

EVOLUTION OF NOVEL MORPHOLOGICAL AND REPRODUCTIVE TRAITS IN A CLADE CONTAINING *ANTIRRHINUM MAJUS* (SCROPHULARIACEAE)¹

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Phylogenetic analysis of DNA sequences of the chloroplast genes *rbcl* and *ndhF* revealed a highly supported clade composed of the families Plantaginaceae, Callitrichaceae, and Hippuridaceae in close association with the model organism *Antirrhinum majus* and other members of family Scrophulariaceae. *Plantago* has miniature actinomorphic wind-pollinated flowers that have evolved from zygomorphic animal-pollinated precursors. The aquatic Hippuridaceae have reduced wind-pollinated flowers with one reproductive organ per whorl, and three, rather than four, whorls. In monoecious aquatic Callitrichaceae, further reduction has occurred such that there is only one whorl per flower containing a single stamen or carpel. Optimization of character states showed that these families descended from an ancestor similar to *Antirrhinum majus*. Recent studies of plant developmental genetics have focused on distantly related species. Differences in the molecular mechanisms controlling floral development between model organisms are difficult to interpret due to phylogenetic distance. In order to understand evolutionary changes in floral morphology in terms of their underlying genetic processes, closely related species exhibiting morphological variation should be examined. Studies of genes that regulate morphogenesis in the clade described here could aid in the elucidation of a general model for such fundamental issues as how changes in floral symmetry, organ number, and whorl number are achieved, as well as providing insight on the evolution of dicliny and associated changes in pollination syndrome.

Key words: *Antirrhinum*; development; evolution; flower morphology; MADS box genes; *ndhF*; phylogeny; *rbcl*; Scrophulariaceae.

One of the fundamental questions in evolutionary biology is the origin of the novel characters that distinguish species. Any genetic change that affects morphology must do so by affecting the interactive and contingent processes of development. For a nascent morphological character to become associated with a discrete evolutionary lineage (e.g., a species or higher taxon), the character must pass through the sieve of selection and the process of speciation, emerging as part of an integrated, successfully adapted organism.

Thus, in order to begin to understand how morphological character differences between taxa have arisen, it is necessary to have an understanding of the character from both a phylogenetic and ontogenetic perspective. Phylogenetic trees are useful in determining homologous structures and processes and in revealing the direction of evolution between character states. Through the comparison of homologous developmental processes at the molecular level, a mechanistic, as opposed to purely descriptive, explanation for observed morphological differences may be discovered.

In plant evolutionary biology, molecular systematics has increased our understanding of phylogeny in many

angiosperm groups. In recent years, plant developmental genetics has made great advances in our knowledge of mechanisms important in floral ontogeny. Much of this research has been focused on a few model systems, most notably *Arabidopsis thaliana* (Brassicaceae) and *Antirrhinum majus* (Scrophulariaceae).

Antirrhinum majus L., the snapdragon, is a common ornamental species characterized by showy zygomorphic flowers arranged in racemes. Its history as a research organism for genetics began with studies on the inheritance of flower color variation (Wheldale, 1907) and the characterization of numerous mutants by Baur (1930). Early genetic analyses culminated with Stubbe's *Genetik und Zytologie von Antirrhinum* (1966). The current status of *Antirrhinum majus* as a model organism for molecular genetics was spurred by the discovery and characterization of several highly mobile transposable elements (Bonas et al., 1984; Sommer et al., 1985; Upadhyaya et al., 1985; Coen and Carpenter, 1986), which were useful for mutagenesis and gene-tagging experiments (Martin et al., 1985; Sommer and Saedler, 1986; Martin et al., 1991). Subsequent studies of floral homeotic mutants led to the identification and characterization of a group of transcription factors, the MADS box gene family, which play a regulatory role during floral morphogenesis, most notably in the specification of organ identity (Carpenter and Coen, 1990; Schwarz-Sommer et al., 1990, 1992).

Studies of floral development in *Arabidopsis thaliana* (Bowman, Smyth, and Meyerowitz, 1989, 1991; Yanofsky et al., 1990) and later examinations in other angiosperms (Pnueli et al., 1991; Angenent et al., 1992; Hansen et al., 1993; Schmidt et al., 1993) confirmed the presence and characteristic expression patterns of a few genes in the MADS family, indicating that the fundamental ge-

¹ Manuscript received 10 June 1997; revision accepted 4 December 1997.

The authors thank Tom Philbrick for providing plant tissue of *Hippuris* and DNA of *Callitriche*, and Tom Philbrick, Jackie Nugent, Michael Frohlich, and Peter Endress for many helpful suggestions on the manuscript. This work was supported in part by a National Science Foundation Undergraduate Research Experience (REU) supplement grant to Richard Olmstead (NSF grant number BSR-9107827) and a grant from the Undergraduate Research Opportunities Program at the University of Colorado to Patrick Reeves.

