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Evolution of G*CYC*, a Gesneriaceae homolog of *CYCLOIDEA*, within Gesnerioideae (Gesneriaceae)

James F. Smith,[a,*] Lena C. Hileman,[b,1] Martyn P. Powell,[c] and David A. Baum[d]

[a] *Department of Biology, Boise State University, 1910 University Drive, Boise, ID 83725, USA*
[b] *Harvard University Herbaria, 22 Divinity Avenue, Cambridge, MA 02138, USA*
[c] *Jodrell Laboratory, Molecular Systematics Section, Royal Botanic Gardens, Kew Richmond, Surrey, TW9 3DS, London, UK*
[d] *Department of Botany, University of Wisconsin-Madison, 430 Lincoln Drive, Madison, WI 53706, USA*

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Abstract

Through recent advances in molecular developmental biology it has become clear that similar morphological traits may sometimes arise from different genetic bases. The molecular developmental biology of floral symmetry has been examined recently in detail and several genes important in controlling floral symmetry in diverse Asteridae have been identified. One of the most important among these is the floral symmetry gene *CYCLOIDEA* (*CYC*). We compared G*CYC* (the Gesneriaceae homolog of *CYC*) sequences in Gesneriaceae genera with the typical bilaterally symmetric flowers and genera with radial or near radial symmetry. Parsimony, Bayesian and maximum likelihood analyses of G*CYC* sequences among members of Gesnerioideae are mostly congruent with previous phylogenetic hypotheses, but suggest two unexpected generic positions: *Diastema* as sister to *Gesneria*, and *Bellonia* within Gloxinieae. In order to evaluate whether these results might be artifactual we obtained new gene sequences from chloroplast and nuclear ribosomal regions. These data disagree with G*CYC* regarding the placement of *Diastema*, but agree with G*CYC* regarding *Bellonia*. We did not find any mutations in G*CYC* that could explain the shift in symmetry and there were no consistent differences in molecular evolution between taxa with bilaterally or radially symmetric flowers. Likewise taxa with radial floral symmetry are not sister to each other showing that the loss of bilateral symmetry has occurred multiple times in parallel. Further investigations of G*CYC* expression will be necessary to determine if any of these independent events involved changes in the regulation of G*CYC*.

Keywords: Gesneriaceae; G*CYC*; *CYCLOIDEA*; Floral symmetry

1. Introduction

Floral symmetry is a trait of systematic and ecological importance in Asteridae (Cronk and Möller, 1997; Donoghue et al., 1998) whose development is thought to be dependent on a small set of floral symmetry genes (Almeida et al., 1997; Luo et al., 1996; Reeves and Olmstead, 1998). By studying mutant plants of *Antirrhinum* (snapdragon) that lack the wild type floral bilateral symmetry, Luo et al. (1996, 1999) identified two loci that are essential for the development of bilaterally symmetric flowers. These are *CYCLOIDEA* (*CYC*) and *DICHOTOMA* (*DICH*). Detailed studies of the expression of these genes indicate that *CYC* and *DICH* are both essential for full bilateral symmetry but that the role of *CYC* is apparently more important and that this gene acts early in floral development. *CYC* and *DICH* have similar amino acid sequences and are part of a larger gene family, the TCP gene family, that also includes *TEOSINTEBRANCHED1*, from maize, *PCF1* and *PCF2* from rice and a few genes isolated from *Arabidopsis* (Cubas et al., 2001). *CYC* expression in the dorsal region of *Antirrhinum* flowers appears necessary for dorsal stamen abortion, perhaps through an effect on the expression of cell-cycle genes, including *CYCLIN D3B* (Gaudin et al., 2000). The developmental aspects of these flower symmetry genes in *Antirrhinum* have

* Corresponding author. Fax: 1-208-426-4267.
E-mail address: jfsmith@boisestate.edu (J.F. Smith).
[1] Present address: Osborn Memorial Labs, Yale University, P.O. Box 8104, New Haven, CT, 06520-8104, USA.

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stimulated interest in the evolution of plant groups with both bilateral and radial floral symmetry (Baum, 1998; Citerne et al., 2000; Coen, 1996; Coen and Nugent, 1994; Coen et al., 1995; Cronk, 2002; Cronk and Möller, 1997; Cubas, 2002; Donoghue et al., 1998; Endress, 1997; Gillies et al., 2002; Hileman and Baum, 2003; Hileman et al., 2003; Möller et al., 1999; Running, 1997; Theißen, 2000).

