Linking floral symmetry genes to breeding system evolution

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Understanding the genetic basis of ecologically important traits is a major focus of evolutionary research. Recent advances in molecular genetic techniques should significantly increase our understanding of how regulatory genes function. By contrast, our understanding of the broader macro-evolutionary implications of developmental gene function lags behind. Here we review published data on the floral symmetry gene network (FSGN), and conduct phylogenetic analyses that provide evidence of a link between floral symmetry and breeding systems in angiosperms via dichogamy. Our results suggest that known genes in the FSGN and those yet to be described underlie this association. We posit that the integration of floral symmetry and the roles of other regulatory genes in plant breeding system evolution will provide new insights about macro-evolutionary patterns and processes in flowering plants.

Introduction

A conspicuous pattern in the angiosperms is the large number of shifts between radial and bilateral floral symmetry [1,2]. Phylogenetic and systematic studies confirm that bilaterally symmetric [also zygomorphic (see Glossary) and monosymmetric] flowers have evolved several times from radially symmetric (also actinomorphic and polysymmetric) ancestors [3–6]; more transitions are likely to be revealed as well-resolved genus level trees become available (e.g. Ref. [7]). Floral symmetry (Figure 1) is associated with noteworthy macro-evolutionary and ecological processes in flowering plants, and has been described as a key innovation [6]. Indeed, transitions to bilateral symmetry are significantly correlated with larger clades in sister-group comparisons [8] and with dramatic shifts in pollination syndrome [9–11]. Bilateral symmetry is also associated with pollinator specialization, another factor implicated in high rates of angiosperm speciation [12–14].

Flowering plants exhibit a staggering amount of sexual diversity in floral form and function, much of which has yet to be studied or explained by evolutionary biologists [15]. For example, the temporal separation of male and female phases within a flower (dichogamy) is generally thought to have evolved to reduce interference between pollen export and pollen import, and to decrease the degree of within-flower self-pollination [16,17], but little is understood about the genetic basis and developmental control of dichogamy. Two forms of dichogamy exist: protandry (male phase precedes female) and protogyny (female phase precedes male). Protogyny is hypothesized to be most effective at reducing within-flower self-pollination [16,18], whereas protandry can be effective at reducing between-flower sexual interference [19–21]. However, we currently lack satisfactory general explanations for the genetic basis and evolutionary significance of these two types of dichogamy.

The maturation of anthers and stigma receptivity are rarely completely simultaneous, hence, dichogamy is extremely common in angiosperms [18]. However, there is significant variation among species in the timing and overlap of the two sex phases, which has been shown to have a heritable component in one species where it was investigated [22]. It has been suggested that protandry can arise as a byproduct of the centripetal development of floral whorls [i.e. within a flower the androecium (third whorl) arises outside of the gynoecium (fourth whorl) and thus

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Glossary

Actinomorphic: flower with multiple planes of symmetry. Also referred to as polysymmetric or radially symmetric.
Adichogamy: breeding system where no temporal separation of pollen dissemination and stigma receptivity exists (compare with dichogamy).
Anthesis: opening of a flower.
Breeding system: floral traits relating to anatomical and/or morphological aspects of sexual reproduction (as defined in Ref. [48]).
Dichogamy: breeding system where temporal separation of pollen presentation and stigma receptivity exists. Two forms exist: protandry, where pollen presentation precedes stigma receptivity, and protogyny, where stigma receptivity precedes pollen presentation.
Didynamous anthers: having four stamens in two pairs of unequal length.
Epi-genetic inheritance: transmission of information to descendants without the information being encoded in the nucleotide sequence of the gene (mechanisms include DNA methylation, histone modifications).
Floral constancy: increased probability that a pollinator transfers pollen between two individuals of the same species.
Mating system: degree to which the seeds of a plant are the result of inbreeding or outcrossing.
Papilionoid legumes: Papilionoideae is a subfamily of the legume family (Fabaceae) that is characterized by floral parts in fives or multiples of five with bilateral pea-like flowers. The petals are differentiated into an adaxial petal (the banner or standard), and abaxial and lateral pairs of petals (the keel and wings, respectively). The keel petals are fused and enclose the stamens and gynoecium.
**Protandry**: form of dichogamy in which pollen release precedes stigma receptivity.
Protogyny: form of dichogamy in which stigma receptivity precedes pollen release.
Zygomorphic: flower with one plane of symmetry where one half mirrors the other half. Also referred to as monosymmetric or bilaterally symmetric.

