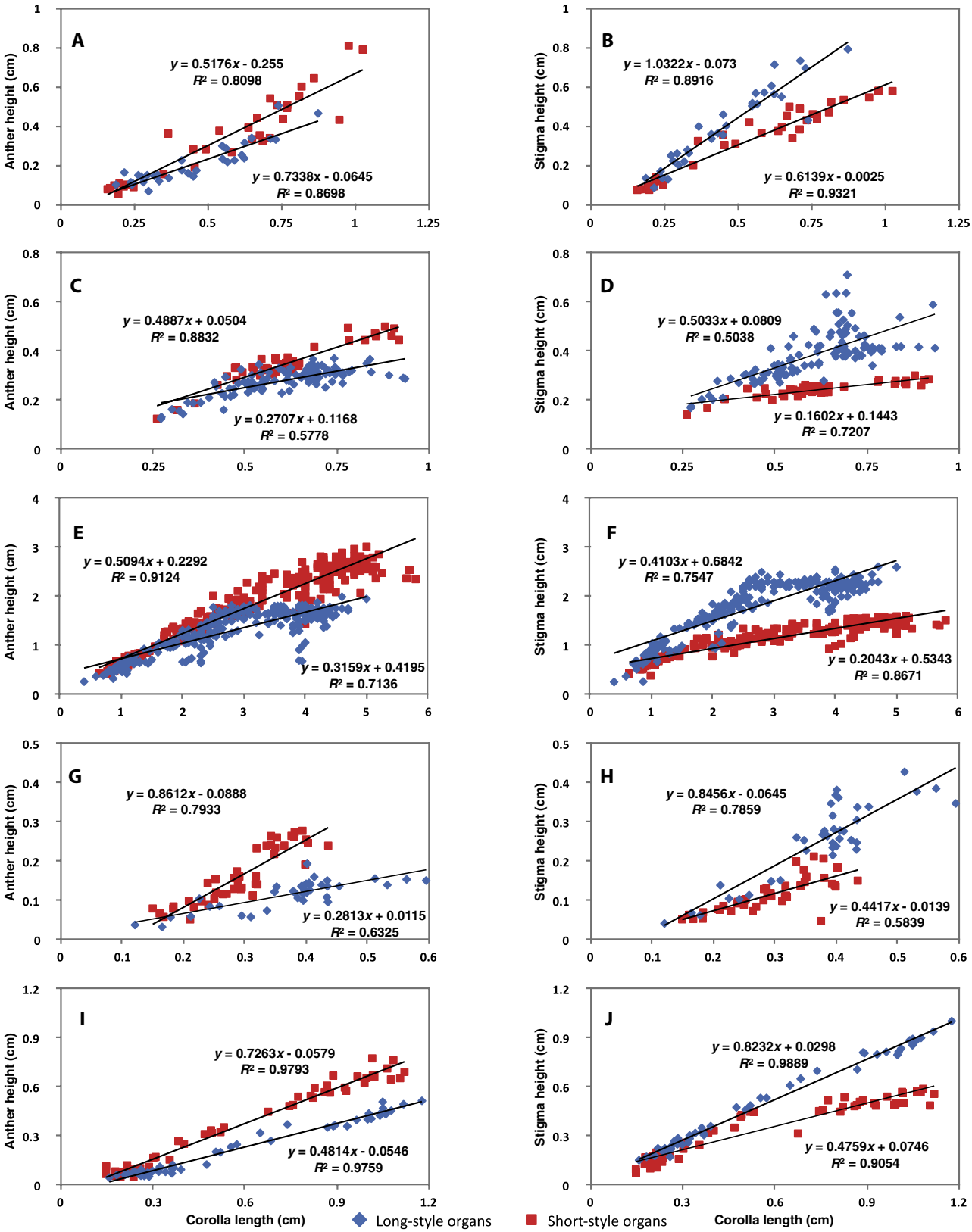

FIGURE 3. Patterns of development for anther and stigma height for (A, B) *Aliciella heterostyla*, (C, D) *Averrhoa carambola*, (E, F) *Cordia boissieri*, (G, H) *Fagopyrum esculentum*, and (I, J) *Houstonia acerosa*. (A), (C), (E), (G), and (I) are for anther height; (B), (D), (F), (H), and (J) are for stigma height. Blue squares: organs of long-style morph; red triangles: organs of short-style morph. Linear regression lines, equations, and R^2 are included for each organ in each morph.

