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Diversity of *cycloidea*-like Genes in Gesneriaceae in Relation to Floral Symmetry

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Homology assessment of *cycloidea*-like genes was carried out in Gesneriaceae, a predominantly zygomorphic family in which several independent reversals to actinomorphy have occurred, as a basis for further investigation of the control and evolution of floral symmetry. Phylogenetic analysis of Gesneriaceae *cycloidea* (*Gcyc*) suggests that independent duplication and gene loss events have occurred during the evolution of this family after the split from Scrophulariaceae. Comparison of *Gcyc* sequences between zygomorphic and naturally occurring actinomorphic taxa does not suggest that reversals to actinomorphy were caused in these cases by loss of function of *cyc*-like genes. Examination of floral development in the nearly actinomorphic *Ramonda myconi* did not reveal any evidence of residual unequal dorso-ventral differentiation indicative of expression of *Gcyc*. This suggests that *Gcyc* may be expressed before primordia initiation in *R. myconi*, or may have additional functions not directly related to floral symmetry.
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Key words: *cycloidea*, developmental gene, floral symmetry, Florist's Gloxinia, *Gcyc*, Gesneriaceae, *Ramonda myconi*, *Sinningia speciosa*.

INTRODUCTION

The genetics of the control of floral symmetry have been extensively examined in *Antirrhinum majus* L. (Scrophulariaceae) (Carpenter and Coen, 1990; Luo *et al.*, 1996, 1999; Almeida *et al.*, 1997). Wild type *Antirrhinum* flowers are strongly zygomorphic with dorso-ventral differentiation most pronounced in the petals and stamens. The organs within each whorl are divided into three types: dorsal, lateral and ventral, based on their respective position from the single axis of symmetry (Coen and Meyerowitz, 1991). In *Antirrhinum*, as in most zygomorphic members of the Lamiales *sensu lato*, bilateral symmetry is characterized by the retardation and subsequent abortion of the dorsal stamen (Luo *et al.*, 1996; Endress, 1997). Actinomorphic mutants of *Antirrhinum*, referred to as peloric mutants, have a ventralized phenotype, i.e. all organ members resemble the ventral member of the wild type. Frequently, the organs in the three outer whorls are more numerous in the peloric *Antirrhinum* (six instead of five members in each whorl) (Luo *et al.*, 1996). Two closely related nuclear genes *cycloidea* (*cyc*) and *dichotoma* (*dich*) expressed in the dorsal region of the flower and controlling dorso-ventral organ differentiation have been isolated (Carpenter and Coen, 1990; Luo *et al.*, 1996, 1999). Of the two, *cyc* has the greatest effect on phenotype although loss of function of both genes is required for a fully radially symmetric development (Luo *et al.*, 1996, 1999; Almeida *et al.*, 1997). *Cyc* has an uninterrupted reading frame encoding a putative protein of 286 amino acids (Luo *et al.*, 1996), and shares certain motifs with DNA binding proteins in rice suggesting that it is involved in transcription regulation (Cubas *et al.*, 1999*b*).

The first naturally occurring peloric mutants to be characterized genetically are from polymorphic populations of *Linaria vulgaris* L. (Scrophulariaceae) (Cubas *et al.*, 1999*a*). Morphologically, the peloric *Linaria* resembles the radial *Antirrhinum* mutants in many respects by having five rather than four functional stamens, and a ventralized phenotype for both petals and stamens. The homologue of *Antirrhinum cyc* in *Linaria*, *Lcyc* was isolated and implicated in the control of floral symmetry. However, loss of function of *Lcyc* in peloric *Linaria* was not caused by a genomic mutation but correlated with extensive methylation (Cubas *et al.*, 1999*a*).

It is still unknown to what extent *cyc*-like genes, defined by certain putatively functional motifs (Cubas *et al.*, 1999*b*), are involved in the control of zygomorphy in more distantly related taxa. It has been proposed that in the Asteridae alone zygomorphy has evolved independently at least eight times, although this scenario assumed equal probability of transformation from one state to another (Donoghue *et al.*, 1998). The predominantly zygomorphic Lamiales *s.l.*, comprising large families such as Scrophulariaceae, Acanthaceae, Bignoniaceae, Lamiaceae and Gesneriaceae, is believed to be ancestrally monosymmetric so that actinomorphic taxa in this group have evolved secondarily (Endress, 1997). It is therefore likely that similar genes controlling floral symmetry are implicated within this taxonomic group.

Among the Lamiales *s.l.*, the highest proportion of genera with actinomorphic members is found within the

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Gesneriaceae (Endress, 1997). Approximately ten of the 120 genera have some or all species which possess (nearly) actinomorphic flowers with five fertile stamens equal in number to the corolla lobes (Burtt, 1970, 1994; Endress, 1997), and are therefore similar in this respect to loss of function *cyc* mutants in *Antirrhinum*. These genera belong to both subfamilies Gesnerioideae and Cyrtandroideae (Burtt and Wiehler, 1995), and have probably evolved this trait independently (Cronk and Möller, 1997; Möller *et al.*, 1999). In addition, peloric mutants with similar characteristics to those of *Antirrhinum* and *Linaria* are known in cultivars of *Saintpaulia* H. Wendl. and *Sinningia speciosa* hybrids (Citerne and Cronk, 1999). This is suggestive of a genetic control of zygomorphy in Gesneriaceae comparable to the model established in *Antirrhinum*. It may therefore be possible to speculate whether naturally occurring actinomorphic taxa in Gesneriaceae have evolved by loss-of-function mutation of a *cyc*-like gene.