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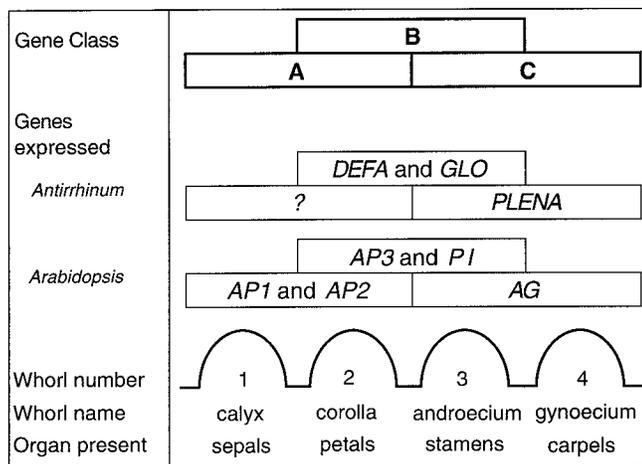


Fig. 1. Model illustrating the pattern of expression of key developmental genes during early flower development in wild-type *Antirrhinum* and *Arabidopsis* (adapted from Coen and Meyerowitz, 1991). Of these, all but *APETALA-2* (*AP2*) belong to the MADS box gene family. There are three distinct gene functions, each given a class designation: A, B, or C. Each gene is expressed in two consecutive whorls. In wild-type developing floral meristems, expression of the B and C class genes as shown is necessary and sufficient for determining the development of the appropriate floral organs in whorls 3 and 4 (Yanofsky, 1995). The A function has not been clearly characterized in *Antirrhinum* (Davies and Schwarz-Sommer, 1994). Phylogenetic analysis suggests that *DEFICIENS* (*DEFA*) and *APETALA-3* (*AP3*), *GLOBOSA* (*GLO*) and *PISTILLATA* (*PI*), and *PLENA* and *AGAMOUS* (*AG*) are orthologous gene pairs (Doyle, 1994).

netic processes governing floral organ differentiation have been conserved over evolutionary time (Fig. 1).

While it is unclear precisely how the whorl-specific expression patterns of MADS box genes are defined, some mechanisms have been suggested. *Antirrhinum majus* *DEFICIENS* protein can move from inner to outer layers in whorl 2 primordia, but not vice versa, and is limited in the degree to which it can move within one cell layer. Thus control over the movement of MADS box proteins through plasmodesmata may be important in maintaining their spatial boundaries (Perbal et al., 1996). MADS box proteins can act alone or in combination as repressors or activators of transcription at other MADS box loci, resulting in spatial regulation. For example, it has been proposed that A and C class genes negatively regulate each other (Drews, Bowman and Meyerowitz, 1991) and that the protein products of B function genes *DEFA* and *GLO* form a heterodimer, which upregulates expression of the two genes (Tröbner et al., 1992). Furthermore, several non-MADS box genes that influence the induction (Coen et al., 1990; Weigel et al., 1992), maintenance (Simon et al., 1994; Lee et al., 1997), and termination (Sakai, Medrano, and Meyerowitz, 1995) of MADS box gene expression in specific whorls have been identified.

In addition to specifying floral organ development within a whorl, MADS box genes also influence determinacy within a flower. In *Arabidopsis*, A class *APETALA-1* (*API*) and C class *AG* genes interact to establish and terminate the floral meristem (Irish and Sussex, 1990; Mizukami and Ma, 1995). In *Antirrhinum majus*, B class *DEFA* inhibits floral termination in the third whorl while

C class *PLENA* promotes termination in the fourth whorl (Tröbner et al., 1992; Davies and Schwarz-Sommer, 1994). As a consequence of their role in meristem determinacy, MADS box genes may be thought of as influencing the number of whorls within a flower. Moreover, genes from all three classes have been shown to influence the number of organs that develop within a whorl (Bowman, Smyth, and Meyerowitz, 1991; Coen and Meyerowitz, 1991).

Several other genes, unrelated to the MADS box family, function in shaping the angiosperm flower. *Antirrhinum majus* has proven to be a valuable system for the study of genes involved in the development of zygomorphic flowers. Mutations in the genes *CYCLOIDEA* and *DICHOTOMA*, which in wild type are expressed only in the dorsal region of the flower, result in the development of actinomorphic *Antirrhinum majus* flowers (Luo et al., 1996). Additional evidence from the gene *DIVARICATA*, which influences the development of features unique to the ventral region of the flower, leads to the suggestion that asymmetry is achieved through the differential expression of such genes along the dorsoventral axis of the developing flower (Almeida, Rocheta and Galego, 1997).

In *Arabidopsis*, numerous genes affect the number of organs that develop within a whorl, but often do so in a stochastic manner (Bowman, Smyth, and Meyerowitz, 1989; Roe et al., 1993; Running and Meyerowitz, 1996; Huang and Ma, 1997). In the *CLAVATA* class of genes, an increase in organ and whorl number is correlated with an increase in floral meristem size (Clark, Running, and Meyerowitz, 1993). This suggests a possible biomechanical influence on the determination of organ and whorl number (Green, 1992).

There is considerable interest in possible roles for MADS box and other developmental genes in the evolution of floral diversity (Coen and Meyerowitz, 1991; Coen and Nugent, 1994; Irish and Yamamoto, 1995). Changes in inflorescence architecture, modification of the basic four-whorled floral ground plan, the appearance of differences between organs within a single whorl, and changes in floral sex expression and symmetry have all been effected via mutations in such genes in model systems (Coen, 1991). These same morphological characteristics often are used to distinguish angiosperm taxonomic groups. It is important to determine whether the differences seen between major groups of plants can be explained by simple differences in the expression of developmental genes.