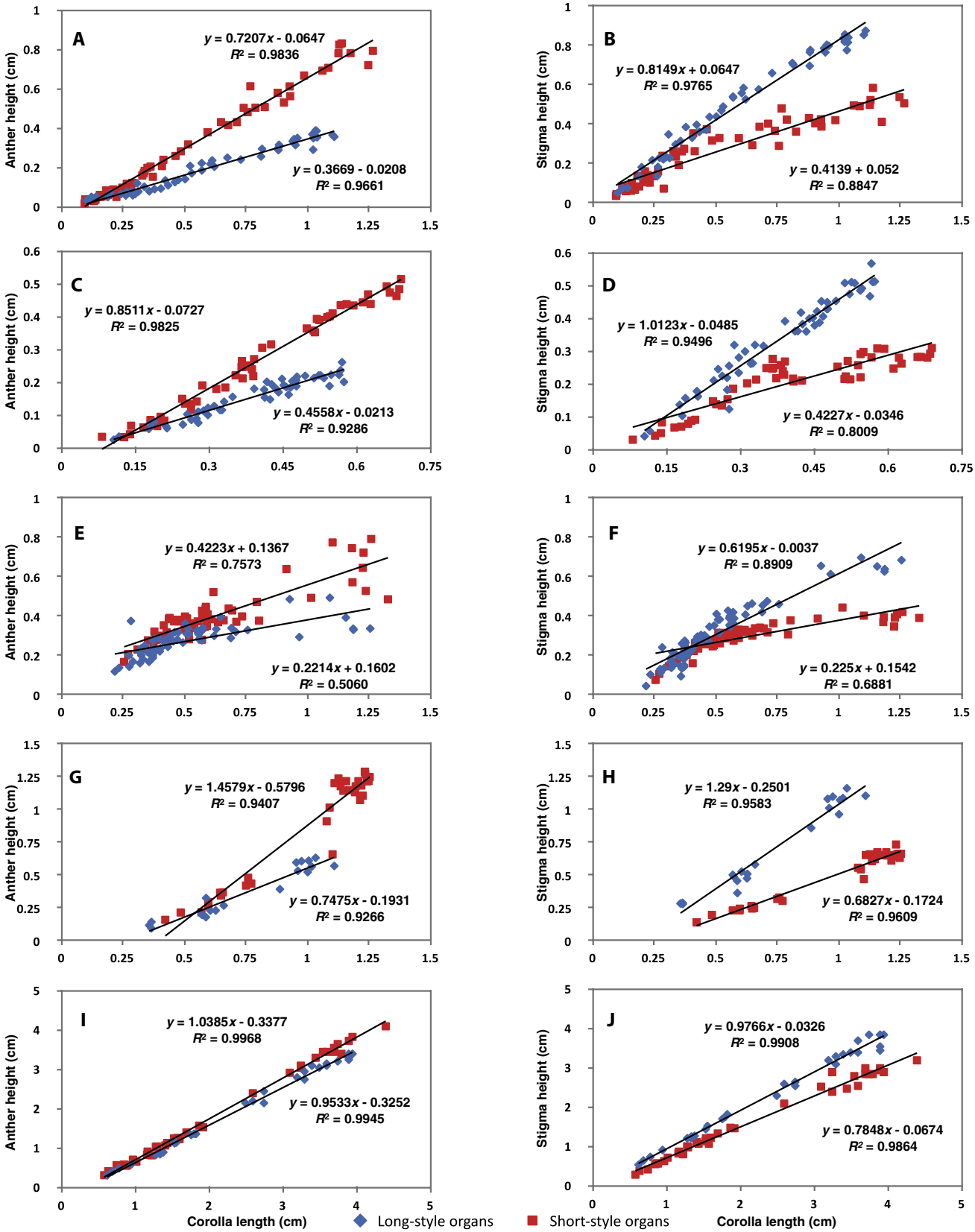

FIGURE 4. Patterns of development for anther and stigma height for (A, B) *Houstonia nigricans*, (C, D) *Houstonia wrightii*, (E, F) *Linum perenne*, (G, H) *Nivenia parviflora*, and (I, J) *N. stenosiphon*. (A), (C), (E), (G), and (I) are for anther height; (B), (D), (F), (H), and (J) are for stigma height. Blue squares: organs of long-style morph; red triangles: organs of short-style morph. Linear regression lines, equations, and R^2 are included for each organ in each morph.