A putative single copy homologue of *cyc*, referred to as Gesneriaceae *cycloidea* (*Gcyc*), has been found in various taxa from both Gesneriaceae subfamilies (Möller *et al.*, 1999). However, *Gcyc* sequence characteristics from three actinomorphic taxa (*Ramonda myconi* Rchb., *Ramonda nathaliae* Pancic & Petrovic, and *Conandron ramondioides* Siebold & Zucc.) had no features indicative of loss of function (Möller *et al.*, 1999). This could suggest that either this phenotypic change was not caused by a loss-of-function mutation in *Gcyc*, or that *Gcyc* is not analogous to *cyc* in function. In addition, *cyc*-like genes are known to belong to a family of genes (Cubas *et al.*, 1999*b*; Vieira *et al.*, 1999). In the African *Streptocarpus*/*Saintpaulia* clade, two copies of *Gcyc* have been isolated (Möller *et al.*, 1999). It is therefore important to ascertain homology relationships between *cyc* and *cyc*-like sequences from Gesneriaceae.

If *Gcyc* is found to be homologous to *cyc*, then it is possible that its expression may only be down-regulated in these actinomorphic taxa. As a result, residual zygomorphy may still be visible early in floral development. Floral organ ontogeny is therefore examined here in the European *Ramonda myconi*, and contrasted with the closely related *Haberlea fernandi-coburgii* Urum. (Möller *et al.*, 1999), the flowers of which differ distinctly at maturity in their shape and pattern of symmetry from those of *R. myconi* (Fig. 1). Although *R. myconi* flowers are nearly radially symmetric and possess five fertile stamens, certain features such as a pronounced downward deflection of the anther cone and style are suggestive of unequal development that may have a genetic and/or gravitropic basis. The assessment of diversity of *cyc*-like genes in Gesneriaceae and their relationship with known Scrophulariaeceae sequences may help set a framework for understanding the control and evolution of floral symmetry in this family.

MATERIALS AND METHODS

Molecular study

Plant material and taxon data set. Sequence data were either retrieved from GenBank (Scrophulariaceae), available from previously sequenced Gesneriaceae taxa (Möller *et al.*, 1999) or newly obtained (Table 1). Gesneriaceae plant material was taken from living plants cultivated at the Royal Botanic Garden Edinburgh (RBGE). A *Sinningia speciosa* hybrid peloric mutant was acquired from the Dobbies Ltd. nursery, Edinburgh, UK. For comparison, *Antirrhinum majus* L., of horticultural origin, was included

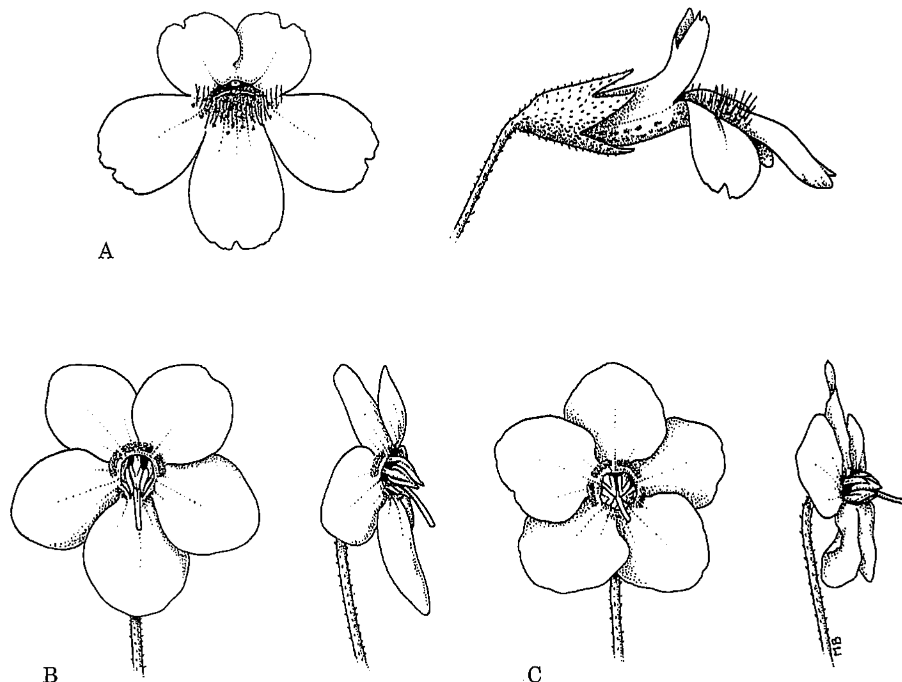

FIG. 1. Front and side view of *Haberlea fernandi-coburgii* (A) and *Ramonda myconi* flowers (B, C), with the two most common floral orientations represented.

TABLE 1. *Accessions and sequences from 13 Gesneriaceae and two Scrophulariaceae taxa examined*