Investigation of the role of developmental genes in plant morphological evolution ultimately may help to answer the critical question: To what degree can differences in gene content and expression pattern account for evolutionary innovation in floral form?

Current research in plant developmental genetics focuses on relatively distantly related dicots (e.g., a few members of the Solanaceae, *Arabidopsis thaliana*, *Antirrhinum majus*) and one monocot (*Zea mays*). These taxa were chosen for technical and historical reasons, but also were thought to be suitable, a posteriori, for evolutionary studies because of their morphological differences. We believe that the use of distantly related taxa to study the evolution of homeotic genes and their effect on the evolution of floral morphology has limited applicability. A

focus on a monophyletic group of closely related species, within which substantial morphological variation exists, is likely to yield more meaningful conclusions.

The principal problem with studying distantly related species is, in the case of the MADS box genes, the likelihood of mistaken identification of orthologous genes given a complex gene family and large phylogenetic distances (Sanderson and Doyle, 1992). The MADS box genes and their binding domains appear to be common to all eukaryotes (Passmore, Elble, and Tye, 1989) and influence a range of processes. In plants, many homologs are expressed in roots, embryos, or otherwise vegetatively and presumably have no role in floral development (Pnueli et al., 1991; Heard and Dunn, 1995; Purugganan et al., 1995). At large phylogenetic distances it has been necessary to screen tissue-specific cDNA libraries at low stringency with highly conserved probes to identify homologous genes. This may give a biased view of the narrowness of function of the gene family and may result in an erroneous assertion of orthology. Related genes with identical expression patterns in two distantly related taxa may not be orthologous or may not regulate the same pathways.

These theoretical difficulties have been demonstrated to be real concerns by Doyle (1994), Purugganan et al. (1995), and Theissen, Kim, and Saedler (1996) who, in a phylogenetic analysis of available MADS gene sequences, accumulated evidence that recent duplications have occurred in some gene lineages of both *Arabidopsis thaliana* and *Zea mays*, or, conversely, that the true orthologs of these pairs had not been identified in *Antirrhinum majus* and other taxa. An alternative hypothesis is that the orthologs do not exist in *Antirrhinum majus* due to gene extinction. Because of the complicating factors of gene duplication and extinction, it may be impossible to identify orthologs confidently in distantly related organisms even if all the MADS genes from the genome were to be characterized.

We contend that the use of closely related species belonging to a single monophyletic group should decrease these problems. In closely related species the probability that any two orthologous genes have undergone functional divergence is lower than with distant relatives. Likewise, the opportunity for gene duplication and extinction to have occurred over the relatively shorter evolutionary time since species divergence is less.

In order for such comparative studies to proceed, accurate homology assessment, at both the molecular and morphological level, and a knowledge of the direction of evolution, is critical. Recognizing this, Endress (1992) described the morphological differences in species related to *Antirrhinum majus* and *Arabidopsis thaliana*. However, his discussion was based on traditional classifications that do not reflect phylogeny among the closest relatives of either species (Price, Palmer, and Al-Shebaz, 1994; Olmstead and Reeves, 1995).

A consequence of the continued reliance on prephylogenetic classifications, in which species are grouped primarily on phenetic similarity, is the belief that there are few major differences between closely related organisms. This leads to the presumption that one necessarily must work at the interordinal or interclass level in order

to study the major types of morphological changes that have occurred during angiosperm diversification.

To the contrary, we have described a well-supported clade belonging to the order Lamiales sensu lato, which contains several groups of plants with divergent morphology and reproductive biology (referred to as the Scroph II clade in Olmstead and Reeves, 1995). The species that make up this clade previously were not known to be closely related to one another. The clade includes, among others, the model organism *Antirrhinum majus*, the actinomorphic wind-pollinated genus *Plantago*, and a series of aquatic plants with unique reproductive strategies and novel floral ground plans missing one whorl in *Hippuris* and three whorls in monoecious *Callitriche*. *Plantago*, *Hippuris*, and *Callitriche* historically have been considered to be sufficiently divergent morphologically to merit classification as distinct families or even orders (Dahlgren, 1980; Cronquist, 1981; Takhtajan, 1987; Thorne, 1992).

In this paper we describe, within a phylogenetic context, the changes that have resulted in diversification of morphological and reproductive characters within the Scroph II clade. We also propose that this clade is well suited for future mechanistic evolutionary studies in developmental genetics.

MATERIALS AND METHODS

A total of 16 species was used for this study. The taxa of interest included six species identified as representatives of the monophyletic Scroph II clade (Olmstead and Reeves, 1995), plus two additional species of *Callitriche*, *C. heterophylla* and *C. verna*. Seven species representing lineages identified as being closely related to the Scroph II clade also were included; three of these belong to the Scroph I clade (Scrophulariaceae sensu stricto), which is distinct from Scroph II. *Nicotiana tabacum* was used as an outgroup.