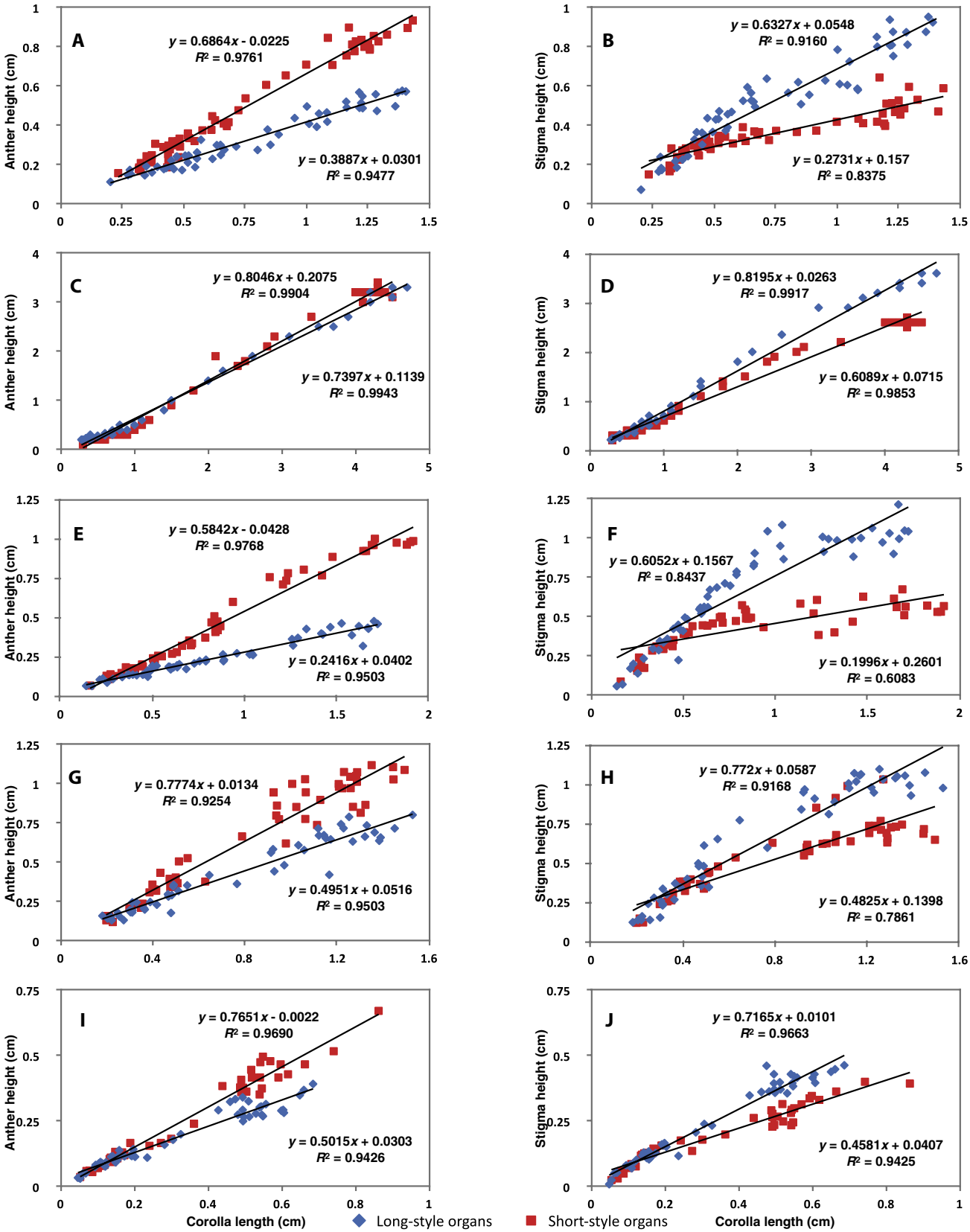

FIGURE 5. Patterns of development for anther and stigma height for (A, B) *Oreocarya paysonii*, (C, D) *Plumbago auriculata*, (E, F) *Pulmonaria mollis*, (G, H) *Salvia brandegeei*, and (I, J) *Turnera diffusa*. (A), (C), (E), (G), and (I) are for anther height; (B), (D), (F), (H), and (J) are for stigma height. Blue squares: organs of long-style morph, and red triangles: organs of short-style morph. Linear regression lines, equations, and R^2 are included for each organ in each morph.

and two species (*F. esculentum* and *S. brandegeei*) showed no intermorph differences during early or later development (Table 3).

Flowers of the LS morph of six species had statistically significant growth rates for the structures contributing to the height of the anthers between early and later development. In these species, rates in early development were faster than in later development in four species, with *A. heterostyla* and *H. nigricans* as the exceptions (Table 3). Flowers of the SS morph of six species had statistically significant growth rates of the structures contributing to the height of the anthers between early and later development. In these species, early development was faster than later development in five species, with *A. heterostyla* as the only exception (Table 3). Both morphs of three species (*A. heterostyla*, *A. carambola*, and *C. boissieri*) had statistically significant differences for the growth rates of the structures that elevate the anthers throughout development, with other species having only one morph displaying this difference. For the structures involved in the height of the stigmas, early growth rates were faster than later growth rates in both morphs, with a few exceptions (the LS morphs of *A. carambola*, *H. wrightii*, *and N. stenosiphon* and both morphs of *F. esculentum* and *N. parviflora*) (Table 3).