Family: subfamily	Taxon	RBGE taxon accession no.	Gene name	Reference	GenBank accession no.	Origin; distribution
SCROPHULARIACEAE	*Antirrhinum majus* L.	—	***cycloidea***	Luo *et al.*, 1996	Y16313	—
		—	*dichotoma*	Luo *et al.*, 1999	AF199465	—
		—	*cycloidea**	This study	AF208341	Horticultural origin
			dichotoma*	This study	AF208494	Horticultural origin
	Linaria vulgaris L.	—	***Lcyc***	Cubas, Vincent & Coen, 1999	AF161252	—
GESNERIACEAE: GESNERIOIDEAE	*Sinningia schiffneri* Fritsch	1978 1514	***Gcyc1***	Möller *et al.*, 1999	AF208327	South America
	Sinningia speciosa hybrid peloric mutant	—	***Gcyc1****	This study	AF208319	Horticultural origin
GESNERIACEAE: CYRTANDROIDEAE	*Conandron ramondioides* Siebold & Zucc.	1969 1267	*Gcyc1*	Möller *et al.*, 1999	AF208329	Japan
		1969 1267	***Gcyc2****	This study	AF208316	
		1969 1267	***Gcyc1****	This study	AF208321	
	Haberlea fernandi-coburgii Urum.	1969 4617B	***Gcyc2****	This study	AF208317	Bulgaria
		1969 4617B	***Gcyc1****	This study	AF208322	
	Haberlea rhodopensis Friv. Symond's form	1975 4106	*Gcyc1*	Möller *et al.*, 1999	AF208330	N.E. Greece, C. and S.C. Bulgaria
	Jankaea heldreichii Boiss.	1979 1939	***Gcyc1***	Möller *et al.*, 1999	AF208332	Greece; Mt. Olympus
	Primulina tabacum Hance	1995 1540	*Gcyc1*	Möller *et al.*, 1999	AF208328	China; Gnangdong, Lian River
		1995 1540	***Gcyc1****	This study	AF208320	
	Ramonda myconi Rchb.	1971 1477A	*Gcyc1*	Möller *et al.*, 1999	AF208331	France; Pyrenees
		1971 1477A	***Gcyc2****	This study	AF208318	
		1971 1477A	***Gcyc1****	This study	AF208323	
	Saintpaulia velutina B.L. Burtt	1987 2179	*Gcyc1B*	Möller *et al.*, 1999	AF208333	Tanzania; W Usambara Mts, Balangai
		1987 2179	*Gcyc1A*	This study	AF208337	
		1997 0100	***Gcyc1B****	This study	AF208324	
		1997 0100	***Gcyc1A****	This study	AF208325	
	Streptocarpus dunnii Hook.	1994 1745	***Gcyc1B***	Möller *et al.*, 1999	AF208335	South Africa; Swaziland, N. Mbabane
		1994 1745	***Gcyc1A***	This study	AF208339	
	Streptocarpus holstii Engl.	1959 2272	***Gcyc1B***	Möller *et al.*, 1999	AF208334	Tanzania; E. Usambara Mts
		1959 2272	***Gcyc1A***	This study	AF208338	
	Streptocarpus primulifolius Gand.	1991 2192	***Gcyc1B***	Möller *et al.*, 1999	AF208336	South Africa; E. Cape, Igoda River
		1991 2192	***Gcyc1A***	This study	AF208340	
	Streptocarpus rexii Lindl.	1991 2547	***Gcyc1B****	This study	AF208326	South Africa; E. Cape, Transkei, Port St Johns

Asterisks (*) indicate sequences obtained from cloned PCR products. Other *Gcyc* sequences were obtained by direct sequencing of PCR products. Gene names in bold indicate sequences used in the phylogenetic analysis (Fig. 3).

in the molecular study. Species and accessions of Gesnericaceae taxa sequenced from cloned PCR products were identical to those from the study of Möller *et al.* (1999) to facilitate comparison of sequences, with the exception of *Saintpaulia velutina* B.L. Burtt (different accession), *Streptocarpus rexii* Lindl., *Haberlea fernandi-coburgii* Urum., and the *Sinningia speciosa* hybrid peloric mutant. Two naturally occurring actinomorphic species from Europe, *Ramonda myconi*, and Asia, *Conandron ramondioides* Siebold. & Zucc., were included in the taxon set.

DNA extraction and PCR conditions. Small scale extraction of total genomic DNA was carried out from fresh leaf material for all taxa following a modified CTAB procedure of Doyle and Doyle (1987). When required, further purification of total genomic DNA was carried out using QIAquick PCR Purification Kit (QIAGEN GmbH, Hilden, Germany).

Approximately 70 % of the uninterrupted open reading frame (ORF) of *cyc*-like sequences was amplified with the polymerase chain reaction (PCR) using the forward *Gcyc*FS (5′-ATG CTA GGT TTC GAC AAG CC-3′) and reverse *Gcyc*R (5′-ATG AAT TTG TGC TGA TCC AAA ATG-3′) primers (Möller *et al.*, 1999). Less stringent PCR conditions were utilized in comparison to the Möller *et al.* (1999) study to maximize chances of amplification of *cyc*-like sequences in Gesneriaceae taxa and *Antirrhinum*. The PCR reaction mixture was as follows and made up to 25 µl with sterile distilled water: 3 µl $MgCl_2$ at 25 mM (Promega, Madison, WI, USA), 2·5 µl 10× $MgCl_2$-free thermophilic DNA Polymerase Buffer (Promega, Madison, WI, USA), 2·5 µl of a mix of each dNTP (final concentration 2 mM) (Boehringer Mannheim, GmbH, Germany), 0·5 µl *Taq* DNA Polymerase (5 U μl^{-1}) (Promega, Madison, WI, USA), 1·25 µl of each primer at 10 µM (Operon, DNA Services, Southampton, UK), and 1·2 µl aliquots of total genomic DNA. PCR cycle parameters consisted of an initial denaturation step at 96°C for 2 min, followed by 32 cycles of denaturation at 95°C for 30 s, primer annealing at 48°C for 30 s, and primer extension at 72°C for 3 min, terminated by a final extension step at 72°C for 4 min. PCR products (20 µl) were separated on a 2 % agarose gel (Gibco BRL, Life Technologies, Paisley, UK) in 1× TBE buffer for 2–3 h at 90 V with size estimated using a 123 bp DNA ladder (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). Bands were excised from the gel and cleaned with QIAEX II Agarose Gel Extraction Kit (QIAGEN GmbH, Hilden, Germany).

Cloning and sequencing. PCR fragments were either sequenced directly or after cloning (Table 1). To distinguish between the two *Gcyc* sequences in the *Streptocarpus*/*Saintpaulia* clade, the forward primers *Gcyc*FL (5′-CAC CCG GAT TCG AGA AAA TC-3′) and *Gcyc*FK (5′-CCC CCA GAT TyC AAG AGG AA-3′) and their respective complement primers were used in *Saintpaulia*/*Streptocarpus holstii*, and the reverse primers *Gcyc*RC (5′-TGA CTA TAG GAG ATG GAT TAG-3′) and *Gcyc* RD (5′-CAT yAG GAG ACG sTA GGG C-3′) in *S. dunnii* and *S. primulifolius*. PCR and direct sequencing were carried out as described in Möller *et al.* (1999).

