Phylogeny and the evolution of flower symmetry in the Asteridae

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Phylogenetic trees imply that flowers with a single plane of symmetry (zygomorphic flowers) have evolved several times independently from radially symmetrical (actinomorphic) ancestors within the Asteridae. However, there also appear to have been reversals to actinomorphy. A few evolutionarily derived actinomorphic flowers resemble mutants caused by loss-of-function mutations in genes such as *CYCLOIDEA*. However, a majority of the shifts from zygomorphy to actinomorphy appear to have entailed a reduction in petal number and flower size, implying a mechanism other than loss of *CYCLOIDEA* function. Within the Asteridae there appear to be three common forms of zygomorphy. An explanation for the virtual absence of other forms rests on the near universality of the basic orientation of the flower in the Asteridae.

The recent dramatic progress in understanding the genetics of flower development has derived largely from studies of just two species, *Arabidopsis thaliana* (Brassicaceae; Rosidae) and *Antirrhinum majus* (Scrophulariaceae; Asteridae). These distantly related plants have a great deal in common with respect to the genes that determine the identity of the major floral organs[1–3]. This implies that the same sets of genes carry out similar functions in Rosidae and Asteridae and probably also in more distantly related groups[4].

Arabidopsis and *Antirrhinum* flowers, although similar in the overall arrangement of parts, differ in other very obvious ways. Improved understanding of the genetic and developmental bases for these differences might shed light on the evolution of the extraordinary diversity of floral forms found in angiosperms. One striking difference is in flower symmetry[5–7]. In *Arabidopsis*, the flowers are actinomorphic (regular, radially symmetrical, or polysymmetric), whereas in *Antirrhinum* they are zygomorphic (irregular, bilaterally symmetrical, or monosymmetric) (Fig. 1). In *Antirrhinum*, and in the vast majority of zygomorphic flowers, zygomorphy is a function of the differentiation of the adaxial (dorsal, or upper) side of the flower from the abaxial (ventral, or lower) side. Focusing on the corolla, there are two adaxial petal lobes in *Antirrhinum*, which differ in size and morphology from the other three petals (two lateral petals, and one medially positioned abaxial petal). There may be associated differences between the adaxial and abaxial halves in other features. In particular, it is common in Asteridae for zygomorphy to be accompanied by the abortion of, or failure to initiate, stamens in certain positions (e.g. in *Antirrhinum* the adaxial stamen is aborted[6,7]).

The genetic basis of dorso-ventral assymmetry has been studied in detail by taking advantage of 'peloric' mutants of *Antirrhinum*, in which the flowers are almost or completely actinomorphic[8–12]. It has been shown that the gene *CYCLOIDEA* (*CYC*) is needed for the normal development of zygomorphic flowers[8] and that it is expressed early in development on the adaxial side of the flower[11]. The differential expression of *CYC* in the young flower meristem is thought to modify the developmental fate of adaxial organs leading to enlarged adaxial petals and abortion of the adaxial stamen[10,11].

Phylogeny and the direction of evolutionary change

Analysis of the development of zygomorphic flowers has raised important questions about the evolution of flower symmetry[9,10]. Peloric mutations appear to be quite common, but mutations have not been described that yield zygomorphic flowers in otherwise actinomorphic plants[9]. This could suggest that the evolutionary transition from actinomorphy to zygomorphy has occurred only rarely, with frequent reversions[9]. This would mean that zygomorphic flowers in widely separated clades could be homologous, having been retained from a distant common ancestor, leading one to expect detailed similarities in the genetic and developmental mechanisms underlying zygomorphy. However, this idea runs counter to the intuition of many systematists, who have tended to view zygomorphy as having originated a number of times independently, with shifts from zygomorphy to actinomorphy being rather rare[13–15].

The Asteridae, a clade of about 65 000 species, which includes *Antirrhinum* and many other zygomorphic taxa, is the obvious group in which to test these two hypotheses. Coen and Nugent[9] illustrated a phylogenetic approach to the problem using a tree of Asteridae containing 16 taxa. They found that a minimum of three changes in flower symmetry had to be invoked when actinomorphy was ancestral and four when zygomorphy was ancestral (Fig. 2). With only a one-step difference between these scenarios, this result leaves open the possibility that zygomorphy originated rather early in angiosperm evolution, and was lost a number of times independently.

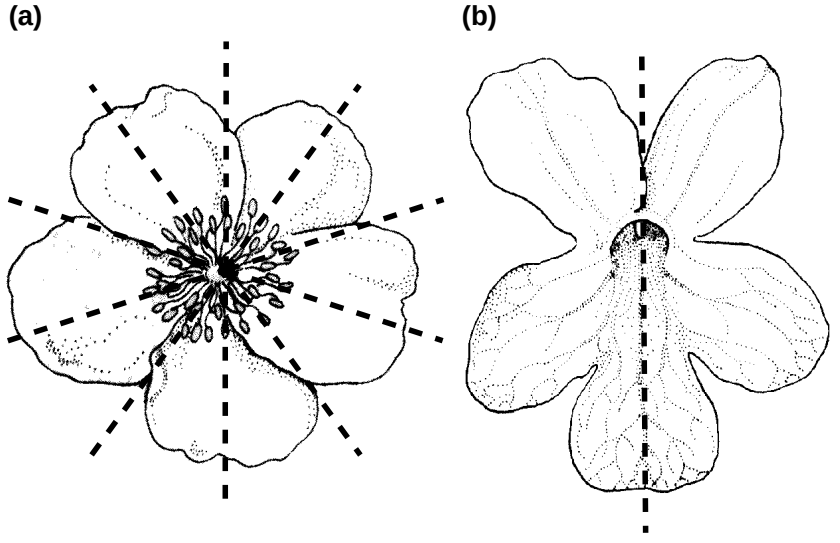

Fig. 1. Types of floral symmetry: (a) Actinomorphy (radial symmetry or polysymmetry); (b) zygomorphy (bilateral symmetry or monosymmetry).