Four patterns were observed for the development of the organs elevating the anthers and stigmas to their morph-specific heights: (1) different growth rates between morphs, (2) development of the shorter organ ceasing in one morph and continuing for the longer organ in the other, (3) different growth rates during later development, and (4) different growth rates and a longer of growth period for the longer organ. For stigma heights, a fifth pattern was also identified: different growth rates established early in development (Table 1). Together, these contribute to 12 combinations of developmental patterns for the collective organs involved in anther and stigma heights among the species. The most common pattern, present in at least seven genera (*Amsinckia*, *Aliciella*, *Guettarda*, *Houstonia*, *Oreocarya*, *Quinchamalium*, and *Salvia*) distributed among five families, involved different growth rates for organs contributing to anther height between morphs and different growth rates during later development for organs involved in stigma heights of the two morphs.

Phylogenetics of distyly

Twelve phylogenies were reconstructed, with some phylogenies including multiple distylous species from the present study (Fig. 6). In general, the MP and two ML phylogenies were quite similar, which was also the case for the different approaches for ancestral character reconstruction (i.e., MP and three models in ML). Distyly was resolved as ancestral in multiple genera and families (Table 4). Of the other species, reverse herkogamy, absence of herkogamy, and approach herkogamy were resolved as ancestral for four, five to seven, and eight to nine origins, respectively (Table 4).