DNA sequences for the chloroplast genes *rbcL* and *ndhF* were determined for *C. verna*; *ndhF* was determined for *C. heterophylla*. GenBank accession numbers for these sequences are L47331, L47330, and L47329, respectively. All other sequences were published previously (Olmstead et al., 1992; Olmstead and Reeves, 1995). Methods for obtaining sequences are described in Olmstead and Sweere (1994), and Olmstead and Reeves (1995).

All of the *rbcL* sequence data and the majority of the *ndhF* data were aligned by eye. A portion of the *ndhF* sequences corresponding to the region between nucleotides 1426 and 1722 of *Nicotiana tabacum* was length variable. Both nucleotide and inferred amino acid sequences for this region were aligned using Clustal V (Higgins, Bleasby, and Fuchs, 1992). Final adjustments were made manually.

Sequence data for both genes were analyzed using PAUP version 3.1 (Swofford, 1993). All nucleotide changes were weighted equally and gaps were scored as missing data. The search settings used were 100 random order entry replicates, TBR branch swapping, and MULPARS "on" to save all equally parsimonious trees from each replicate. A bootstrap analysis was performed with 100 replicates to assess the stability of clades on the resulting tree.

RESULTS

A total of 3530 bases of aligned sequence was analyzed. Of this, 489 positions were phylogenetically informative. Although not used in the primary parsimony analysis as characters, 11 gaps were required to align the sequences, three of which were informative.

The analysis yielded two most parsimonious trees with

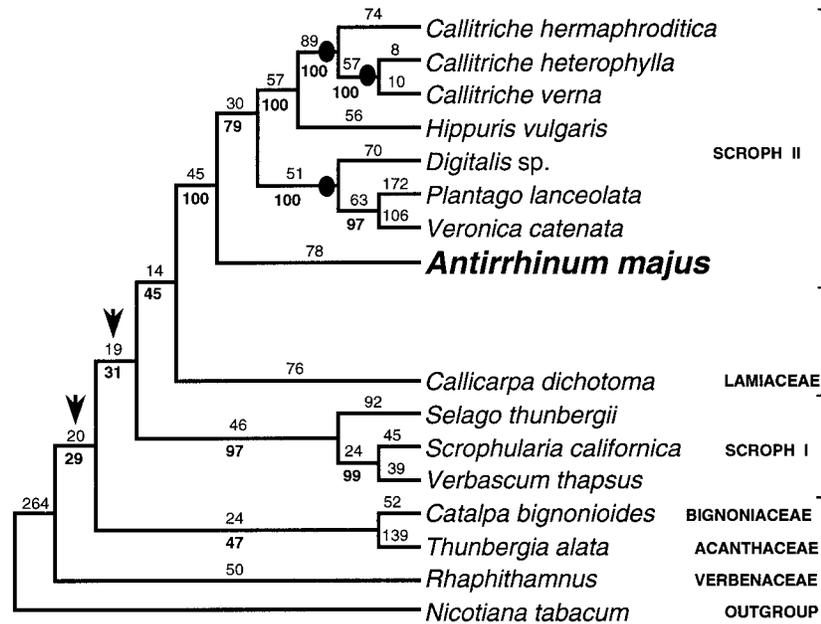


Fig. 2. Bootstrap consensus tree for combined analysis of *rbcL* and *ndhF*. Branch lengths are above nodes, bootstrap support values are below nodes. Arrowheads identify nodes that collapse in the strict consensus of the two most parsimonious trees found. Bullets mark clades that are supported additionally by deletions in the *ndhF* sequence relative to the outgroup.

a total length of 1870 steps and a consistency index, excluding uninformative characters, of 0.569. The bootstrap consensus tree, which was identical to one of the two shortest trees, is shown in Fig. 2.

DISCUSSION

Tree support—Within the Scroph II clade, the branching pattern shown in Fig. 2 is congruent with previous studies (Olmstead and Reeves, 1995). The addition of two more species of *Callitriche* has the effect of splitting the long terminal branch of *C. hermaphroditica* found in previous studies, thus diminishing the possibility of an artifactual branch position (Felsenstein, 1978). The relationship among species of *Callitriche* found here is consistent with the RFLP analysis of Philbrick and Jansen (1991).

The relationships between the Scroph II clade and the other taxa included in this study differ in some ways from previous analyses. However, taxonomic sampling also differs, and the branches that are inconsistent are only weakly supported, as evidenced by both short branch lengths and low bootstrap values. For the purposes of examining morphological and reproductive evolution in the clade, all branches with bootstrap values less than 50% have been collapsed (Figs. 3, 4). The resulting tree is entirely consistent with that found by Olmstead and Reeves (1995).

Evolution of reproductive biology—An explicit phylogenetic hypothesis is necessary in any study of character evolution. By optimizing terminal character states onto a tree, a reasoned hypothesis of the character states present in the common ancestor of the clade can be constructed. Once done, the polarity of character change can be inferred and the appearance of characters in extant

taxa can be understood as direct modifications from an extinct ancestral type.