For other taxa, to ensure that single copies were sequenced, the PCR products were isolated and cloned into the pCR-4 plasmid vector using the TOPO® TA Cloning® Kit (Invitrogen, Carlsbad, CA, USA). One clone from each excised band was sequenced using an ABI PRISM Big Dye terminator cycle-sequencing ready reaction kit (Perkin Elmer, Applied Biosystems Division, Foster City, CA, USA) and analysed on an Applied Biosystems model 377 Automatic DNA sequencing system. The forward and reverse M13 priming sites of the pCR-4 vector were used for sequencing.

Sequence analysis. Initial sequence similarity to *cyc* was confirmed by BLAST search. Multiple nucleotide sequence alignment was performed using Clustal X version 1.8 (Thompson *et al.*, 1997). An amino acid matrix was generated from the aligned nucleotide sequence matrix with MacClade version 3.01 (Maddison and Maddison, 1992). The nucleotide alignment was optimized manually using the amino acid matrix. Nucleotide and amino acid sequence divergence among taxa was calculated using the DISTANCE MATRIX option in PAUP version 4.0b2a (Swofford, 1998), based on the entire alignment matrix. The G + C content and transitions/tranversions ratio were obtained using MacClade 3.01.

Phylogenetic analysis. Phylogenetic analysis of the aligned nucleotide data matrix was carried out using PAUP version 4.0b2a. In all analyses characters were unordered and of equal weight, gaps were treated as missing. Initially, all sequences available were included in the analysis. For simplicity, only a subset of the sequences available are presented here in the phylogenetic analysis representing all taxa and *cyc*-like sequence types. In preference, the cloned sequences obtained were utilized in the analysis with the exception of *Antirrhinum cyc* (see Results), available from GenBank. The number of taxa in the data matrix required a heuristic search strategy to generate the most parsimonious trees. One hundred starting tree replicates generated by RANDOM addition followed by TBR (tree bisection-reconnection) branch swapping on the best trees was carried out. The options COLLAPSE (max.), MULTREES on and ACCTRAN optimization were selected. The effect of alignment variable regions on topology and branch support was investigated by sensitivity analysis excluding ambiguous regions. No differences in taxic relationships or their support were observed. Thus, all characters were included in the analysis. The amino acid matrix was also analysed by PAUP 4.0b2a with a heuristic maximum parsimony search described above. Bootstrap analyses (Felsenstein, 1985) were performed using PAUP 4.0b2a, set to HEURISTIC search option and SIMPLE addition sequence. Bootstrap values were calculated using 1000 replicates. Decay indices (DI) (Bremer, 1988; Donoghue *et al.*, 1992) of individual clades were obtained by comparing the strict consensus of all equal length trees up to 12 steps (nucleotide matrix) or six steps (amino acid matrix) longer, using SIMPLE addition sequence and TBR

swapping in PAUP 4.0b2a. Other descriptive statistics, i.e. consistency index (CI) (Kluge and Farris, 1969), retention index (RI) (Farris, 1989), rescaled consistency index (RC) and homoplasy index (HI) (Swofford, 1998) were calculated with PAUP 4.0b2a.

Developmental study

Floral bud development of *Haberlea fernandi-coburgii* (RBGE accession number 1969 4617B) and *Ramonda myconi* (RBGE accession number 1971 1477A) were examined by scanning electron microscopy (SEM). Buds at different development stages were collected and fixed in Kopenhagen Mixture (3·5 parts methyl alcohol, 5·5 parts water, 0·5 parts glycerol). These were then dehydrated through an ethanol/acetone series into 100 % acetone dried with a molecular sieve. Material was then dried in an Emitech K850 critical point dryer. Desiccated buds were dissected and mounted with Acheson Electrodag 1415M (Agar Scientific Ltd., Stansted, UK) on Agar Scientific 1·25 cm aluminium stubs. These were coated with gold-palladium using an Emscope SC500 sputter coater. Buds were examined with a Zeiss DSM962 SEM at a working distance of 8–10 mm at 5 kV.

RESULTS

Sequence analysis

The aligned nucleotide matrix from the 494-656 base pair (bp) long sequences of the diverse taxa was 842 characters in length. Alignment required the insertion of 117 indels with gap sizes varying in multiples of three from three to 75 characters, with the sole exception of a single base deletion found in the *Sinningia speciosa* peloric mutant sequence (Fig. 2). The aligned matrix was characterized by conserved regions found across the ORF separated by highly variable regions which apparently evolved by insertion/deletion events as well as base substitutions (aligned matrix available from authors on request and has been deposited in GenBank). In the latter regions, alignment was difficult particularly between Scrophulariaceae and Gesneriaceae sequences. Of all the characters in the nucleotide matrix, 43·0 % were constant and 39·6 % were informative. Translation to amino acids resulted in a 280 codon matrix, with a slight increase in the proportion of phylogenetically informative sites (47·5 %). The characteristics of both data matrices are summarized in Table 2.

Two *cyc*-like sequences were detected for all taxa examined in this study with the exception of *Sinningia speciosa* hybrid peloric mutant, *Primulina tabacum* and *Streptocarpus rexii*, where only one sequence was detected. Comparison of the cloned sequences with pre-existing sequences obtained by direct sequencing of PCR products (Möller *et al.*, 1999) revealed that these, with the exception of *Ramonda Gcyc2*, *Haberlea Gcyc2* and *Conandron Gcyc2*, were very similar to known *cyc*-like sequences from the same taxa. Percent sequence divergence was low between *Ramonda Gcyc1* AF208331—AF208323 (0·94 %), *Primulina Gcyc1* AF208328—AF208320 (0·65 %) and *Saintpaulia Gcyc1A* AF208337—AF208325 (0·34 %), whereas sequences were identical between *Conandron Gcyc1* AF208329—AF208321 and *Saintpaulia Gcyc1B* AF 208333—AF208324. Sequence divergence was also low between the two closely related *Haberlea* species: *H. fernandi-coburgii Gcyc1*—*H. rhodopensis Gcyc1* (0·31 %). Comparison of the two *Antirrhinum cyc*-like sequences from this study with sequences available from GenBank revealed low sequence divergence between one sequence (AF208341) and the first published *cycloidea* sequence (1·82 %), whereas the other sequence (AF208494) was found to be similar to *dichotoma* (Luo *et al.*, 1999), with only 0·52 % sequence divergence. Pairwise sequence divergence between respective *Ramonda Gcyc2*, *Conandron Gcyc2* and *Haberlea Gcyc2* paralogues was much higher (24·7–27·0 %).