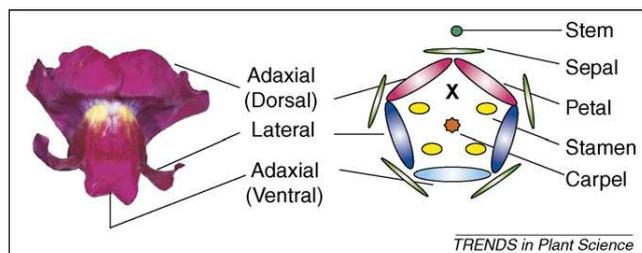*Corresponding author:* Kalisz, S. (kalisz@pitt.edu).
Available online 9 November 2006.
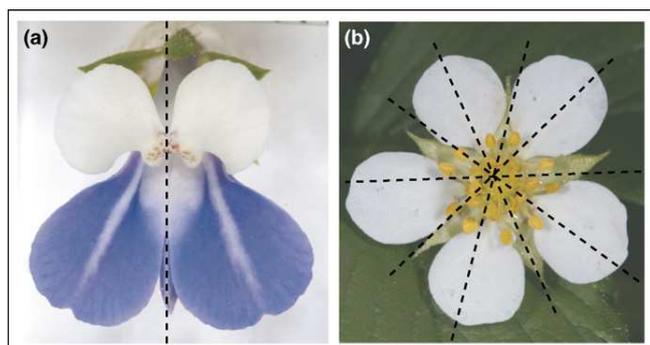
Figure 1. Axes of symmetry for bilaterally versus radially symmetrical flowers. **(a)** Bilaterally symmetrical flower of *Collinsia verna* (blue-eyed Mary, Veronicaceae). **(b)** Radially symmetrical flower of *Fragaria virginiana* (strawberry, Rosaceae).

Figure 2. Schematic of the floral whorls in the distinctly bilaterally symmetric flower of *Antirrhinum majus*. The five petals of *A. majus* exhibit marked differences in shape and size along the symmetry axis. The upper lip of the flower is formed from the two larger adaxial petals, whereas the lower lip is formed from the smaller lateral (2) and abaxial (1) petals. In *A. majus* and many other bilaterally symmetric flowers, the stamen whorl also exhibits bilateral symmetry – the arrested adaxial stamen is a staminode ($\times$), the two lateral stamens develop to a shorter length than the two long abaxial stamens (i.e. didynamous stamens). Both *A. majus* and its close relative *Linaria vulgaris* are self-incompatible; dichogamy has not been investigated in either of these species.

stamens would develop first] [23] (Figure 2). Under this model, protandry is expected to be the 'default' condition, irrespective of flower symmetry.

Interestingly, genes that control bilateral corolla symmetry have also been demonstrated to affect stamen development and size (Figure 3) [11,24,25], making these excellent candidates for studying genetic control of breeding system traits such as protandry (Box 1). Moreover, macroevolutionary evidence suggests that bilateral floral

symmetry and protandry can be linked via the selective regime of specialized animal pollination: shifts from biotic to abiotic pollination are associated with shifts from bilateral to radial floral symmetry [26] as well as shifts away from protandry [27]. These findings lead us to hypothesize that the evolution of protandry and floral symmetry is correlated via a common genetic network.