One plant family that manifests shifts in floral symmetry is Gesneriaceae (Cronk and Möller, 1997). Gesneriaceae and nearly all close relatives of the family (placed in Lamiales sensu APG, 1998) have bilaterally symmetric flowers. Phylogenetic analyses (Smith, 1996, 2000a; Smith et al., 1997) indicate that the ancestral condition for Gesneriaceae is bilateral symmetry and that radially symmetric flowered plants have been derived several times from within otherwise bilaterally symmetric flowered clades (Cronk and Möller, 1997; Möller et al., 1999; Smith, 2000a). Among the radially symmetric flowered genera in Gesneriaceae are *Tengia*, *Conandron*, *Ramonda*, *Niphaea*, *Phinaea*, and *Bellonia* as well as particular species of *Cyrtandra*, *Napeanthus*, and *Fieldia* (Burtt, 1992; Gillett, 1970, 1973; Wiehler, 1983). Further, in one cultivated species (*Sinningia speciosa*) peloric mutants are known (Citerne et al., 2000; Coen and Nugent, 1994; Möller et al., 1999). The degree of radial symmetry varies from "full" radial symmetry where there are five (sometimes six) petals in a radially symmetric corolla and five equal stamens, to flowers with a radial corolla, but only four stamens (the dorsal stamen failing to fully develop). All of these forms are referred to as radial herein. *Tengia*, *Conandron*, *Cyrtandra*, and *Ramonda* are members of the old world Cyrtandroideae whereas *Niphaea*, *Phinaea*, *Bellonia*, *Fieldia*, *Napeanthus*, and *Sinningia* are in the new world Gesnerioideae (Burtt and Wiehler, 1995; Smith, 1996, 2000a; Smith et al., 1997; Weng et al., 1998). Relationships in the latter subfamily are well known (Perret et al., 2003; Smith, 2000a,b,c; Smith, 2001; Smith and Atkinson, 1998; Smith and Carroll, 1997; Smith et al., 1997; Zimmer et al., 2002) and indicate that *Napeanthus* (tribe Napeantheae) and *Fieldia* (tribe Coronanthereae) are separate from each other and from the remaining species with naturally occurring radial flowers, which are in the Gloxinieae.

Studies of the Gesneriaceae *CYC* homolog, G*CYC*, within Cyrtandroideae failed to find evidence that radially symmetric species had anomalous coding region sequences, either in terms of nonsense mutations or accelerated substitution rates (Citerne et al., 2000; Möller et al., 1999). These data suggest that G*CYC* is still maintained by selection in radial taxa and imply that the switch to radial symmetry did not entail significant changes in G*CYC* function (Citerne et al., 2000). The present study undertakes a parallel examination of G*CYC* gene sequences within Gesnerioideae. As a point of reference, we also conducted new phylogenetic analyses of Gesnerioideae based on G*CYC* and four additional genetic markers.

2. Methods

Taxa sampled in this investigation, their voucher numbers and GenBank Accession numbers for sequences are listed in Table 1.

DNA was extracted using a modified CTAB procedure (Smith et al., 1992) or Qiagen DNeasy extraction kits following the manufacturer's instructions. Five genes were selected for examination; low copy number nuclear gene G*CYC* (Citerne et al., 2000; Möller et al., 1999), the chloroplast genes *ndh*F (Smith, 2000a,c, 2001; Smith and Atkinson, 1998; Smith and Carroll, 1997; Smith et al., 1997), the *trn*L intron and the *trn*L–*trn*F spacer (Brouat et al., 2001; Eldenäs and Linder, 2000; Manktelow et al., 2001; Möller et al., 1999; Salatino et al., 2001; Sweeney and Price, 2000; Yen and Olmstead, 2000), and the nuclear ribosomal internal transcribed spacers, ITS1 and ITS2 (Baldwin et al., 1995; Baum et al., 1994; Möller and Cronk, 1997; Smith, 2000b; Smith et al., 1998; Soltis and Kuzoff, 1995; Suh et al., 1993; Yuan et al., 1996). For *ndh*F and ITS, PCR was performed according to Smith et al. (1997), and Smith (2000b), respectively. The *trn*L intron and *trn*L–*trn*F spacer were amplified using the primers of Taberlet et al. (1991) and cycling parameters of Smith et al. (1997). G*CYC* was amplified according to Möller et al. (1999). The G*CYC* primers of Möller et al. (1999) were not successful for all taxa and a new forward primer G*CYC* FS1 (5′-AMTGGTTSCTCACTARATC-3′) was designed. To verify that this did not amplify a paralogue of G*CYC*, this new forward primer was used with a subset of other taxa that were amplified with the primers of Möller et al. (1999). A subset of the *ndh*F and ITS sequences were from previous publications (Smith, 2000b,c; Smith and Atkinson, 1998; Smith and Carroll, 1997; Smith et al., 1997) and all G*CYC* sequences for Cyrtandroideae, *Antirrhinum* and *Linaria* were from previously published results (Citerne et al., 2000; Möller et al., 1999).