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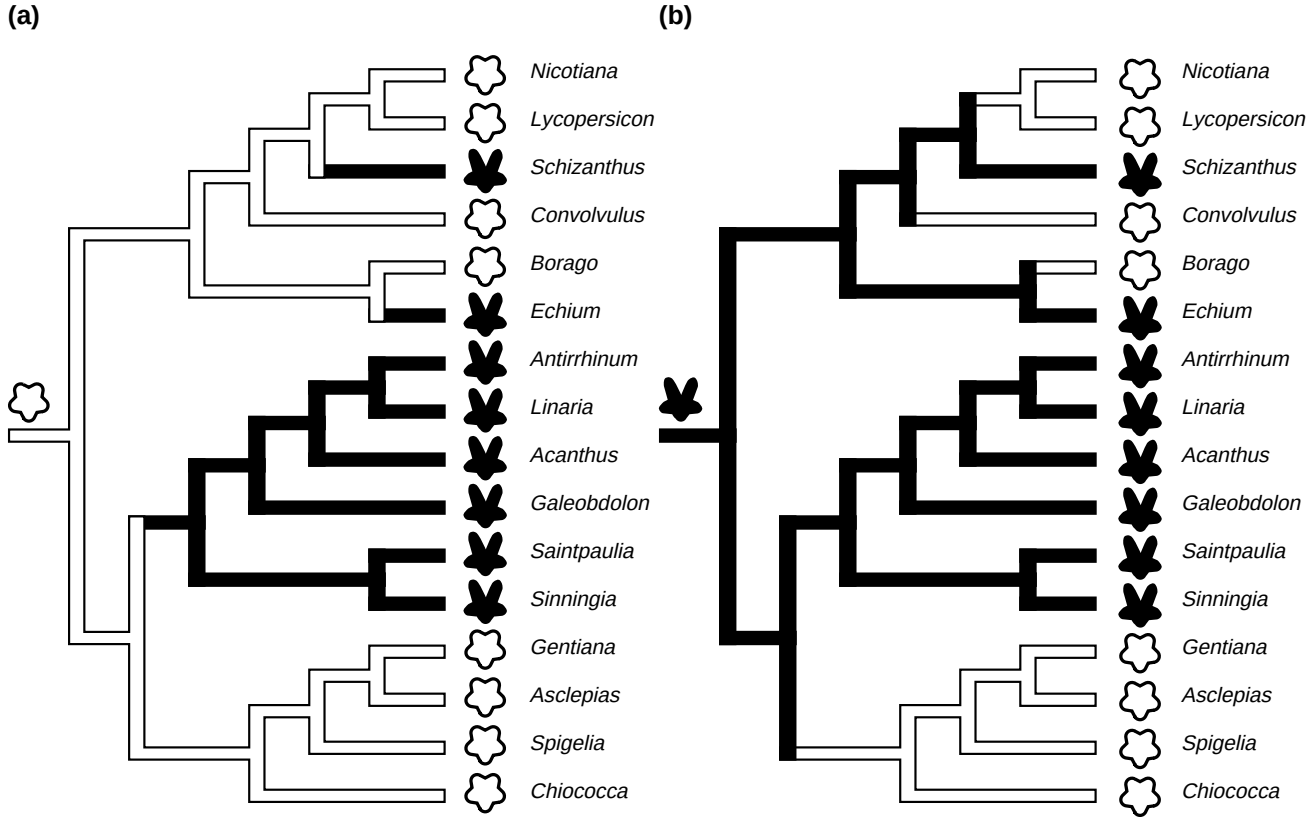

Fig. 2. Coen and Nugent's example of the use of a phylogenetic tree of Asteridae in evaluating the evolution of flower symmetry[9]. (a) A minimum of three changes to zygomorphy (black) are required when actinomorphy (white) is considered ancestral in Asteridae. When zygomorphy is forced to be ancestral in Asteridae, there are three most-parsimonious reconstructions, each involving four changes. The reconstruction shown in (b) entails four independent changes from zygomorphy to actinomorphy. The other two optimizations involve more than one independent change to zygomorphy (i.e. zygomorphic flowers are not all homologous).