Figure 3. Stamens are not didynamous in *CYCLOIDEA* mutants, one of the floral symmetry genes. Center: *Antirrhinum majus* wild type (WT 75R) exhibits didynamous stamens (two long abaxial, two shorter lateral). (a–d) The *cyc* mutants of *A. majus* express phenotypes in which four to six fertile stamens in the mutants often develop with equal length. **(a)** 650: four to five equal stamens. **(b)** 721-cyc: four long stamens and two short adaxial stamens. **(c)** G25: six equal stamens. **(d)** 705-backpetal-cyc: one staminode, four equal stamens. Stock numbers from the John Innes Centre Antirrhinum stock collection.
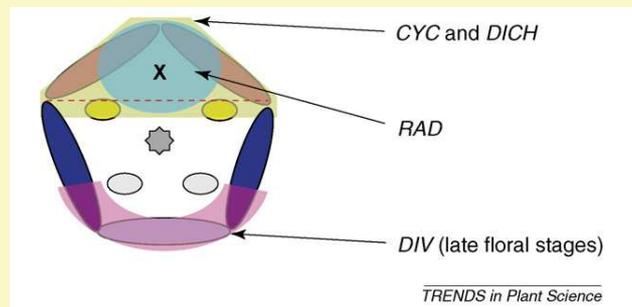
Box 1. Floral symmetry gene network interacts to control bilateral symmetry

A network of gene interactions establishes bilateral flower symmetry in petal and stamen whorls of *Antirrhinum majus* and its relatives. The genes identified to date include the TCP family of transcription factors [43] *CYCLOIDEA* (*CYC*) and *DICHOTOMA* (*DICH*), and the MYB-type transcription factors *RADIALIS* (*RAD*) and *DIVARICATA* (*DIV*). RNA *in situ* hybridization studies of *CYC* and *DICH* reveal that *CYC* and *DICH* expression is localized to the adaxial petals and adaxial stamen (broken red line in Figure I) in *A. majus* [24] and *Linaria vulgaris* (toadflax) [43] or adaxial petals plus adaxial and lateral stamens in *Mohavea confertifolia* (desert ghost flower) [11]. In *M. confertifolia* flowers, *CYC* expression extends further down the symmetry plane, such that only the abaxial stamens are fertile – the adaxial and lateral stamens develop as staminodes but the lateral petals are not affected [11]. These and other studies with *cyc* and *dich* mutants have shown that the expression of *CYC* and *DICH* in developing flower tissue can overlap, are partially redundant and can affect development of the petal and stamen whorls differently. *RAD* expression is more or less identical to that of *CYC* across the adaxial portion of the flower, including the adaxial staminode [29]. A major mechanism by which *CYC* and *DICH* establish adaxial petal identity is through activating *RAD* [29]. Therefore, in addition to specifying adaxial petal identity, *RAD* could play some role in reducing the adaxial stamen. However, other, as yet undiscovered, *RAD*-independent mechanisms could mediate adaxial stamen abortion via *CYC*. Together, *CYC*, *DICH* and *RAD* are necessary for establishing adaxial flower identity [24,31,44].

DIV is responsible for abaxial petal identity [45,46]. During early stages of flower development, *DIV* is expressed in all *A. majus* petals, but, by late stages, *DIV* expression is restricted to the abaxial petals where *DIV* protein appears to function [46]. *DIV* is thought to be negatively regulated in adaxial petals by *RAD* competing with *DIV* for downstream targets. One of these downstream targets is likely to be the *DIV* promoter itself because the *DIV* protein is necessary for *DIV* expression [46]. Therefore, *DIV* activation does not occur in adaxial petals where *RAD* is present, but is continuously activated in the abaxial petals where its promoter is not bound by *RAD* protein. *RAD* and *DIV* encode similar MYB-type proteins, but *RAD* lacks recognizable transcriptional activation motifs. It is likely that *RAD* gene products specify adaxial petal identity by negatively regulating *DIV* [29].