Sequences were obtained from both strands of all gene regions examined in this study via PCR amplification and sequencing either manually (Smith et al., 1997), or automatically on an ABI 377, or a Li-Cor LongreadIR 4200 automated sequencer. For all genes, and for most copies of G*CYC*, direct sequencing provided clear sequences with few ambiguities. However, for some species of Gesnerioideae, direct sequencing was not successful and the amplified gene had to be cloned prior to sequencing. The pGEM-T vector system kit (Promega) was used to clone G*CYC* from *Fieldia*, *Asteranthera*, *Coronanthera*, *Sarmienta*, *Rhabdothamnus*,

Table 1
Species sequenced in this study with GenBank submission numbers and voucher specimens

Species	Voucher	*ndh*F	ITS	*trn*L intron	*trn*L–*trn*F spacer	G*CYC*
Alloplectus panamensis C. V. Morton	Skog et al. 7641 (US)	AF013685*	AF272160/ AF272161*	AY364283	AY364305	AY363933
Asteranthera ovata (Cav.) Hanst.	Stewart 12234 (SRP)	NA	NA	NA	NA	AY363949/ AY363950
Bellonia spinosa Sw	Smith 4111 (SRP)	AF040144*	AY372334/ AY372351	AY364278	AY364300	AY363924
Besleria sp.	Amaya & Smith 525 (COL)	AF176626*	AY372330/ AY372348	AY364274	AY364296	AY363943
Besleria aggregata (Mart.) Hanst.	Smith 3377 (US)	NA	NA	NA	NA	AY363945
Columnea byrsina (Wiehler) Kvist & L. Skog	Smith 3408 (SRP)	AY364308	AF272176/ AF272177*	AY364282	AY364304	AY363931
Codonanthe elegans Wiehler	Smith 3932 (SRP)	NA	NA	NA	NA	AY363929
Coronanthera clarkeana Schlechter	T. Motley 2191 (NY)	NA	NA	NA	NA	AY363952
Cremosperma ecuadoranum Kvist & L. Skog	Smith 3400 (SRP)	NA	NA	NA	NA	AY363944
Diastema racemiferum Benth.	Skog 7574 (US)	U62156*	AY372324/ AY372342	AY364268	AY364290	AY363916
Episcia lilicina Hanst	SI 92-001	NA	NA	NA	NA	AY363930
Fieldia australis Cunn.	Stewart s.n. (SRP)	NA	NA	NA	NA	AY363954
Gasteranthus sp.	Amaya & Smith 515 (COL)	AF176629*	AY372331	AY364275	AY364297	AY363946
Gesneria christii Urban	SI 94-507	U62191*	AY372336/ AY372353	AY364280	AY364302	AY363923
Gloxinia sylvatica (H.B.K.) Kunth	Dunn 9012051 (SRP)	U62157*	AY372325/ AY372343	AY364269	AY364291	AY363917
Goyazia rupicola Taubert	Smith et al. 3722 (SRP)	AF257485*	AY372329/ AY372347	AY364273	AY364295	AY363922
Heppiella verticillata (Cav.) Cuatr.	Smith 3427 (SRP)	NA	NA	NA	NA	AY363921
Kohleria spicata (Kunth) Oerst.	Skog 7701 (US)	U62181*	AY372327/ AY372345	AY364271	AY364293	AY363919
Mitraria coccinea Cav.	Smith 3936 (SRP)	U62193*	AY372321/ AY372340	AY364265	AY364287	AY363953
Napeanthus apodemus J. D. Smith	Amaya & Smith 605 (COL)	AF176623*	AY372332/ AY372349	AY364276	AY364298	AY363947
Napeanthus macrostoma Leeuwenberg	Feuillet s. n. (US)	U62161*	AY372333/ AY372350	AY364277	AY364299	AY363948
Nautilocalyx adenosiphon (Leeuw.) Wiehler	Skog 7897 (US)	NA	NA	NA	NA	AY363934
Nematanthus albus Chautems (ined.)	Smith et al. 3726 (SRP)	AF206197*	AF272212/ AF2722143*	AY364281	AY364303	AY363928
Niphaea oblonga Lindl.	Skog 7564 (US)	U62160*	AY372326/ AY372344	AY364270	AY364292	AY363918
Paradrymonia aurea Wiehler	Skog 7979 (US)	NA	NA	NA	NA	AY363932
Pearcea hypocyrtiflora Regel	Smith 3943 (SRP)	NA	NA	NA	NA	AY363913
Phinaea albiflora Rusby	SI 94-503	AF040151*	AY372322	AY364266	AY364288	AY363914
Rhabdothamnus solandri A. Cunn.	Smith 4393 (SRP)	NA	NA	NA	NA	AY363955/ AY363956
Rhytidophyllum auriculatum Hook.	SI 94-524	U62199*	AY372335/ AY372352	AY364279	AY364301	AY363927
R. leucomallon Hanst.	Smith 3949 (SRP)	NA	NA	NA	NA	AY363925
R. tomentosum (L.) Mart.	SI 77-235	NA	NA	NA	NA	AY363926