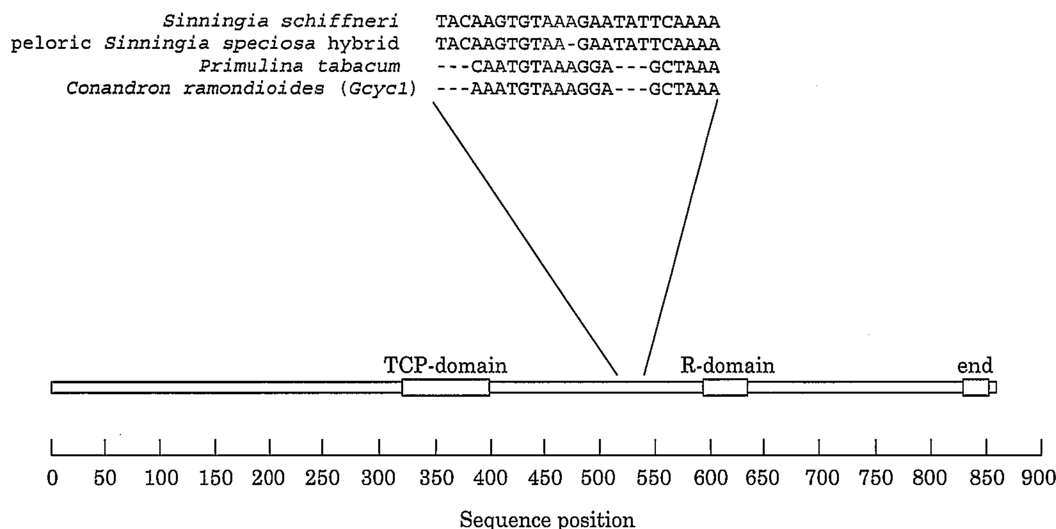

FIG. 2. Comparison of sequences of *Sinningia speciosa* hybrid peloric mutant, *Sinningia schiffneri*, *Primulina tabacum* and *Conandron ramondioides Gcyc1* showing the single base deletion in the peloric *Sinningia* clone and its location with respect to a diagrammatic representation of the *Antirrhinum cyc* locus.

TABLE 2. *Nucleotide and amino acid sequence characteristics of 22 cyc-like sequences from 12 Gesneriaceae and two Scrophulariaceae taxa*

Parameter	Nucleotide matrix	Amino acid matrix
Length range (total), bp	494–656	164–218
Length mean (total), bp	596·5	198·2
Length range (Gesneriaceae), bp	530–656	176–218
Length mean (Gesneriaceae), bp	603·8	200·6
Length range (Scrophulariaceae), bp	494–578	164–192
Length mean (Scrophulariaceae), bp	550	182·7
Aligned length, bp	842	280
G + C content range (%)	38·49–45·12	—
G + C content mean (%)	42·52	—
Number of indels (total)	117	117
Size of indels*	3–75	1–25
Number of constant sites (%)	42·99	34·64
Number of variable sites (%)	57·01	65·36
Number of autapomorphic sites (%)	17·46	17·86
Number of informative sites (%)	39·55	47·50
Sequence divergence (Gesneriaceae)	1·5–29·1	3·0–39·9
Sequence divergence (Scrophulariaceae)	12·3–24·2	10·5–26·5
Sequence divergence (Gesneriaceae/Scrophulariaceae)	27·8–37·0	27·0–47·2
Transitions (minimum)	320	—
Transversions (minimum)	250	—
Transitions/transversions†	1·28	—

* With the exclusion of a single base deletion in the *Sinningia speciosa* clone

† based on number of unambiguous transitions and transversions.

Sequences from naturally occurring actinomorphic taxa (i.e. *Ramonda myconi* and *Conandron ramondiodes*) were not found to have any frameshift mutations (all indels were in multiple of three nucleotides) or stop codons. However, the sequence isolated from the *Sinningia speciosa* hybrid peloric mutant· had a single adenine base deletion at position 242 (position 315 in the aligned matrix, not shown) resulting in an apparent shift in the reading frame, as evident compared with sequences from *Sinningia schiffneri*, *Primulina tabacum* and *Conandron ramondioides Gcycl* (Fig. 2).

Phylogenetic analysis

Parsimony analysis of the reduced nucleotide data matrix resulted in a single most parsimonious tree of 974 steps in length (CI = 0·73, HI = 0·27, RI = 0·76, RC = 0·55). The tree was rooted using midpoint rooting. In the rooted phylogram the Gesneriaceae sequences form a strongly supported monophyletic group [bootstrap value (BS) = 100 %, decay index (DI) ⩾ + 12] (Fig. 3). Different clades are formed within the Gesneriaceae clade, one of which comprises one of the sequences (*Gcyc2*) of the European *Ramonda myconi* and *Haberlea fernandi-coburgii*, and the Asian *Conandron ramondioides* (BS = 100, DI ⩾ + 12) and is sister to the rest of the Gesneriaceae sequences. The resolution within this clade, however, is poor (BS = 58 %, DI = +1). The support for the position of the South American *Sinningia* spp. clade (BS = 100, DI ⩾ + 12) within and basal to the clade comprising the rest of the Gesneriaceae sequences is moderate (BS = 65, DI = +2). The European and Asian taxa form well supported sister clades (BS = 81, DI = +3 and BS = 96, DI = +5, respectively), and the latter is sister to all sequences of the African *Streptocarpus/Saintpaulia*. The *Streptocarpus/Saintpaulia* clade forms two highly supported monophyletic groups (BS = 97, DI = +5 and BS = 96, DI = +6) each with one sequence from each taxon and with identical topology.