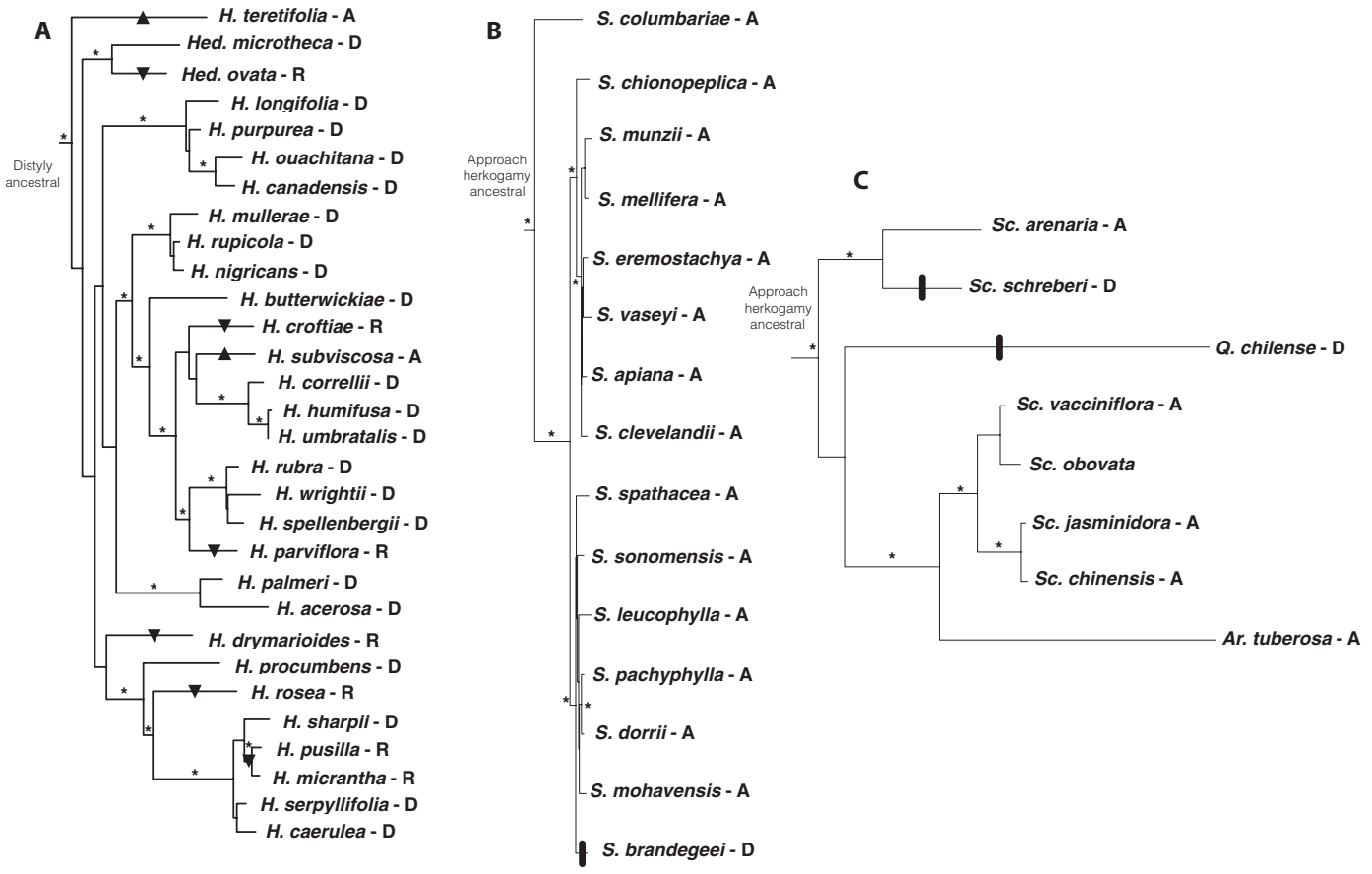

FIGURE 6. Maximum likelihood phylogenetic trees, based on GTR+G model, of (A) *Houstonia* (*H.*) and *Hedyotis* (*Hed.*), (B) *Salvia* (*S.*), and (C) *Schoepfia* (*Sc.*), *Quinchamalium* (*Q.*), and *Arjona* (*Ar.*) showing evolutionary patterns and distribution of types of herkogamy. D, distyly; R, reverse herkogamy; A, approach herkogamy. Symbols denote reconstruction of distyly (ellipses), approach herkogamy (triangles), and reverse herkogamy (downward-pointing triangles), respectively. Asterisks represent 70% or greater maximum parsimony jackknife support and maximum likelihood bootstrap support.

TABLE 4. Summary of results of phylogenetic analyses of distylous species, including number of species, DNA regions, and aligned length of DNA regions, in base pairs (bp). Ancestral type of herkogamy noted, for each species, via ancestral character reconstruction in maximum parsimony (MP) and maximum likelihood (ML) frameworks.

Species	Family	No. of species	No. of DNA regions	Alignment length (bp)	Ancestral type of herkogamy	
					MP	ML
Aliciella heterostyla	Polemoniaceae	336	13	16,876	Approach/Absence of herkogamy	Absence of herkogamy
Averrhoa carambola	Oxalidaceae	298	11	14,209	Heterostyly ancestral	
Cordia boissieri	Cordiaceae	1200	15	27,666	Reverse herkogamy	
Fagopyrum esculentum	Polygonaceae	544	21	29,658	Absence of herkogamy	Heterostyly most likely ancestral; absence of less likely
Houstonia acerosa	Rubiaceae	3377	16	46,326	Heterostyly ancestral	
Houstonia nigricans	Rubiaceae	3377	16	46,326	Heterostyly ancestral	
Houstonia wrightii	Rubiaceae	3377	16	46,326	Heterostyly ancestral; approach and reverse herkogamy resolved in ancestors and close relatives	Heterostyly ancestral; reverse herkogamy less likely
Linum perenne	Linaceae	97	11	21,660	Heterostyly ancestral; absence of herkogamy resolved in ancestor of clade of heterostylous species	Heterostyly ancestral; other types of herkogamy also likely
Nivenia parviflora	Iridaceae	1025	20	27,697	Approach herkogamy	
Nivenia stenosiphon	Iridaceae	1025	20	27,697	Approach herkogamy	
Oreocaraya paysonii	Boraginaceae	1200	15	27,666	Heterostyly ancestral; absence of herkogamy in close relatives	
Plumbago auriculata	Plumbaginaceae	308	11	12,764	Heterostyly ancestral; reverse herkogamy resolved in ancestors	Heterostyly ancestral; reverse or approach herkogamy resolved in ancestors
Pulmonaria mollis	Boraginaceae	1200	15	27,666	Heterostyly ancestral; approach herkogamy resolved in closest ancestor	
Salvia brandegeei	Labiatae	486	15	19,220	Approach herkogamy	
Turnera diffusa	Passifloraceae	473	13	21,905	Heterostyly ancestral	
Lithospermum canescens	Boraginaceae	1200	15	27,666	Approach herkogamy	
Psychotria chiapensis	Rubiaceae	3377	16	46,326	Not included in phylogenetic analyses	
Psychotria poeppigiana	Rubiaceae	3377	16	46,326	Heterostyly ancestral	
Bouvardia ternifolia	Rubiaceae	3377	16	46,326	Heterostyly ancestral	
Guettarda scabra	Rubiaceae	3377	16	46,326	Approach herkogamy	Heterostyly ancestral or approach herkogamy
Amsinckia	Boraginaceae	1200	15	27,666	Absence of herkogamy	Heterostyly ancestral
Houstonia caerulea	Rubiaceae	3377	16	46,326	Heterostyly ancestral; reverse herkogamy resolved in close relatives	Heterostyly ancestral; reverse herkogamy resolved in close relatives and possible in ancestors
Quinchamalium chilense	Schoepfiaceae	159	12	17,343	Approach herkogamy	
Oreocarya crassipes	Boraginaceae	1200	15	27,666	Heterostyly ancestral; close relatives lacking herkogamy	
Polygonum jucundum	Polygonaceae	544	21	29,658	Absence of herkogamy	Heterostyly ancestral most likely; absence of herkogamy less likely
Primula vulgaris	Primulaceae	734	13	19,802	Heterostyly ancestral	