An expanded analysis of the Asteridae can be carried out by grafting recently published phylogenies for major subgroups of Asteridae [e.g. Apiales[16], Dipscales (A. Backlund, PhD thesis, Uppsala University, 1996), Ericales[17], Hydrangeaceae[18], Lamiales[19], Rubiaceae[20] and Solanaceae[21]] onto a backbone tree based on the chloroplast gene *rbc*L (Refs 22 and 23). We assembled such a composite tree, and scored the 379 terminal taxa as having either actinomorphic or zygomorphic corollas. The computer program MacClade (W. Maddison and D. Maddison, Sinauer Associates, Sunderland, MA, USA) was then used to find the most parsimonious optimization of character evolution and to infer the number of character state changes in both directions. Although many of the phylogenies upon which the composite tree is based contained polytomies (i.e. the relationships are not fully resolved either because of conflict among equally supported trees or because of a lack of data), the complexities of reconstructing character evolution in this situation were avoided by working primarily with a completely resolved tree[24]. However, alternative fully resolved trees were also considered in order to explore the sensitivity of the results to different tree topologies[25]. The resolved composite tree and our scorings can be obtained from the World Wide Web (http://www.herbaria.harvard.edu/~mdonoghu/zygotree/).

According to this analysis, it is most parsimonious to suppose that: the first Asteridae were actinomorphic; zygomorphic flowers originated at least eight times independently (e.g. in Asterales, Dipsacales, Solanales, Ericales, and a large clade including the Lamiaceae, Scrophulariaceae and several other groups); and there were at least nine reversals to actinomorphy (mostly within the Lamiaceae–Scrophulariaceae clade). Only slight deviations from these numbers occur when alternative resolutions of the original supertree are considered.

An important concern about this sort of phylogenetic parsimony test is that it assumes an equal probability of evolutionary transitions in the two directions[9]. A different answer might be obtained if one made the assumption that it was easier to evolve actinomorphy from zygomorphy than the reverse. The impact of unequal transitions can be explored with MacClade by iteratively changing the assumed costs of the two transitions, making use of a step-matrix[26]. The results are quite robust to different weighting schemes (Fig. 3). The transition from zygomorphy to actinomorphy needs to be considered ten times easier than the reverse in order for the hypothesis that zygomorphy evolved only once in Asteridae, with all cases of zygomorphy being retentions of this ancestral condition, to be preferred. Such a scenario would entail at least 47 independent origins of actinomorphy.

These analyses do not tell us about the frequency of shifts between actinomorphy and zygomorphy unless a range of plausible costs is specified. It could be argued that because simple genetic mutations can cause the loss of zygomorphy, frequent transitions to actinomorphy are more likely than frequent origins of zygomorphy[9]. This may well be correct but it does not necessarily mean that the frequency of evolutionary transitions will be similarly biased. In order to become fixed in a lineage, an attribute must both appear by mutation (whether a single genetic change or multiple changes over a longer period of time) and confer sufficient fitness that it can go to fixation and be maintained long enough to be seen in a living species. Most

zygomorphic asterids are pollinated by specialist bees that rely on visual cues during foraging. Therefore, actinomorphic mutants in populations of zygomorphic plants might be at a selective disadvantage[9]. Bees visiting actinomorphic flowers tend to do so inefficiently[27]. Furthermore, reversals to actinomorphy tend to occur in lineages that have previously switched to generalist pollinators[27]. Thus, although genetic and developmental factors might favor zygomorphy-to-actinomorphy transitions, ecological factors might well favor the reverse. Although we do not know how these conflicting forces balance out in evolution, the extreme weights needed to force all changes to occur in one direction appear unlikely. The phylogenetic analysis discussed here therefore seems to support the view of several independent origins of zygomorphy in the Asteridae, while also supporting the suggestion that there have been a number of independent reversals from zygomorphy to actinomorphy[9].

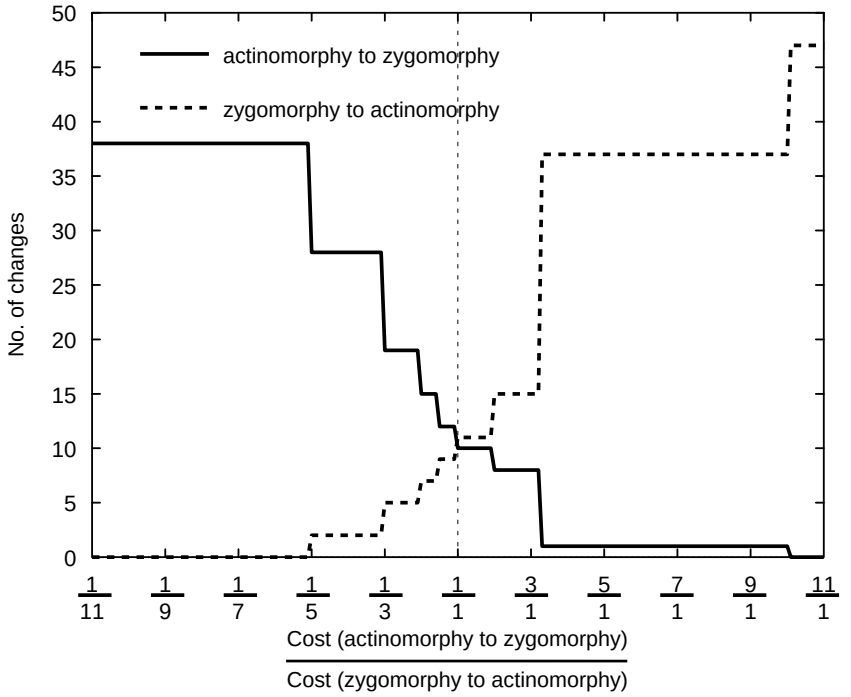

Fig. 3. A 'cost–change graph'[26] based on a composite tree of Asteridae containing 379 terminal taxa. The x-axis shows the ratio of the cost associated with changing from actinomorphy to zygomorphy versus the reverse. For a given cost ratio, the number of changes in the two directions found under the optimal reconstruction (i.e. that with the lowest overall cost) on the asterid supertree is plotted on the y-axis. For example, when actinomorphy-to-zygomorphy changes cost five times as much as zygomorphy-to-actinomorphy changes (i.e. actinomorphy-to-zygomorphy changes are judged less likely) the optimal reconstruction entailed 37 zygomorphy-to-actinomorphy changes and one actinomorphy-to-zygomorphy change.