The ancestor of the Scroph II clade is inferred to have had bisexual, animal-pollinated flowers. In the aquatic clade identified in Fig. 3, wind pollination has evolved along with miniscule flowers. *Hippuris* has bisexual flowers and emergent inflorescences that facilitate wind-mediated pollination. Monoecy has evolved in *Callitriche* from the bisexual arrangement present in the most recent common ancestor with *Hippuris*. In *Callitriche*, the terminal rosette of leaves floating at the water's surface is an impediment to wind pollination because the flowers lie in the axils of tightly arranged leaves and because wind speed decreases dramatically at the air/water interface.

Monoecy often is interpreted as a means of promoting outcrossing. However, Philbrick and Anderson (1992) have shown that, while some outcrossing via water-mediated pollen transfer may occur (Philbrick, 1993), *Callitriche* is primarily a selfing genus and that mechanisms have evolved to guarantee geitonogamy. In *C. hermaphroditica* the styles are reflexed and extend to the node below to contact the stamens. Pollen tubes grow directly from stamen to stigma with no pollen transfer. In *C. verna* and *C. heterophylla* a unique fertilization mechanism, termed internal geitonogamy, has evolved wherein pollen germination occurs precociously, in the anthers, and pollen tubes grow through vegetative tissues to pistillate flowers at adjacent nodes where fertilization is effected through the base of the ovary (Philbrick, 1984).

Plantago exhibits a wide range of reproductive strategies, from wind pollination to insect pollination to cleistogamy, resulting in levels of outcrossing from 0 to 100% depending on the species (Wolff, Friso, and Van Damme, 1988; Sharma, Koul, and Koul, 1992).

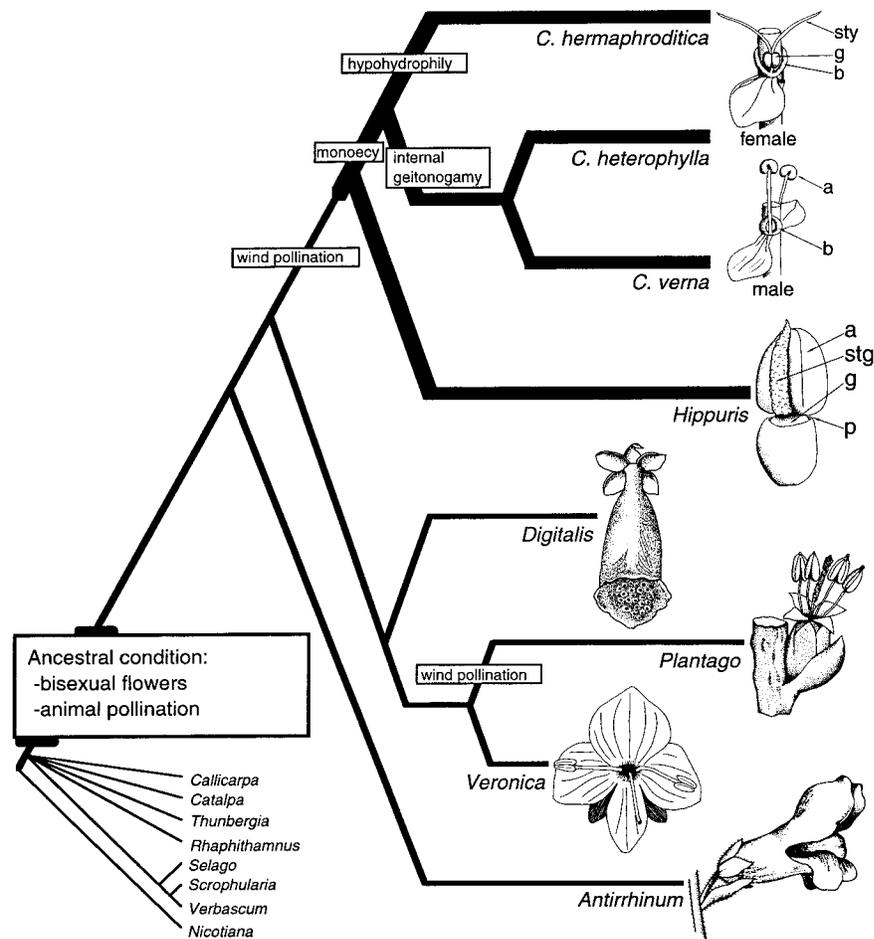


Fig. 3. Phylogenetic tree depicting evolution of selected reproductive characters in the Scroph II clade. The branches of the aquatic clade are in boldface. Because floral morphology is similar among all three species of *Callitriche*, single representative male and female flowers are shown for simplicity. Illustrations are not to same scale. a, androecium/stamen; b, bract; g, gynoecium/carpel; p, corolla/petal; stg, stigma; sty, style.

Evolution of floral morphology—Preliminary evidence from the phylogenetic analysis of chloroplast DNA from additional members of the traditionally circumscribed Scrophulariaceae suggests that *Tetranema*, *Collinsia*, and *Chelone* are basal members of the Scroph II clade (R. Olmstead, A. Wolfe, and C. DePamplis, unpublished data). The floral morphology of these taxa is similar to that of *Antirrhinum majus*. Thus *Antirrhinum majus* retains many features of the ancestral morphology of the Scroph II clade.