Recent studies with *Lotus japonicus* have extended the floral symmetry gene network (FSGN) [30] to include a lateral petal identity factor. In *L. japonicus*, *LjCYC2* functions in a similar fashion to CYC in floral primordia. An allele of *LjCYC2*, termed *squ1*, has squared adaxial petals and adaxial petal epidermal cells shaped like those of the lateral petals. Another floral mutant, *kew1*, exhibits lateral petals that are abaxial in shape and in epidermal cell type. Analysis of the data also suggests that *Kew1* is a specific factor that functions late in petal development to control lateral petal development and interacts with *LjCYC* to affect petal shape along the symmetry axis [30].

Figure I. Known floral symmetry genes of *Antirrhinum majus* – expression domains and phenotypic effects. × indicates the arrested adaxial stamen (a staminode).

A floral symmetry gene network controls bilateral symmetry

Antirrhinum majus (Veronicaceae), a model plant for studies of floral development and symmetry [28], exhibits a floral morphology that is common in bilaterally symmetrical species (Figure 2). A decade of research on this model and on related wild species has sharpened our understanding of the genetic network that underpins bilateral symmetry [29]. More recent work using the model legume *Lotus japonicus* has led to the identification of two orthologs of CYCLOIDEA (CYC; *LegCYC1* and *LegCYC2*) that control bilateral symmetry in papilionoid legumes [30]. To date, four genes in two transcription factor families are known to interact and affect floral symmetry through their distinct effects on petal versus stamen development in *A. majus*. A lateral identity gene *Kew1* has been described in the model legume *L. japonicus* [30]. The expression patterns controlling floral symmetry and the cross-regulatory interactions among the known floral symmetry gene network (FSGN) genes and gene products are detailed in Boxes 1 and 2, respectively.

Briefly, *CYC* and its paralog *DICHOTOMA* (*DICH*) were identified in *A. majus* [24,31] and derive from a recent gene duplication event [11]. *CYC* expression results in bilateral symmetry in petals and creates a gradient in stamen size along the symmetry axis. In the wild type, development of the adaxial (dorsal) stamen is arrested and the lateral stamens are shorter than the longer abaxial (ventral) stamens (Figures 1–3). Mutations at the *CYC* locus can result in *A. majus* flowers with both reduced

bilateral petal symmetry and a fertile stamen in place of the adaxial staminode. In *CYC* mutants, adaxial and lateral petals and stamens express abaxial identity (Box 2) [24] and the lateral and abaxial stamens develop to an approximately equal length (Box 2). *CYC* and *DICH* gene products are necessary to establish adaxial flower identity, and *CYC*, but not *DICH*, is necessary to specify petal and stamen number as well as patterns of stamen abortion in *A. majus*.

Floral symmetry appears to be controlled by the same fundamental set of related genes in many distantly related taxa, suggesting a common genetic basis for the trait [30,32–34]. In addition, *CYCLOIDEA* and *DICHOTAMA*-like genes have recently been found in several plant clades that are more distantly related to *A. majus*, including other members of Veronicaceae, Fabaceae (the pea family), Gesneriaceae (the tropical snapdragons), Asteraceae (the sunflowers) and Lamiaceae (the mints) (reviewed in Refs [6,30,34], T. Yi, L. Hileman and S. Kalisz unpublished). Recent work with floral mutants of *L. japonicus* suggests that *Kew1* is a specific factor that controls lateral petal identity and interacts with *LjCYC2* in determining floral bilateral symmetry [30], adding to the FSGN for legumes (Box 1). Furthermore, in the radially symmetrical legume, *Cadia purpurea*, the domain of expression of *LegCYC* is expanded to include the lateral and abaxial portions of the floral primordia. Here radial symmetry is achieved not through the loss of *CYC* expression, which is the situation in *Linaria* and *Antirrhinum*, but because all petals and stamens express adaxial identity [34]. Future research on

Box 2. Floral symmetry gene mutants reveal distinct effects on petal versus stamen phenotypes