Phylogeny and the evolutionary loss of zygomorphy

The vast majority of actinomorphic Asteridae, such as most species of Rubiaceae, Apocynaceae, Apiales and Ericales, appear to have simply retained the ancestral condition. However, the phylogenetic analyses already discussed indicate that some species with actinomorphic flowers are derived from zygomorphic ancestors. These reversals are scattered among the several major zygomorphic clades, but the greatest number, and many of the clearest cases, are found in the clade that includes Lamiaceae, Scrophulariaceae and Acanthaceae (Fig. 4). This clade can be used to examine the types of floral changes that have been associated with shifts to actinomorphy.

In order to evaluate the developmental mechanisms that might underlie reversals to actinomorphy, it is necessary to focus on cases that are deeply nested within zygomorphic clades such that instances of retained actinomorphy can be ruled out. *Plantago* provides a prime example, being well nested within a zygomorphic clade. To suppose that the actinomorphic flowers of *Plantago* were retained from a more distant actinomorphic ancestor would require that zygomorphy-to-actinomorphy transitions be more than five times more costly. A number of the inferred cases of reversal within mints are equally robust to changes in weighting.

Compared to their presumed close relatives, such as *Veronica*, *Plantago* flowers are smaller, have more exserted stamens and are basically four-merous, with four sepals, four petals and usually four stamens[28] (Fig. 5). Similar changes are observed in several other cases (Fig. 4; e.g. *Callicarpa* and *Petitia*). In particular, shifts to actinomorphy commonly seem to co-occur with shifts to four-merousness.

How do these observations relate to the possibility that actinomorphy was derived from zygomorphy via the mechanism demonstrated for peloric forms in *Antirrhinum* – that is, via loss of function of *CYC* or similar genes? In the case of *CYC* mutants, the adaxial side of the flower proceeds to develop like the abaxial side. The usual outcome of this 'ventralization' process is that the adaxial side ends up with three petals (one medial and two lateral), and the flower has six petals in all[11]. If this process were to operate in the evolution of actinomorphy, the expected outcome would be an increase in the number of petals from five to six, but in *Plantago* and similar instances a decrease from five to four occurred.

These observations suggest that reversal to actinomorphy in *Plantago* and other such plants came about through some other genetic or developmental changes. One possibility is that the flower of *Plantago* is a 'dorsalized' peloric mutant, arising either by loss of function of an abaxial identity gene (possibly *DIVARICATA*; Ref. 12) or overexpression of an adaxial identity gene (*CYC* or *DICHOTOMA*). In this interpretation, the three petals in the abaxial half of the flower are 'replaced' by the two adaxial petals. This model can probably be ruled out, however, because it would predict that the petals are positioned on either side of the medial plane, as is normal for the adaxial petals, whereas *Plantago* has both the adaxial and abaxial petals positioned medially. The second, more likely, interpretation is that actinomorphy in *Plantago* arises via fusion of the adaxial petals accompanied by a shift in the orientation of the two lateral petals. This interpretation gains credence by consideration of the related genus *Veronica*. In these flowers a shift towards four-merousness occurs, but the adaxial petal is enlarged and sometimes notched, suggesting evolutionary fusion (Fig. 5; Ref. 28). Scanning electron micrographs of young *Veronica* flowers do not show two primordia, which later fuse, but instead indicate a much enlarged single adaxial primordium[29], occupying the area of the meristem where two separate primordia occur in five-merous flowers.

The most plausible explanations for the origin of actinomorphy in *Plantago* predict that developing *Plantago* flowers will show normal or increased expression of *CYC*. Scanning electron micrographs of *Plantago*[7] are consistent