DISCUSSION

Overall patterns of floral development

Distyly is a compelling example of convergent evolution of a suite of floral traits, and the present study provides evidence of multiple developmental changes and ancestral types of herkogamy associated with the evolution of the floral morphs. Four and five morph-specific patterns of development were observed for organs elevating the anthers and for positioning the stigmas, respectively, among species. While multiple possibilities exist for the intermorph development of floral organs of the two distylous morphs (Fig. 1), 12 distinct patterns for producing the anther and stigma heights of the two morphs were identified among species (Table 1). Seven of the floral developmental patterns are only known from one species, with three other patterns described among pairs of close relatives. Only two patterns of floral development are known from multiple species from different families (Table 1). Consequently, most of the known origins of distyly are unique in their integration of floral developmental patterns for sexual organ height. Most of the patterns for intermorph organ development depicted in Fig. 1 were identified among studied species; however, some of these patterns, such as morph-specific timing of organ initiation or size of organs, may be more frequent but occur earlier in development than the present study was able to identify (e.g., Bull-Hereñu et al., 2016).

The most common pattern of development, known from at least eight species from seven genera, involves morph-specific growth rates for filament (and frequently the corolla) elongation for anther heights and different growth rates between morphs, during later gynoecial development, for stigma height. The individual patterns for

the positioning of the anthers and of the stigmas are even more common across the angiosperms. Indeed, five other distylous species exhibit the specific pattern for raising the anthers (13 species in total), and six studied distylous species display the particular pattern for the development of stigma height (14 species in total) (Table 1). These results suggest that particular patterns for elevating anther and stigma heights may be favored across the angiosperms, even if the patterns do not occur together in a species. Most of the species that bear the most common pattern are members of Lamiidae (i.e., the lamiids sensu APG 4; Asterid I) (Chase et al., 2016), and while distyly is not frequent among this group, of all subclasses across the angiosperms, Lamiidae has the most distylous species (Ganders, 1979a; Barrett, 1992).

Most species of Lamiidae included in the present study have small stigmatic receptive surfaces as well as inferior ovaries or gynobasic styles, which result in the majority, if not all, of the differences in stigma heights due to divergent stylar growth between morphs. A shift in gynoecial length is one of the earliest stages in the evolution of distyly (Anderson, 1973; Lloyd and Webb, 1992), and restricting shifts in length to only the style, rather than other or multiple areas of the gynoecium, may facilitate the origin of distyly among these species because it minimizes the number of gynoecial structures that need to be modified to attain the distinct intermorph stigma heights.

Primula vulgaris and *Aliciella heterostyla*, members of Ericales (the clade sister to the one that includes both Lamiidae and Campanuliidae) (Chase et al., 2016), also exhibit the most common pattern of development for anther and stigma heights (morph-specific growth rates for anther heights and different growth rates, between morphs, during later development for stigma height). Among the asterids, distyly originated multiple times and from different types of ancestral herkogamy, demonstrating that the common developmental pattern is the result of convergent evolution. The prevalence of this pattern among the various origins in Ericales and Lamiidae could suggest a similar, underlying pattern of floral development, in the asterids, from which these distylous species evolved. Studies of floral development of distylous species in Menyanthaceae can shed additional light on this possibility because Menyanthaceae is the only member of the group sister to Lamiidae, Campanuliidae (i.e., the Campanuliids sensu APG 4; asterid II), known to include distylous species (Ganders, 1979a). Studies on distylous species of Menyanthaceae could identify whether the pattern in this family is similar to, or different from, that of other members of the asterids and, therefore, which members of the asterids exhibit this pattern: all of them or only those in Ericales and Lamiidae. The common pattern also has been identified in *Quinchamalium chilense*, a member of Santalales, demonstrating that this type of development is not limited to Lamiidae or asterids. All of the species with the most common pattern of floral development (Table 1) bear stamens adnate to a sympetalous corolla, an arrangement of sexual organs that impacts the likelihood for the evolution of distyly.

The developmental pathways in Lamiidae may have a greater ability to respond to selection in particular groups, such as Boraginales, an order with at least 13 independent origins of distyly (cf. Cohen, 2014), and Rubiaceae, a family with at least 20 origins of the breeding system (Jones, 2012). In both groups, divergent patterns of growth for sexual organ height were identified (i.e., *Cordia* and *Lithospermum* in Boraginales; *Bouvardia*, *Psychotria*, and three derived species of *Houstonia* in Rubiaceae [Table 1]). In some situations, such as with species of *Houstonia*, the modifications to the common pattern of development are the result of additional growth to elevate anthers in the SS morph compared to in the LS morph. Faivre (2000) hypothesized that this type of additional organ growth in one morph contributes to increased precision of reciprocal herkogamy between morphs. In other species, such as *Cordia boissieri*, the floral development pattern differs completely from that of relatives. In this species, the different patterns of development may reflect the divergent evolutionary history of the genus relative to other distylous Boraginales. *Cordia boissieri* also develops corollas that are much larger and more funnelform in shape than other studied species in the order, and these differences could influence its pattern of development.