We infer the ancestral floral morphology to have been strongly zygomorphic with four distinct whorls: calyx (whorl 1), corolla (whorl 2), androecium (whorl 3), and gynoecium (whorl 4). The flowers were pentamerous, with five sepals and a corolla of five fused petals. The correct ancestral state for stamen number is not clear because the number of stamens in whorl 3 varies in the basal taxa. In the Scrophulariaceae, stamen number changes frequently between five stamens, four stamens and a staminode, and four stamens (Endress, 1997). The ancestral gynoecium consisted of two fused carpels, each having a single locule. Given this ancestral morphology, it is likely that, on the molecular level, a developmental

program much like that of the model organism *Antirrhinum majus* was also present.

In the aquatic clade a variety of interesting evolutionary changes have occurred (Fig. 4). In both *Callitriche* and *Hippuris*, the stamen number has been reduced to one. Because of the reduction in organ number, primordia no longer initiate in whorls. However, for the purposes of discussion, we have retained the terminology, defining “whorl” not as an arrangement of organs, but rather as a physical region of the floral meristem wherein differential gene expression leads to the development of specific floral organs.

In *Hippuris*, a three-whorled floral ground plan has evolved. The floral organs associated with one perianth whorl have been lost over evolutionary time. The perianth whorl that remains contains a collar-like, entire to lobed structure without a clear number of organs (Leins and Erbar, 1988). It is not evident whether the remaining floral organs are sepals, petals, or a chimeric structure.

The loss of showy floral parts, typically petals, is commonly associated with the evolution of wind pollination. If this trend has been followed during the evolution of wind pollination in *Hippuris*, the remaining perianth

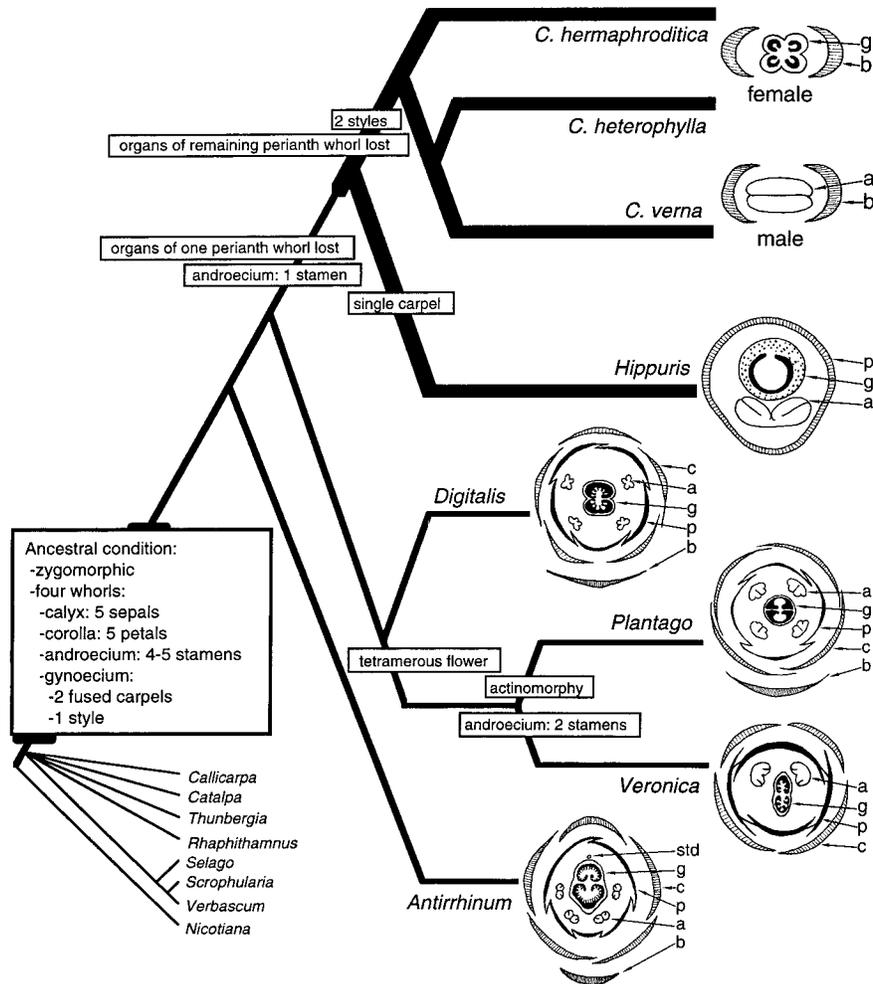


Fig. 4. Phylogenetic tree depicting evolution of selected morphological characters in the Scroph II clade. An equally parsimonious alternative to the pattern of perianth reduction and loss shown is one in which the loss of one perianth whorl is unique to *Hippuris*, and the loss of the entire perianth in a single step is a shared trait for the genus *Callitriche*. Single representative male and female flowers are shown for species of *Callitriche*. Illustrations are not to same scale. a, androecium/stamen; b, bract; c, calyx/sepals; g, gynoecium/carpel; p, corolla/petal; std, staminode.

whorl may contain one or more fused sepals. Alternatively, given that the ancestral state can be inferred to be free sepals and fused petals, the perianth may consist of one or more petals. In this case, fusion into a single tubular structure is a retained ancestral state. Because B class MADS box genes are expressed in petals and not sepals, these hypotheses could be tested by in situ hybridization studies using the *Hippuris* orthologs of *Antirrhinum majus* *DEFA* and *GLO*.