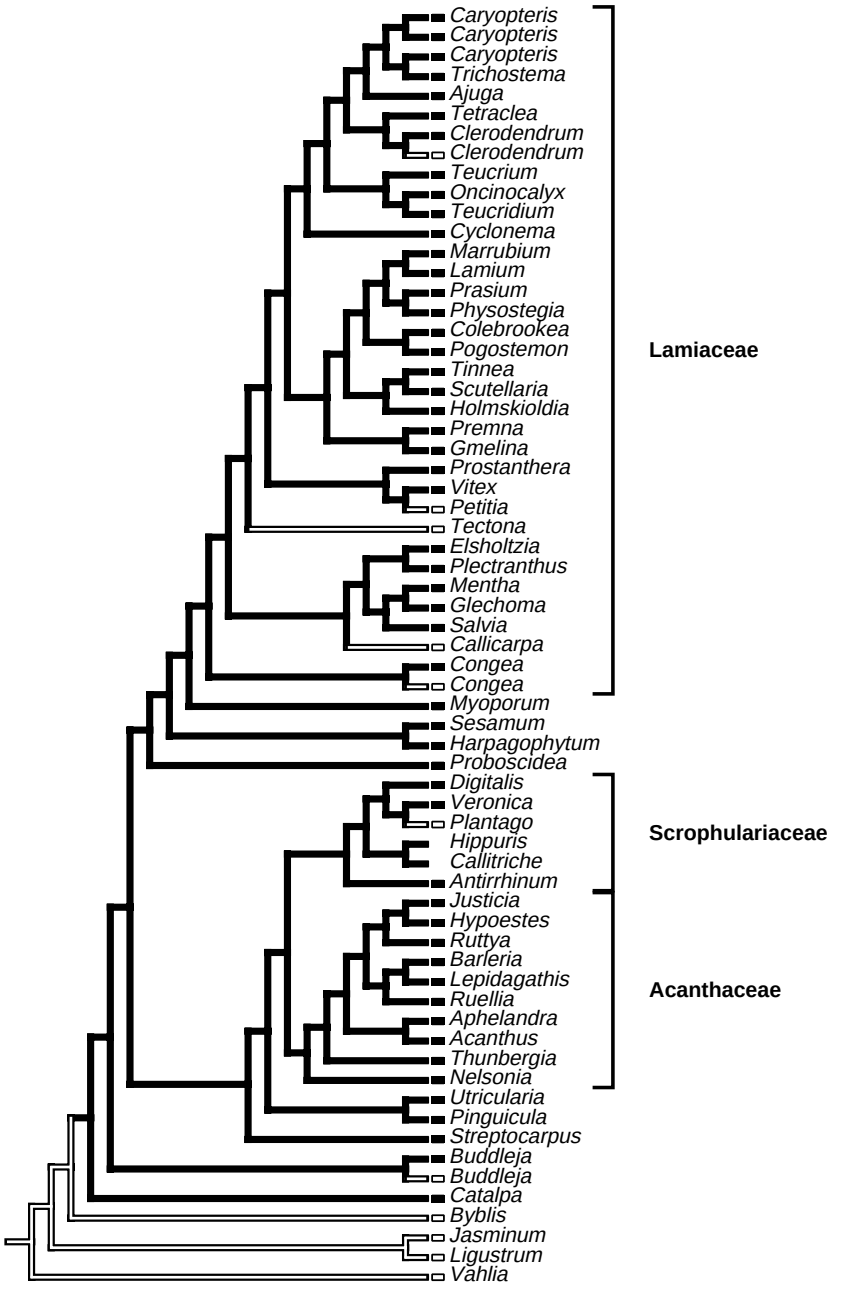

Fig. 4. A phylogeny of the asterid clade including Lamiaceae, Scrophulariaceae and Acanthaceae. Some genera are represented by more than one species. Species with actinomorphic corollas are shown with a white box and species with zygomorphic corollas with a black box. *Hippuris* and *Callitriche* lack a corolla and are scored as missing. The most-parsimonious reconstruction of ancestral states (with equal costs) is shown, with white branches representing actinomorphy and black branches representing zygomorphy. Seven reversals from zygomorphy to actinomorphy are shown: *Plantago*, *Petitia*, *Tectona*, *Callicarpa* and some species within *Clerodendrum*, *Congea* and *Buddleja*.

with this interpretation in that they show differential rates of development of petal (and stamen) primordia in abaxial and adaxial positions. Some of the effects of this early activity may be seen in mature flowers in which the adaxial petal differs somewhat from the other three, and the two adaxial sepals are not fused to the same level as the rest of the calyx.

Loss of function of the *CYC* pathway may also have occurred in a few instances. In fact, there are several actinomorphic Asteridae, not included in the phylogeny assembled here, that do show tell-tale signs of having originated through this mechanism. Several genera of Gesneriaceae have four- to six-merous and nearly actinomorphic flowers (e.g. *Ramonda* and *Protocyrtandra*)[5,30], and some resemble 'natural pelories' (e.g. *Tengia* and *Marssonia*)[5]. Similarly, in the Scrophulariaceae the genus *Sibthorpia* has actinomorphic flowers with four to eight petals[7]. The development of these flowers merits special attention. However, even in these cases, it is possible that the resemblance to *cyc* mutants is coincidental, with the relevant genetic changes being located in genes that are either upstream or downstream in the *CYC* developmental pathway or in an entirely parallel pathway.

The finding that the evolution of actinomorphy from zygomorphy need not involve simple loss-of-function mutations has general significance. It shows that evolutionary shifts to a particular morphological end-point need not come about via the same mechanisms that underlie mutant phenotypes in model genetic systems. The types of mutations studied by geneticists are mainly single-event substitutions or deletions, whereas evolutionary changes may often involve genomic rearrangements[31] or a series of mutations that accumulate over time to generate the phenotype of interest. Also, the success of different mutant forms, and consequently the likelihood of observing different evolutionary pathways, is tied ultimately to function in the wild. It has been emphasized that in the case of floral symmetry the connection to pollinator behavior may be critical[27]. In particular, a shift from zygomorphy to actinomorphy may be disadvantageous when mature anthers remain within a tubular flower, whereas this shift may be more successful in lineages in which there has already been a shift to more open flower morphology, with exserted stamens. It seems likely that detailed studies of the several types of derived actinomorphic flowers will reveal a number of different pathways to actinomorphy, involving different suites of genes, acting at different times during development.