Divergent patterns of filament length development are observed between morphs in all species, except those of Boraginaceae, and the height of filament attachment to the corolla differs in all species with filaments adnate to the corolla, except for species of *Aliciella* and *Nivenia* (Table 2). In species in which the filaments are adnate to the corolla and the filaments are not of negligible length, filament length plays a larger role in the resulting morph-specific anther heights, throughout development and at anthesis, than the height of filament attachment to the corolla, except in species of *Nivenia* (Appendices S1 and S2). Therefore, a lack of integrated development exists between free filament length and corollas in most distylous species, at least in the Asterids. Rather than the corolla and the filaments collectively impacting the heights of the anthers, it either appears to be one or the other.

Evolution of floral development of distyly

Even after more than 150 years of study since Darwin's (1877) foundational book on distyly, the evolution of the breeding system remains an open question. Patterns of floral development, in conjunction with phylogenetic analyses and the ancestral type of herkogamy, aid in understanding the diverse origins of distyly, particularly when examined in the context of the two most common models for the evolution of the breeding system. The two models each invoke a different ancestral type of herkogamy as well as distinct orders of events for the origin of distyly: Charlesworth and Charlesworth (1979) hypothesized that an ancestor lacking herkogamy and with self- and intramorph incompatibility arose before one with reciprocal herkogamy, while Lloyd and Webb (1992) inferred an ancestor that was approach (or, less frequently, reverse) herkogamous and that reciprocal herkogamy originated before self- and intramorph incompatibility.

Ancestors of most of the species in the present study exhibit some separation between the anthers and stigmas, either displaying approach or reverse herkogamy (Fig. 6, Table 4). Most of the inferred origins are congruent with the model of Lloyd and Webb (1992). Consequently, most examined species, including *Q. chilense* and *S. brandegeei* (Fig. 6), would develop the morphological component of distyly (i.e., reciprocal herkogamy) before the physiological aspect (i.e., self- and intramorph incompatibility), but some, such as *A. heterostyla* and *L. perenne*, may undergo the opposite order of events. Both *Q. chilense* and *S. brandegeei* are resolved to each have an approach herkogamous ancestor (Fig. 6B and C), similar to the LS morph of these distylous species, so the SS morph would be the derived condition. During the origin of distyly in both species, flowers of the SS morph would result from the rate of filament elongation increasing throughout development and stylar growth slowing during later development.

In a small number of species, such as *Cordia boissieri*, reverse, not approach, herkogamy, is resolved as the ancestral condition (Table 4), which is the opposite type proposed by Lloyd and Webb (1992). In this situation, the LS morph would be derived. In *C. boissieri*, stylar growth would have slowed or halted in the ancestor,

similar to floral development in SS morph, and the derived pattern would represent stylar growth continuing throughout development, while staminal growth rates would decrease (Figs. 3–5).

In *Houstonia*, distyly is resolved as ancestral (Fig. 6A), with multiple losses of the breeding system, and two developmental patterns for anther height have been identified. In *H. acerosa*, different growth rates contribute to the distinct heights of the anthers, while in *H. caerulea* L. (Sampson and Krebs, 2013), *H. nigricans*, and *H. wrightii*, different growth rates and durations result in the final anther heights in the two morphs (Figs. 3I and 4A, C). Phylogenetic evidence resolves *H. acerosa* in a clade sister to one that includes *H. nigricans* and *H. wrightii* (Fig. 6A) (Shanks, 2015). Given the phylogenetic distribution of the most common pattern of anther height development (i.e., different growth rates between morphs), this pattern can be hypothesized as ancestral to one that includes differences in both growth rates and durations, which is congruent with the current phylogeny of the genus. *Houstonia caerulea* is a member of a different clade from that of the other three species included in the present study, so its pattern of development may represent an independent origin of this derived condition. Further investigations of members of this clade, as well as of distylous species in the clade that includes *H. purpurea* L. and *H. canadensis* Willd. ex Roem. & Schult., can identify whether the same hypothesized sequence of events resulted in the similar evolutionary development of distyly.