Phylogenetic analysis including all sequences available resulted in one most parsimonious tree of 991 steps (CI = 0·72, HI = 0·28, RI = 0·84, RC = 0·61) (tree not shown). This analysis did not provide any additional informative relationships other than confirming the similarity between certain sequence pairs from the same taxa as shown in pairwise sequence divergence.

The topology described above was broadly recovered in the amino acid matrix analysis, which resulted in two most parsimonious trees of 481 steps (CI = 0·82, HI = 0·18, RI = 0·79, RC = 0·65). The topology of the strict consensus tree was identical to the nucleotide tree with the exception of the *Ramonda Gcyc2*, *Haberlea Gcyc2* and *Conandron Gcyc2* clade forming a polytomy (tree not shown).

Differential substitution rates of *cyc*-like genes in actinomorphic and zygomorphic taxa was investigated by Möller *et al.* (1999) in part by quantifying the partition of variation in the first and second *vs.* the third codon position at the terminal branches of the tree. Similar values between the three positions are consistent with a lack of functional constraint. Further examination was necessary for the new *cyc*-like sequence (*Gcyc2*) in the actinomorphic *Ramonda* and *Conandron* and the zygomorphic *Haberlea*. A procedure similar to that of Möller *et al.* (1999) was carried out with this phylogenetic analysis. First and second codons were plotted against the third codon (Fig. 4). Results show that the actinomorphic taxa are no different from zygomorphic taxa, both having a higher rate of substitution at the third codon position as would be expected for a functionally constrained gene.

Floral development

As both *cyc*-like genes in *Ramonda myconi* are likely to be functional, it may be that residual zygomorphy is visible during floral development. Comparison of floral ontogeny at stage 6 (initiation of petal and stamen primordia; Harrison *et al.*, 1999) in the closely related taxa *Haberlea fernandi-coburgii* and *Ramonda myconi* revealed that this difference is evident early in development (Fig. 5). In *Haberlea*, the dorsal stamen, lying in the plane of symmetry, is initiated but its development is arrested early (Fig. 5A, C, E). The corolla primordia initiate as a ring, and unequal development is apparent at a later stage than within the androecial whorl. *Ramonda* shows no sign of residual zygomorphy at these stages of development (Fig. 5B, D, F). The five petals and stamens develop equally after initiation, with the corolla primordia arising separately, implying that the highly reduced corolla tube is formed later in development.

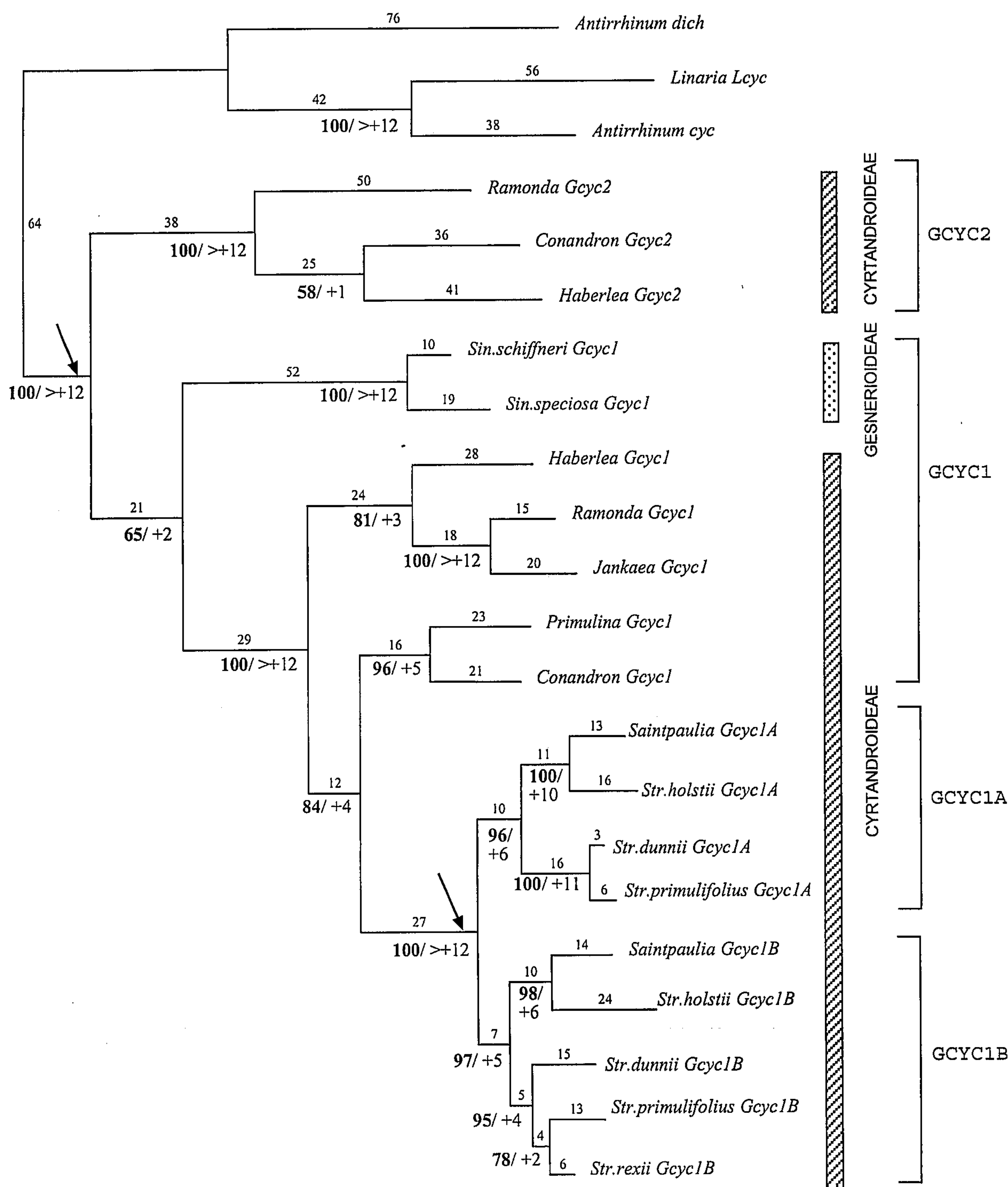

FIG. 3. Midpoint rooted phylogram of the most parsimonious tree of 974 steps based on the nucleotide data set of 22 sequences. Bootstrap values of 1000 replicates (bold) and decay indices as the number of steps required to collapse the corresponding clade, are given below the branch. Branch lengths are given above branches. Arrows indicate duplication events. *Sin*, *Sinningia*; *Str*, *Streptocarpus*.