Phylogeny and different forms of zygomorphy

In scoring floral symmetry for the groups represented in the asterid supertree, three basic forms of corolla zygomorphy were observed (Fig. 6a–c). The most common form is the one found in *Antirrhinum*, in which the two adaxial petals are differentiated from the three other petals (two lateral and the medial abaxial petal). A second pattern is also reasonably common, in which four adaxial petals are differentiated from the medial abaxial petal. In a third and perhaps rarer form, all five petals are shifted towards the abaxial side of the flower. These can be termed the 2 : 3, 4 : 1 and 0 : 5 patterns, respectively. Although the existence of these three forms has been noted before[6,32], little is known of their phylogenetic distribution, or the pattern of evolutionary change among them.

Two, or often all three, patterns are found in each of the major zygomorphic clades of Asteridae. All three floral forms are found among the Lamiaceae, for example: *Lamium* shows the 2 : 3 pattern; *Coleus* possesses the 4 : 1 pattern; and *Teucrium* shows the 0 : 5 pattern. Likewise, in the Asteraceae the zygomorphic flowers of *Mutisia*, for example, are 2 : 3, whereas those of *Barnadesia* and other Barnadesineae are 4 : 1, and *Cichorium* and other Lactuceae are 0 : 5. In the Scrophulariaceae, the 2 : 3 form is most common, but *Nemesia* and related South African genera show the 4 : 1 pattern. In the Dipsacales, members of the Linnaeeae (e.g. *Kolkwitzia* and *Dipelta*) and Diervilleae (*Weigela* and *Diervilla*) have 2 : 3 flowers, whereas zygomorphic Lonicereae (e.g. *Lonicera* and *Triosteum*) exhibit the 4 : 1 pattern.

These observations raise a number of questions:

- Why is it that these particular forms of zygomorphy are repeated again and again?
- Why are other forms only rarely observed?
- How is it, from a genetic and developmental standpoint, that transitions among the types occur?

Answers to these questions depend on a critical observation: that the ancestral, and nearly universal orientation of the asterid flower results in two adaxial petals, two lateral petals and a medial abaxial petal[33]. Hence, thinking in terms of the initiation of flower parts on the developing flower primordium, the standard sequence begins with a medially positioned sepal on the adaxial side of the flower (opposite the bract that usually subtends the flower).

If this basic arrangement is maintained, along with five petals, then there are only three options for dividing the flower perpendicular to the dorso-ventral axis into adaxial and abaxial portions (Fig. 6a–c). Dividing the flower between the two adaxial petals and the rest gives the 2 : 3 pattern, whereas dividing it between the medial abaxial petal and the rest yields the 4 : 1 pattern. In the 0 : 5 pattern, all five petals are shifted abaxially, so that, in effect, a 'division' occurs between the corolla and the axis. The opposite of this, shifting all of the petals adaxially, is not a possibility without splitting the medial abaxial petal, thereby increasing the number of petals. All other possible divisions between petals would be oblique. It appears, then, that the basic number and orientation of flower parts on the primordium constrains the ways in which the flower can be differentiated into adaxial and abaxial portions.

In the Asteridae there are a few alternative forms of zygomorphy known, aside from the three common forms. These are particularly interesting because they offer a means of evaluating the hypothesis that a structural constraint, the conserved floral orientation, might play a role in restricting the types of zygomorphy generated. One of the most obvious alternative forms of zygomorphy is found within the large genus *Rhododendron* of the Ericaceae. Many species of *Rhododendron* have actinomorphic flowers, as is characteristic of other ericads. However, there are some clearly zygomorphic species (e.g. *R. canadense*), and these have a 3 : 2 arrangement with three adaxial petals (one medial and two lateral) differentiated from two abaxial petals (Fig. 6d). How has this come about? Studies of early flower development have shown that in *Rhododendron* and some other Ericaceae (e.g. some *Vaccinium* species[34]) the normal asterid orientation is not manifested. From its earliest inception, the medial petal is in the adaxial rather than abaxial position (and the medial sepal is abaxial rather than adaxial). The 'inverted' flower orientation of *Rhododendron* explains the unusual form of zygomorphy found in this group. In fact, within the constraints of this new orientation, it should be impossible to obtain the standard patterns seen elsewhere in the Asteridae.

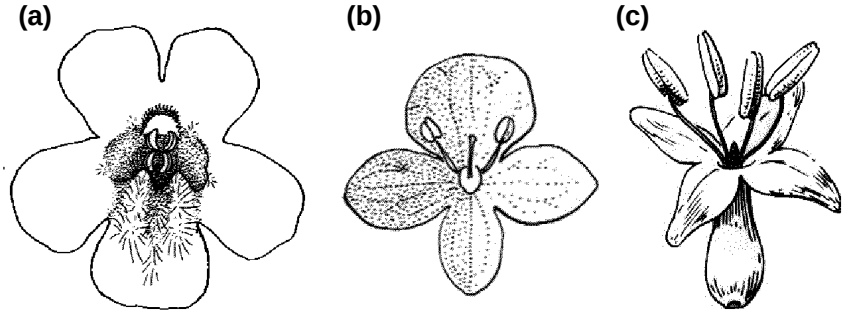

Fig. 5. Comparison of (a) a typical five-merous, actinomorphic Scrophulariaceae flower (*Mimulus* spp.); (b) a four-merous, zygomorphic *Veronica* flower; and (c) a four-merous, actinomorphic *Plantago* flower.