Coen (1991) points out that in homeotic mutants of *Antirrhinum majus*, congenital organ fusion is a property of the organ type, rather than a property of the whorl the organ occupies. This provides support for the hypothesis that the fused perianth structure of *Hippuris* contains one or more petals because the ancestral condition of the Scroph II clade was fused petals and free sepals. However, it raises the question of which whorl contains the perianth structure because, in principle, sepals or petals could develop in either whorl 1 or 2 depending on the pattern of MADS box gene expression.

Evidence from *Arabidopsis* and *Antirrhinum majus* suggests that the order of appearance of developing organ

primordia is a property of the whorl they occupy rather than the organ type. For example, in *Arabidopsis ap2* mutants the development of stamens in whorl 2 occurs on the same time course as wild-type petals, after the organs of the other whorls of the flower. Likewise, in mutants of *ap3* and its *Antirrhinum majus* ortholog *defA*, sepals develop in whorl 2 on a time course similar to wild-type petals (Bowman, Smyth, and Meyerowitz, 1989; Sommer et al., 1990). This implies that whorl 2 has an identity (e.g., delayed development) that is independent of the processes that specify which organs develop there. Therefore, although the first morphological indication of whorl initiation is the development of organ primordia, whorl identity and organ identity can be considered as distinct characters.

Studies of early development in *Antirrhinum majus*, *Veronica*, and several other members of the Veroniceae show that whorl 1 always initiates prior to whorl 2, which, depending on the species, appears after or at the same time as whorl 3 (Awasthi, Kumar, and Murty, 1984; Kampny, Dickinson, and Dengler, 1993; Hufford, 1995). In *Hippuris*, the stamen primordium, inferred to occupy

	whorl 1 "lost"	whorl 2 "lost"	whorl 3 "lost"	whorl 4 "lost"
sepals present				
petals present				

Fig. 5. Alternative hypotheses of the expression of MADS box genes in the three-whorled flower of *Hippuris*. Assumptions regarding the identity of the single perianth whorl are shown to the left. Assumptions regarding the identity of the whorls in which organs develop are shown across the top. The term "lost" is used to describe the absence of organ initiation in one of the four possible whorls. As such, "loss" could occur through three possible scenarios: (1) expression of genes before the induction of the MADS box organ identity genes that prevent the expression of the MADS box genes in a specific whorl; (2) expression of genes after the induction of the MADS box genes that affect the ability of the MADS box genes to influence downstream processes; (3) modification of the ancestral pattern of MADS box gene expression. Because no A class genes have been identified from *Antirrhinum*, their appearance in the figure should be considered putative. The necessary evolutionary modifications from the ancestral developmental plan shown in Fig. 1 are listed below each diagram. Due to the large number of changes required, it is unlikely that the stamen and carpel develop in whorls other than 3 and 4, respectively. From the perspective of parsimony, the most likely alternative is the one in which petals are present and whorl 1 has been "lost."

whorl 3 (see Fig. 5), appears prior to the perianth primordium (Leins and Erbar, 1988). Therefore, if we assume that the relative order of appearance of the first three floral whorls has been conserved in the Scroph II clade (with whorls 2 and 3 always being initiated after whorl 1), then the perianth organ(s) of *Hippuris*, which appear subsequent to whorl 3 stamen initiation, must develop in whorl 2. Figure 5 summarizes arguments regarding the identity of the perianth and the evolutionary loss of one floral whorl in *Hippuris* using parsimony as an arbiter.

In *Callitriche* no perianth organs are present, and the adoption of a monoecious sexual system has resulted in male flowers containing a solitary stamen and female flowers containing two fused carpels. It is unclear whether perianth loss has occurred as a single evolutionary step in *Callitriche*, or as a modification of a putative three-whorled ancestral floral ground plan similar to that seen

in extant *Hippuris*. The alternatives are equally parsimonious.

Modification of C class expression patterns may be important in the evolution of unisexuality in *Callitriche* flowers. In dioecious *Rumex acetosa*, development of stamens in female flowers ceases very soon after initiation, coincident with the disappearance of C class gene expression. In male flowers, in the position normally occupied by the carpels, there is no proliferation of cells, and C class gene expression becomes undetectable as soon as the stamen primordia begin to enlarge significantly (Ainsworth et al., 1995). However, it is not clear whether the cessation of C class gene expression in the affected whorl is a cause or a consequence of the arrested development of organs in the whorl. In dioecious *Silene latifolia*, development of stamens in female flowers, and carpels in male flowers, ceases somewhat later after initiation, when anthers have differentiated from the fila-

ment, and the rudimentary gynoecium is ~3 mm long (Grant, Hunkirchen, and Saedler, 1994). C class genes are expressed in both stamens and carpels throughout subsequent development, implying that, in *Silene*, other loci are important in sex determination (Hardenack et al., 1994). Likewise, in monoecious *Zea mays*, male flower sex determination occurs via abortion of the developing gynoecium. Development of the terminal whorl is repressed by the action of the *TASSELSEED2* gene, which codes for a short-chain alcohol dehydrogenase and may program organ death shortly after initiation (DeLong, Calderon-Urrea, and Dellaporta, 1993).