Mutations at the *CYC* locus result in flowers with reduced bilateral petal symmetry, a fertile stamen in place of the adaxial staminode (×), and the lateral and abaxial stamens develop to approximately equal length (Figure I). Interestingly, *CYC* function also controls floral organ number. In *cyc* mutants, petals have ventral identity (light blue) and can develop with six petals and six stamens instead of the typical five [24] (e.g. G25 in Figure 3c). Similarly, *dich* mutants also lose adaxial organ identity, although the phenotype is less severe than for *cyc* mutants. The adaxial petals are more symmetrical than in the wild type, but the lateral and abaxial petals and the stamens are like the wild type [24]. The phenotype of *cyc dich* double mutants is more severe than either of the single mutants, indicating that *CYC* and *DICH* functions are partially redundant in establishing adaxial flower identity. The *cyc dich* double mutants form 6-merous flowers with complete radial symmetry: all six stamens develop completely, and all petals have abaxial identity [31]. The *RAD* mutant results in a similar phenotype to that of *cyc dich* double mutants, but differs in that the dorsal-most part of adaxial petals gains lateral petal identity. Unlike *cyc* or *cyc dich* double mutants, in *rad* mutants the adaxial stamen remains arrested as a staminode but with a longer filament than in the wild type. In *div* mutants, abaxial petals have lateral identity [45,46]. In *cyc dich* double mutants (and presumably *rad* mutants), *DIV* expression does not become restricted to the abaxial petals [46]. This expanded *DIV* expression in *cyc dich* double mutants transforms adaxial organs to abaxial identity in the *cyc dich* double mutant background. The *div* mutations only affect abaxial petal development.

In summary, *CYC, DICH* and *RAD* are necessary for establishing adaxial flower identity [24,29,31]. However, *CYC* plays a role in determining second and third whorl organ number, and is necessary

for reducing the size of the adaxial stamen [24] and lateral stamens in *Mohavea* (desert ghost flower) [47], whereas *DIV* gene products specify abaxial petal identity [45,46], and can increase filament length where expressed. There are no data comparing the rate of development of stamens in different positions within the flowers of either the wild type or mutants for any species, but we hypothesize that the abaxial stamens of wild-type flowers will mature before those in lateral stamens.
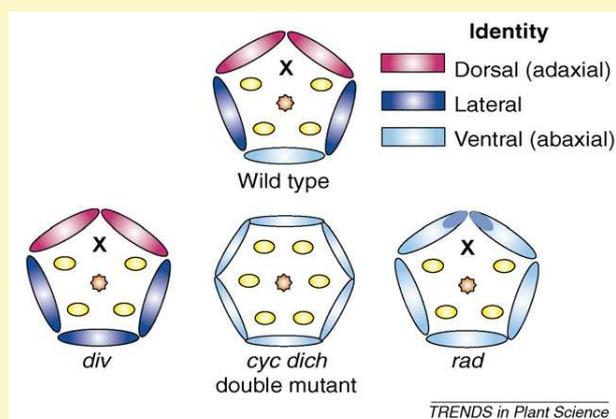
Figure I. The floral symmetry gene network mutants of *Antirrhinum majus* for *CYC, RAD, DICH* and *DIV* affect petal and stamen whorl symmetry in distinct ways.

FSGN is likely to uncover novel genes, gene expression patterns and gene interactions, and enrich our understanding of the genetic basis of floral morphology.

Floral symmetry genes – are they also breeding system genes?