DISCUSSION

Gcyc *diversity in Gesneriaceae*

The nuclear gene *Gcyc* was initially believed to be putatively single copy (*sensu* Doyle and Doyle, 1999). However, Möller *et al.* (1999) indicated the presence of a closely related gene in *Streptocarpus* and *Saintpaulia*. This study unequivocally reveals that several *cycloidea* paralogues exist in Gesneriaceae. Under less stringent PCR conditions, more than one sequence type was isolated in some taxa (Fig. 3). Homology assessment from phylogenetic analysis suggests that *Gcyc* belongs to a small gene family which has undergone several duplication and putative gene loss events during the evolution of the Gesneriaceae. The monophyly

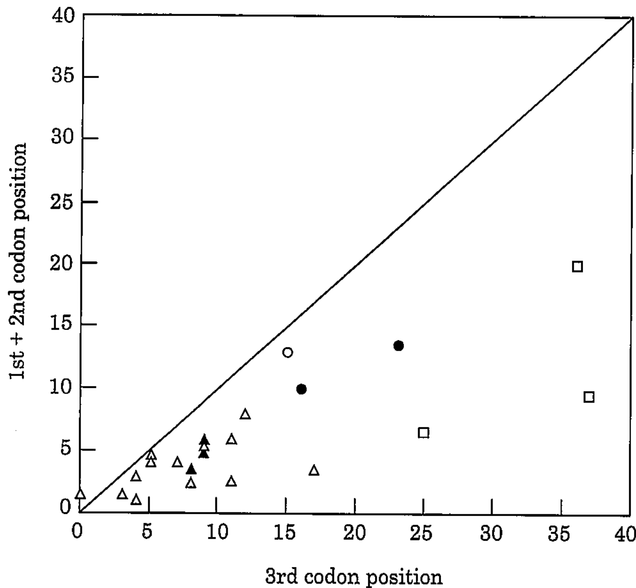

FIG. 4. Relation between branch lengths for actinomorphic (*Ramonda Gcyc2*, *Conandron Gcyc2* (⊖), *Ramonda Gcycl*, *Conandron Gcycl*, peloric *Sinningia speciosa* hybrid (▲)) and zygomorphic (Scrophulariaceae (□), *Haberlea Gcyc2* (○), other Gesneriaceae sequences (△)) taxa, of trees based on the first and second or the third codon position of the nucleotide data set.

of the Gesneriaceae sequences suggests that the *Gcyc* gene family has probably evolved from a single copy ancestor after the split between Gesneriaceae and Scrophulariaceae. With the primers designed previously (Möller *et al.*, 1999), two *Antirrhinum cyc*-like genes were isolated here, suggesting that duplication of *cyc*-like genes may be a common and iterative phenomenon. Two separate duplication events of *cyc*-like genes were apparent in the Gesneriaceae taxa examined. One ancient duplication has seemingly occurred prior to the split between the predominantly New World Gesnerioideae and Old World Cyrtandroideae, as suggested by the sister group relationship of the '*Ramonda Gcyc2*, *Conandron Gcyc2*, *Haberlea Gcyc2*' clade and the clade within which the *Sinningia* spp. sequences occur (Fig. 3). Within the former clade, although the poorly supported resolution in the nucleotide analysis is not congruent with other phylogenetic estimates e.g. ITS, *trn*L + F (Möller *et al.*, 1999), the three sequences are believed to be homologous and are referred to as the gene *Gcyc2* (Fig. 3). Poor resolution may be accounted for by inadequate sampling. *Gcyc2* was not found in the other Gesneriaceae taxa, suggesting it has either been lost repeatedly, has undergone gene conversion or is as yet undetected. The relationship between the remaining sequences from South American, European and Asian Gesneriaceae taxa is congruent with ITS and *trn*L + F phylogenies (Möller *et al.*, 1999) and these sequences are therefore believed to be homologous. Consequently, these are referred to as *Gcycl* (Fig. 3). The identical topology and short branch lengths of the two clades of the African *Streptocarpus*/*Saintpaulia* species is suggestive of a second, more recent duplication. These two genes are both homologous to *Gcycl*, as they have seemingly evolved from this gene, and are referred to as *Gcycl A* and *Gcycl B* (Fig. 3).

Putative function of Gcyc

Although it is possible that the frameshift mutation in the peloric *Sinningia Gcycl* sequence is a PCR artefact, it is interesting as it is situated before the R domain, a conserved and putatively functional region (Cubas *et al.*, 1999*b*), and would, if genuine, suppress gene function. As *Gcycl* appears to be single copy in *Sinningia* spp., it is possible that this gene is implicated in the control of floral symmetry in Gesneriaceae. Further work is needed to determine whether this mutation is found in other peloric *Sinningia* and whether it is the cause of phenotypic change in the peloric *Sinningia* cultivar or is a by-product of the loss of functional constraint.