In contrast to *Rumex*, *Silene*, and *Zea mays*, in *Callitriche* there is no morphological evidence of stamen or carpel initiation in female or male flowers, respectively (Leins and Erbar, 1988). It has been demonstrated in *Nicotiana tabacum* and *Arabidopsis thaliana* that corolla and androecium development can be terminated by genetic ablation prior to organ initiation with no effect on subsequent gynoecial development (Day, Galgoci, and Irish, 1995). Therefore, in tobacco and *Arabidopsis*, organ development in the first and fourth whorls is not dependent on information from adjacent second and third whorl primordia. These findings suggest that it may be possible for organ initiation to be repressed in a specific whorl without affecting the further development of the flower. A system of repressors that act before any morphological evidence of organ initiation and that do not affect later floral development may have evolved in *Hippuris* and *Callitriche* as an elaboration of the ancestral developmental program.

The prevailing model of MADS box gene expression (Fig. 1) predicts that C class gene expression alone should be sufficient to cause the production of a normal female *Callitriche* flower from a floral meristem. In male flowers, however, both B and C class gene expression is necessary. In *Antirrhinum majus*, B and C class gene expression begins when sepal primordia first appear, prior to the appearance of petal, stamen, or carpel primordia (Schwarz-Sommer et al., 1992; Bradley et al., 1993), thus it is conceivable that B class expression could occur in female *Callitriche* flowers, in spite of the lack of morphological evidence of stamen initiation. It would be interesting to determine whether the mechanism that prevents the development of stamens in female flowers acts before or after the induction of B class gene expression. Because it is possible to distinguish between male and female floral meristems at an early stage in *Callitriche* (Leins and Erbar, 1988), in situ hybridization studies of the early expression of the *Callitriche* orthologs of *Antirrhinum majus* *DEFA* and *GLO* in developing female *Callitriche* flowers could resolve this issue.

The ancestral bicarpellate bilocular gynoecium has been modified to a single unilocular carpel in *Hippuris*. The *Callitriche* gynoecium consists of an ovary with four locules, each containing a single ovule. The *Callitriche* ovary may have evolved by the division of two carpels into four locules via development of false septa, a common modification throughout the Lamiales (Wagstaff and Olmstead, 1997).

Figure 4 implies that the ancestor of *Plantago* and *Veronica* had evolved a four-parted calyx and corolla from the five-parted common ancestor with *Digitalis*. In *Ve-*

ronica and other Veroniceae this appears to have occurred by the approach and fusion of two upper petals and simultaneous disappearance of the upper sepal (Kampny, Dickinson, and Dengler, 1993; Hufford, 1995; Endress, 1997). It is important to note that some members of tribe Veroniceae have five-parted perianth whorls (Hong, 1984). Thus, tetramerous flowers of *Plantago* and *Veronica* may have evolved in parallel and the underlying genetic mechanism may be different. Further systematic sampling could help resolve whether there is a single origin of four-parted flowers in this portion of the phylogeny.

Stamen number has been reduced to two in *Veronica* and throughout most of the Veroniceae. In *Plantago* the number is four, similar to the basal members of tribe Veroniceae suggested by Hong (1984). *Plantago* flowers are reduced in size and are radially symmetrical in contrast to the zygomorphy of the hypothesized ancestor. In *Antirrhinum majus*, mutations in the genes *CYCLOIDEA* and *DICHOTOMA* cause the development of radially symmetrical flowers (Luo et al., 1996). It would be interesting to determine whether orthologs of these genes influence the development of actinomorphic flowers in *Plantago*. As in wind-pollinated *Hippuris* and *Callitriche*, ovule number in many species of *Plantago* has been reduced to one per locule (Sharma, Koul, and Koul, 1992).

Summary—Advances in our understanding of the genes involved in floral ontogeny, and in phylogenetic relationships among plant species, have encouraged the study of the genetic mechanisms that cause the changes in floral morphology seen over evolutionary time. Most developmental genetics studies have focused on distantly related model organisms for which phylogenetic relationships and ancestral character states are not clear. We have identified a clade of plants closely related to the model organism *Antirrhinum majus* that exhibit profound morphological variation. We can be confident of our reconstruction of ancestral character states due to shared ancestral characters among the ingroup taxa. We can infer, for example, that the ancestral developmental program that has been modified to create the variety of floral morphologies in the Scroph II clade was a program very much like that of *Antirrhinum majus*. This is critical in understanding the nature and direction of the character transformations that have occurred during evolution.

Within the Scroph II clade, some of the most distinctive, yet repeatedly occurring, characteristics of angiosperm evolution are exhibited. These include changes in floral symmetry and pollination syndrome, modifications in the number of organs in a whorl, and in the number of whorls in a flower, and the evolution of dicliny. By determining the specifics of these transformations a general model for major issues in angiosperm evolution may be built.

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