The effect of floral symmetry genes on both corolla and androecium development and parallel macro-evolutionary patterns involving protandry and floral symmetry [8,27] support our hypothesis of an association between bilateral symmetry and protandry. The FSGN creates size asymmetry in the mature androecium whorl by affecting the development of stamens. In wild-type *A. majus*, *CYC–DICH–RAD* in conjunction with *DIV* expression result in a short, sterile adaxial stamen (staminode), lateral stamens reduced in length and long abaxial stamens. We hypothesize that the FSGN expression affects the rate of stamen development along the symmetry axis, with the abaxial stamens developing first. Is it possible that the FSGN also affects post-anthesis stamen developmental timing traits that affect dichogamy? Currently, there are no published data on the relative rates of stamen development pre- or post floral anthesis for bilaterally symmetrical flowers. Given that the FSGN creates a gradient in mature stamen size in *A. majus* that could represent a gradient in development, we speculate that the FSGN could also influence stamen maturation rates after the flower is open to pollinators – arrested in the (shorter) adaxial staminode, slowed in lateral stamens and unaffected in the (longer) abaxial stamens. By contrast, in the radially symmetrical *Cadia purpurea*, the expression domain of *LegCYC* encompasses the adaxial, lateral and abaxial stamens [34] and the stamens are equal in length.

Thus, the FSGN can alter or influence the centripetal development of floral whorls by staggering stamen development within a flower, allowing the timing of male versus female phases to be evolutionary dynamic. Furthermore, along the symmetry axis of a typical 5-merous bilateral flower, the gynoecium is positioned adaxially (i.e. above) the abaxial stamens and in the same plane as the lateral organs. Although *CYC–DICH* and *DIV* expression have not been detected in late-stage gynoecium primordia in *A. majus*, we speculate that the floral primordia cells that give rise to the gynoecium could be influenced by their action and/or interaction at an early developmental stage, as are the lateral petals and stamens. If so, bilateral flowers could be predisposed to a delayed female phase and thus express protandry. Finally, we speculate that unknown players in the FSGN could be responsible for initiating post-floral anthesis stamen development in an abaxial (ventral) to adaxial (dorsal) direction. Whether by known or unknown factors in the FSGN, or via direct or downstream factors to FSGN expression, the condition of bilateral symmetry can 'set the stage' for the evolution (or reinforcement) of protandry.

Because FSGN produces dimorphic anthers (Boxes 1 and 2), our second expectation is for a general association between petal bilateral symmetry and didynamous stamens if the FSGN is responsible for the symmetry. We tested these predictions by conducting a phylogenetic analysis (Box 3; see also online Supplementary data).

Testing the association between bilateral symmetry and protandry

We explored these novel hypotheses through a phylogenetic analysis of floral symmetry and protandry in 1458

Box 3. Correlated evolution of bilateral symmetry and protandry

Using phylogenetic methods for inferring character evolution, we tested whether protandry has been more frequently gained and less frequently lost in bilaterally symmetrical lineages and, conversely, more frequently lost and less frequently gained in radial lineages, compared with null expectations if protandry had evolved independently of flower symmetry (Table I). Over a set of candidate phylogenies, we compared frequencies of change inferred from the observed data with those inferred from simulated data (see online Supplementary data: phylogenetic methods, data and results). Gains and losses of protandry in radial lineages show the expected trend, with 89% and 78% of simulations yielding the predicted outcome, respectively. However, the results are mixed for bilaterally symmetrical lineages. Gains of protandry conformed to expectation in 64% of the simulations, whereas only 40% of the simulations for losses of protandry yielded the expected outcome. Although not uniformly upholding our predictions, this analysis suggests that the evolution of dichogamy is indeed correlated with flower symmetry.

Table I. Macroevolutionary predictions for change in protandry in the context of flower symmetry compared with null expectations if traits evolved independently[a]

Corolla symmetry	Gains of protandry	Losses of protandry
Radial	Less frequent (0.8912)	More frequent (0.7824)
Bilateral	More frequent (0.6436)	Less frequent (0.4048)

[a]Experimental support for predictions shown in parentheses.

species in the angiosperm clade Asteridae (Box 3) and demonstrated that transitions to protandry have been less frequent, and losses of protandry more frequent than expected in radial clades; gains of protandry are also more frequent than expected in bilateral clades. These results are consistent with the hypothesis that flower symmetry and protandry are correlated and potentially controlled by the same regulatory genes. This suggests that exploring the role of the FSGN in breeding system evolution will be fruitful in the pursuit of understanding the link between regulatory genes and macro-evolutionary patterns. We also predict that independent evolutionary forces on organs under common genetic control, such as those acting on flower symmetry and dichogamy or stamen number, are likely to be common in morphological development, a consideration that can influence the direction and focus of experimental studies on the evolution of development.