Another fascinating case concerns *Lobelia*, *Downingia*, and their relatives in the Lobeliaceae. In these taxa the mature flowers appear to conform to the standard 2 : 3 pattern (Fig. 6f). However, closer examination shows that the flower stalk rotates through 180° rather late in flower development, a phenomenon known as resupination. This means that early in development (before resupination) the medial abaxial petal of the mature flower occupied the atypical adaxial position (Fig. 6e). Thus, as in *Rhododendron*, the basic orientation of the flower is inverted. In this case, one might imagine that resupination is a mechanism to compensate for the basic shift in orientation, perhaps related to pollination by bees accustomed to the 2 : 3 form of zygomorphy. Although this may sometimes be the case, some members of this group, such as *Nemacladus* and its relatives, are non-resupinate and therefore do show the 3 : 2 pattern in mature flowers.

Those Asteridae that differ from the three common forms of zygomorphy show inverted floral orientation, which supports the hypothesis that floral orientation is a developmental constraint on the evolution of dorso-ventral asymmetry. It therefore becomes important to understand how floral orientation is itself regulated and why shifts in floral orientation are apparently uncommon. In this connection it would be useful to clarify the role of genes that are involved in determining the position of initiation of floral organs[35]. Additionally, it will be important to look at floral symmetry genes, such as *CYC*, to assess whether they play a more general role in determining floral orientation[36]. Indeed, *CYC*-like genes have been found in ESTs from the actinomorphic flower of *Arabidopsis* (P. Cubas *et al.*, unpublished). However, until there is information on the spatial and temporal pattern of gene expression and/or knockouts are obtained, it will be difficult to evaluate the possible role of such genes in floral orientation.

Progress and prospects

What insights are gained by taking a phylogenetic perspective on flower symmetry? In expanding Coen and Nugent's phylogenetic approach[9], it can be concluded that the first Asteridae were likely to have been actinomorphic, and that zygomorphy evolved several times independently within the group. There are also some well-supported instances of reversal to actinomorphy within each of the large zygomorphic clades, several of which seem to have involved an evolutionary pathway other than a loss of *CYC* activity. For example, in the case of *Plantago*, it appears that actinomorphy was achieved through fusion of the two adaxial petals. This observation serves to emphasize that evolutionary paths to a particular morphology need not involve the same mutations that generate such a phenotype in genetic model systems.