Genetic change in naturally actinomorphic Gesneriaceae

It appears from the above that at least one member of the *Gcyc* gene family is involved in the establishment of zygomorphy in Gesneriaceae. Although only approximately 70 % of the ORF of *Gcyc* genes was isolated, various lines of evidence suggest that both *Gcycl* and *Gcyc2* seem functional in *Ramonda* or *Conandron*, as indicated by the absence of frameshifts and premature stop codons, and the apparent constraints to substitutions at the first and second *vs.* third codon positions. This is consistent in the case of the former with the secondary evolution of zygomorphy in *Jankaea* from within the *Ramonda* clade (Möller *et al.*, 1999). It has been suggested that modifications in the promoters of transcription regulators are an important source of evolutionary change (Baum, 1998; Doebley and Lukens, 1998; Wang *et al.*, 1999). It is therefore possible that down-regulation of *Gcyc* genes is responsible for actinomorphy in these taxa, although one cannot rule out changes upstream or downstream of the *Gcyc* genes' expression pathway. No residual zygomorphy was found early in the floral development of *R. myconi* (see also Endress, 1997), suggesting that *Gcyc* expression may be restricted to the first stages of floral development, before organ initiation. Alternatively, expression may be restricted to late stages of floral development being responsible for the asymmetric pigmentation pattern or the 'nodding' posture of the flower (Fig. 1). This latter feature is altered in the peloric mutants of *Sinningia* which have more upright flowers than the wild type. Although *cyc* function is believed to be restricted to the determination of floral shape, additional functions such as the control of flower orientation have been suggested (Baum, 1998; Donoghue *et al.*, 1998). Interestingly, *R. myconi* is known to be variable in this feature by having either one or two dorsal petals (Fig. 1). Investigation of the role and the expression pattern of *Gcyc* genes in naturally occurring actinomorphic Gesneriaceae taxa such as *Ramonda* and *Conandron* will provide further insight into the importance of these genes in floral development.

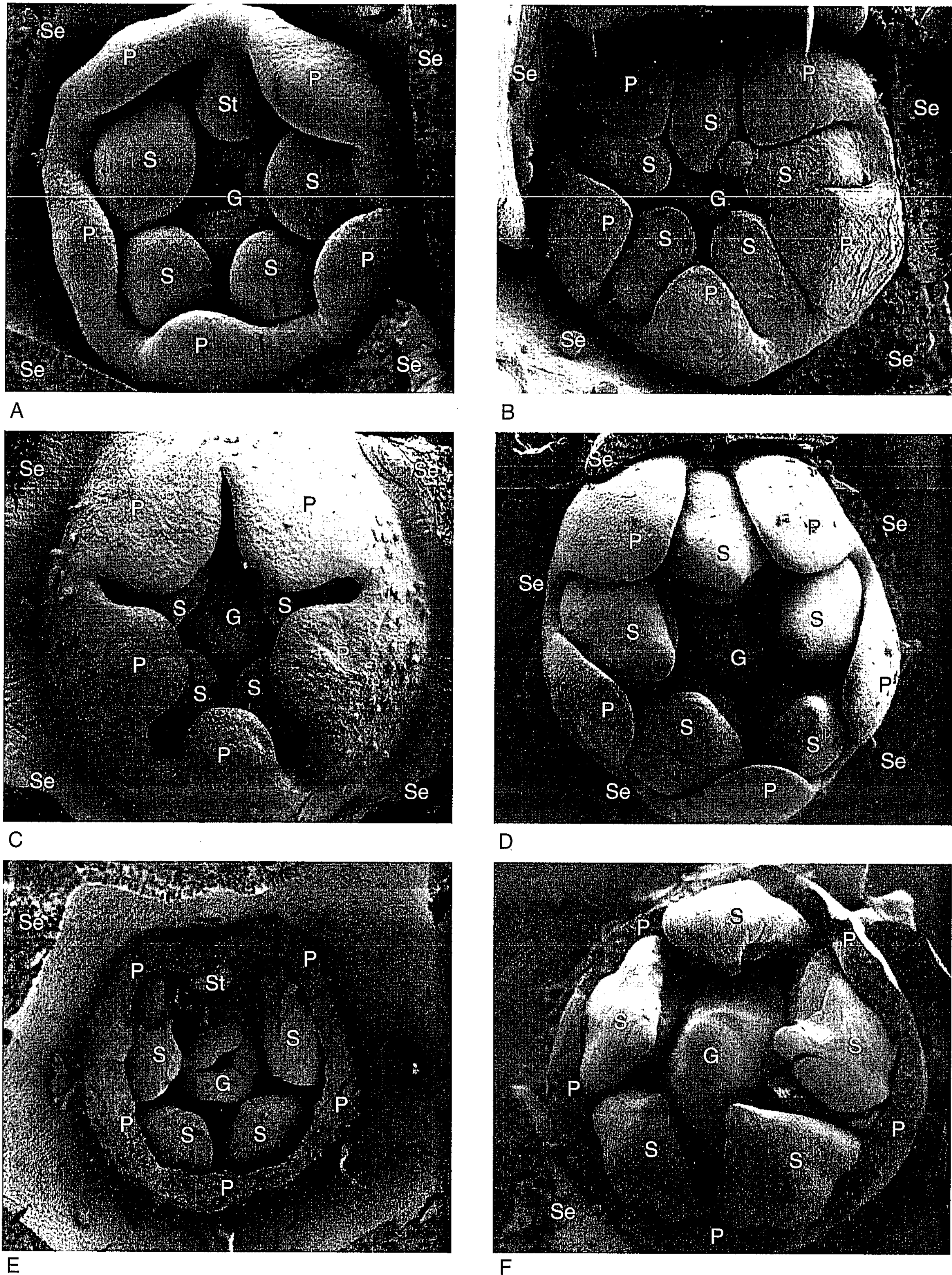

FIG. 5. Stages of floral development in *Haberlea fernandi-coburgii* (A, C, E) and *Ramonda myconi* (B, D, F). A and B (×190), Stage 6, gynoecial primordium initiation. C and D (×100), Stage 7A, corolla growth. E and F (×70), Stage 7B, androecium and gynoecium development (based on Harrison *et al*, 1999). Se, Sepals; P, petals; St, staminode; S, stamen; G, gynoecium.

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