Associations between floral symmetry and plant breeding system traits have been hypothesized previously: in *The Different Forms of Flowers on Plants of the Same Species* (p. 259) [35], Charles Darwin noted that heterostyly is not common in bilateral lineages; this pattern has been confirmed by recent phylogenetic analysis [36].

Linking dichogamy, floral symmetry and pollination ecology

Early pollination ecologists hypothesized that there is an association between floral symmetry and pollination of plants by animals [10]. Numerous investigations have since confirmed that floral symmetry plays a role in the preference and perception of flowers by pollinators. For example, it has been demonstrated that bumblebees have an innate preference for bilateral flowers [37], whereas

beetles, honeybees, moths and butterflies appear to prefer radial flowers [38]. Behavioral studies have shown that structurally complex flowers (i.e. those with zygomorphy and a long and/or narrow corolla tube) [10] require an additional learning and handling time investment on the part of bumblebee pollinators [39]. This finding suggests that zygomorphy can promote pollinator fidelity in general [10].

In addition to its effect on pollinator preference, floral symmetry affects the direction of approach of a pollinator to a flower. Bilateral symmetry restricts the direction of approach to a single plane, which can allow more specific placement of pollen on the pollinator [40]. By contrast, radial flowers can be approached from more than one direction, decreasing the consistency of pollen placement [10]. Bilateral symmetry can also reduce pollen wastage [41]. It can also allow greater opportunity for floral diversification, the idea being that a bilaterally symmetrical image has more elements and, therefore, more potential variants [42].

The association between specialist pollinators and bilateral symmetry has been predicted to yield higher diversification rates for clades [9]. A recent analysis of 19 sister groups confirmed that bilateral lineages tend to be more diverse than their radial counterparts [8]. Evolutionary transitions between radial and bilateral symmetry have important consequences for pollination syndrome, accurate pollen transfer and, ultimately, speciation.

Dichogamy has also been functionally linked to pollination ecology. Robert Bertin and Christian Newman [17] found that protandry was associated with animal pollination (although not beetle pollination) and that protogyny was associated with wind pollination. Although a recent phylogenetic analysis does not support the former association (animal pollination was found to be equally common in protandrous and adichogamous species [27]), other empirical evidence suggests that protandry evolved, at least in part, to reduce transfer of self-pollen in animal-pollinated plants with large floral displays [19,20], suggesting that the association can be at a finer scale than Risa Sargent and Sarah Otto [27] were able to detect with their analysis.

Conclusions

Could a common genetic system such as the FSGN explain the association between bilateral flowers and protandry? Did the innovation of bilateral symmetry, in concert with specialized pollinators, set the stage for the evolution of reduced floral interference via protandry? Our phylogenetic analysis provides preliminary support for these conjectures; further exploration along these lines should improve our understanding of the evolutionary connections between floral traits, breeding system and pollination ecology.

Supplementary data

The following three supplementary data files associated with this article can be found, in the online version, at doi:10.1016/j.tplants.2006.10.005.

- A pdf file describing the methods, data and results of the phylogenetic analysis of the correlated evolution of floral symmetry and protandry.
- A NEXUS-formatted text file (for use in Mesquite) containing the floral symmetry and dichogamy data and base phylogeny for 1458 species of asterid angiosperms used in the phylogenetic analysis of correlated evolution.
- An Excel spreadsheet file containing the floral symmetry and dichogamy data, with references to source literature used in scoring species for corolla and androecium symmetry.

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