There appear to be three main forms of zygomorphy, occurring in each of several

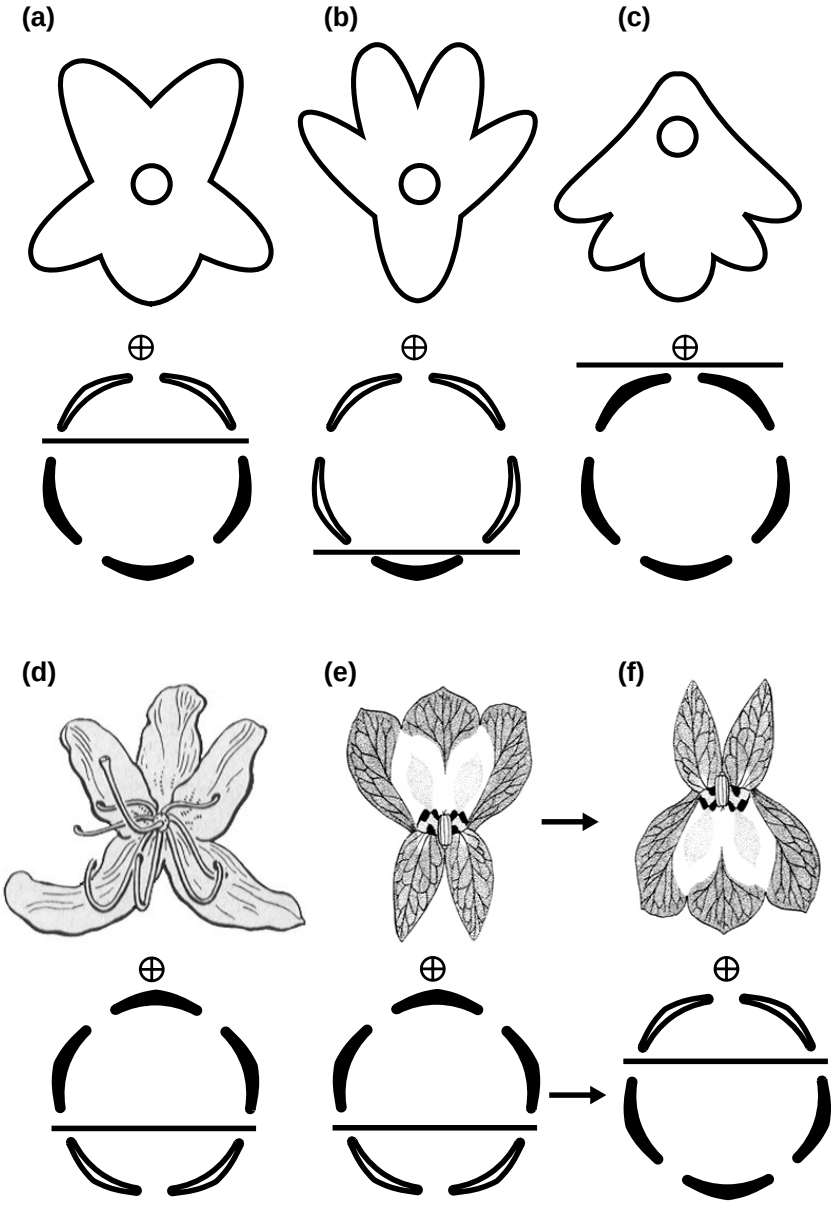

Fig. 6. Three common types of zygomorphy in Asteridae: (a) 2 : 3, (b) 4 : 1 and (c) 0 : 5. Below each diagram of a corolla is a schematic transverse section of the corolla whorl in the standard asterid orientation (stem axis marked by a cross). The three types of zygomorphy correspond to passing a line through the flower that separates an adaxial from an abaxial region. Petals in the abaxial region are shaded. Exceptional cases are shown in (d) to (f). The flowers of *Rhododendron* (d), and *Downingia* (e) have a floral orientation that is the inverse of most Asteridae, with the medial petal positioned on the adaxial side. This explains the unusual 3 : 2 form of zygomorphy found in those taxa. The situation is complicated in *Downingia*, because the flower undergoes resupination (torsion late in development, represented by the arrow) resulting in an apparently normal 2 : 3 form of zygomorphy (f).

major clades. These patterns can be accounted for by the near universality of the basic orientation of the flower in Asteridae. The presence of a medial petal in the abaxial position places constraints on the form of zygomorphy that can evolve, and it is only in those rare instances in which the constraint breaks down that other patterns seem to have been possible.

A comparison is needed of the genetic and developmental mechanisms underlying zygomorphy where it appears to have evolved independently. Have the same pathways been used in each case, or are there significant differences? Another possibility is to study derived actinomorphic flowers such as *Plantago* to see if *CYC* expression is more or less normal. Additionally, there is a need for genetic studies of the regulation of flower orientation. Finally, it should be clear that there are many fascinating evolutionary patterns in flower symmetry outside of the Asteridae, and a general theory of floral evolution will ultimately need to accommodate all of these.

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technical focus

GFP for *in vivo* imaging of subcellular structures in plant cells

A novel marker, green fluorescent protein (GFP), has initiated a new era in cell biology because it permits real-time observations of dynamic changes in living cells and organisms. GFP-tagged fusion proteins are used to study gene expression, subcellular localization, and trafficking of proteins and organelles *in vivo*. GFP fusions have also revealed novel subcellular structures and provided new insights into compartmentation and protein exchange between compartments.

Imaging of subcellular structures

In vivo observations of subcellular structures, compartments and organelles have been restricted in the past by the resolution of light microscopy. Cell components of a size at or below the limit of light resolution have mostly been studied in fixed dead material by electron microscopy or immunocytochemistry. Both methods are time consuming and make it difficult to study and understand the dynamic changes that are a part of all living cells. More recently, membrane-permeable fluorescent dyes have facilitated observation of organelles, such as mitochondria and the endoplasmic reticulum (ER), in living cells[1]. However, properties such as concentration-dependent toxicity, poor penetration and fast photobleaching have restricted their value for many applications.

GFP, a novel marker protein isolated from the jellyfish *Aequorea victoria*, has generated a lot of excitement, because it permits direct observation of the localization and intracellular trafficking of fusion proteins *in vivo*[2]. The remarkable ability of GFPs to emit green light (509 nm) when excited by either blue (475 nm) or UV (395 nm) light is defined by its primary sequence[3]. Formation of the fluorophore of GFP requires molecular oxygen, but is otherwise independent of co-factors and occurs autocatalytically[3]. Studies from a wide range of different organisms, including bacteria, *Drosophila*, *Xenopus*, mice, mammalian cells and plants, have demonstrated that GFP has little or no toxicity, and that its ability to fluoresce is preserved in N- or C-terminal fusion proteins[3]. Furthermore, the fluorescence of GFP and its derivatives is resistant to photobleaching and remains stable under a wide variety of conditions[3,4], permitting extended observation of GFP fusion proteins and observation of GFP in different environments.

Imaging of GFP in plant cells

Initial problems in expressing and visualizing GFP in plant cells have been overcome by the elimination of cryptic splice sites and optimizing codon usage[5–8]. Further modifications of the *GFP* gene that improve the use and detection of the protein in plants allow increased expression, improved folding during post-translational maturation, faster maturation, increased fluorescence intensity and altered spectral properties[8–10]. Transient or stable expression of these modified *GFP* genes leads to bright-green fluorescence in both monocot and dicot cells[5,6,10].

Chloroplasts emit red autofluorescence when excited. The red autofluorescence and green GFP fluorescence can be separated with appropriate filter sets. Figures 1–3 show confocal laser scanning microscope (CLSM) images, where red and green fluorescence were collected in separate channels and merged electronically. The red and green are pseudo colors that resemble the fluorescence observable by epifluorescence microscopy.

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