In *Nivenia*, the ancestral species is resolved as approach herkogamous, with the LS morph more similar to the ancestral condition than the SS morph, and two patterns of floral development are observed, one in *N. parviflora* and one in *N. stenosiphon* (Table 1). In both morphs of *N. parviflora*, the growth rates of the structures elevating the higher anthers and stigmas increase during later development (Fig. 4G). In general, during later development, growth of organs contributing to anther and stigma height slows (Tables 1–3, Figs. 3–5), so it is unusual to observe this increase in later-stage growth. This pattern for anther elevation has only been noted previously in distylous species of Polygonaceae, in which the later growth rate of the filaments, particularly the outer whorl of filaments (Huang et al., 2014), increases in the SS morph compared to the LS morph (Tables 1–3), and in the tristylous species *Eichhornia paniculata* Solms (Pontederiaceae) (Richards and Barrett, 1992). In the other species, *N. stenosiphon*, the organs contributing to anther height in the SS morph appear have a longer period of growth than in the LS morph, which also occurs in some species of *Houstonia*. Given that *Nivenia* is a small genus (ca. 10 species), it is unusual that two patterns of development were identified, and these patterns may provide evidence for independent origins of the breeding system in the genus. Alternatively, the presence of multiple, distinct floral developmental patterns in *Nivenia* may result from continual adaptation, via organ positioning, throughout the evolution and maintenance of reciprocal herkogamy for effective intermorph pollination.

Distyly originated independently in multiple species of Polygonaceae (cf. Schuster et al., 2011). Two are included in the present study, *Polygonum jucundum* Meisn. (Huang et al., 2014) and *Fagopyrum esculentum*, and both arose from an ancestor lacking herkogamy, providing evidence of similar pathways for the evolution of the breeding system across the family. However, in *P. jucundum*, filament elongation increases during later development in the SS morph, but in *F. esculentum*, filament elongation slows during later development in the LS morph. These differences in developmental patterns illustrate that the same breeding system may have different origins, even within the same family. Distyly is unusual in Polygonaceae due to the presence of bowl-shaped flowers (i.e., no corolla tube) and two whorls of anthers (Barrett, 1992) because most distylous species bear corollas with a floral tube and only one whorl of anthers. Different patterns of filament elongation, within and between morphs (Huang et al., 2014), provide evidence that in the family filament growth may be more evolutionarily labile than gynoecial growth, patterns of which do not differ between the species. Patterns of floral development in both *Nivenia* and Polygonaceae may differ from those of other distylous species because the breeding system is uncommon not only in these families but also monocots and Caryophyllales. Consequently, in these taxa, the origin of distyly may have involved developmental and genetic pathways distinct from other distylous species in which the breeding system is more common (e.g., Lamiidae).

In Boraginaceae, two different patterns for floral development were identified (Table 1). Species of *Lithospermum* and *Pulmonaria* exhibit similar overall patterns of floral development, and both are resolved as having an approach herkogamous ancestor (Tables 1, 4). Although these results might suggest similarities for the evolution of distyly in Boraginoideae, the subfamily to which both species belong, differences observed in stylar elongation indicate diverse developmental pathways. In *Lithospermum*, the style continues to elongate throughout floral development in the LS morph but ceases in the SS morph. In *Pulmonaria*, stylar growth ceases in both morphs while the corolla continues to elongate during development (Fig. 5F). These two patterns provide evidence for distinct types of origins within the subfamily and modifications of the evolution of distyly in these groups, even with origins via the same type of ancestral herkogamy. Similar patterns are also recognized among independent origins of related species in Cynoglossoideae, including *Oreocarya* and *Amsinckia* (Hasenstab-Lehman and Simpson, 2012; Cohen, 2014; Simpson et al., 2017; J. I. Cohen, H. Rodriguez and H. Hutcheson, unpublished manuscript). Additionally, the type of heteromorphic incompatibility differs among species in the three tribes (Ganders, 1979b; Philipp and Schou, 1981; Schou and Philipp, 1984; Casper, 1985), and the distinct patterns of floral development provide further evidence of diverse origins of distyly across Boraginaceae.

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AUTHOR CONTRIBUTION

J.I.C. designed the project, performed and managed floral developmental studies, conducted statistical analyses, and wrote the manuscript.

DATA AVAILABILITY

Raw data, log-transformed data, and graphs of floral organ heights and lengths for all studied species and aligned sequence data,

GenBank numbers, phylogenies, and character coding for and results from ancestral character reconstruction are available as Appendices.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

APPENDIX S1. Raw data and log-transformed data for each distylous species, and graphs of patterns of organs contributing to anther and stigma height development.

APPENDIX S2. Descriptions of patterns of floral development for each species.

APPENDIX S3. Sequence alignments, GenBank numbers, and data on type of herkogamy for distylous species and relatives.

APPENDIX S4. Results of maximum likelihood ancestral character reconstructions, based on three models of character evolution (all rates different [ARD], equal rates [ER], and symmetrical rates [SYM]), for each taxon, with trees from two models of sequence evolution (GTR+Γ and GTR+Γ+I).

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