Table 1 (*continued*)

Species	Voucher	*ndh*F	ITS	*trn*L intron	*trn*L–*trn*F spacer	G*CYC*
Sarmienta repens R. & P.	Smith 3933 (SRP)	U62194*	AY372320/ AY372339	AY364264	AY364286	AY363951
Sinningia defoliata (Malme) A. Chautems	Chautems 01-031 (G)	NA	NA	NA	NA	AY363940
S. lindleyi Schau.	Chautems 97-016 (G)	NA	NA	NA	NA	AY363939
S. richii Clayb.	SI 94-554	U62186*	AY372338/ AY372355	AY364285	AY364307	AY363935
S. speciosa Hiern., peloric cultivar	Smith 4512 (SRP)	AY364309	AY372337/ AY372354	AY364284	AY364306	AY363942
Smithiantha cinnabarina (Linden) Kuntze	SI 94-484	AF040152*	AY372323/ AY372341	AY364267	AY364289	AY363915
Solenophora obliqua D.L.Denham & D.N.Gibson	Breedlove 71542 (CAS)	U62202*	AY372328/ AY372346	AY364272	AY364294	AY363921
Vanhouttea brueggeri Chautems	F. S. Pires et al. AC501 (CESJ)	NA	NA	NA	NA	AY363938
Vanhouttea gardneri (Hook.) Fritsch	Chautems 01-029 (G)	NA	NA	NA	NA	AY363936
Vanhouttea hilariana Chautems	F. S. Pires et al. AC506 (CESJ)	NA	NA	NA	NA	AY363937

SI, Smithsonian Institution living collection. Letters in parentheses indicate herbarium acronyms where vouchers are deposited. Asterisks indicate sequences from previous publications. *ndh*F, Smith and Atkinson, 1998; Smith and Carroll, 1997; Smith et al., 1997; Smith, 2000b,c; ITS – Smith, 2000b.

and *Mitraria* for which it was essential, as well as for *Goyazia*, *Diastema*, *Gesneria*, *Sinningia* spp., *Napeanthus macrostoma*, *Vanhouttea* spp., *Columnea*, and *Nematanthus* to verify that multiple copies did not exist in other members of Gesnerioideae. Cloning also was essential for several of the ITS sequences including *Besleria* spp., *Smithiantha*, *Gasteranthus*, *Diastema*, *Phinaea*, *Sarmienta*, *Mitraria*, *Asteranthera*, *Coronanthera*, *Rhabdothamnus*, and *Bellonia*. Four to eight clones were sequenced from each ligation. Sequences were aligned manually. Small insertions and deletions (indels) were necessary for some sequences. Indels were excluded completely, or (for G*CYC* only) rescored as multi-state characters (Baum et al., 1994).

Three different sets of analyses were run with G*CYC* sequences using different outgroups (herein referred to as OG options), OG option (1) *Antirrhinum* and *Linaria* as outgroup using sequences from both Cyrtandroideae (Citerne et al., 2000; Möller et al., 1999) and Gesnerioideae as ingroups, OG option (2) Cyrtandroideae as outgroup with Gesnerioideae as ingroup, and OG option (3) Coronanthereae as outgroup with representatives of the remaining tribes of Gesnerioideae as ingroup. The latter approach is justified since previous analyses (Smith, 1996, 2000a; Smith et al., 1997) have all indicated Coronanthereae (represented by *Mitraria* and *Sarmienta*) are sister to the remainder of Gesnerioideae. Outgroup options 1 and 2 were used to examine the monophyly of the G*CYC* sequences from Gesnerioideae and to verify that there were no duplicate copies that matched the duplicates found in Cyrtandroideae (Citerne et al., 2000; Möller et al., 1999). Outgroup option 3 minimized the number of taxa and allowed us the maximum ability to analyze the data while minimizing potential errors in the alignment and was used for all analyses of the other four DNA regions sampled in this study.

The data were analyzed using PAUP* 4.0b8 (Swofford, 2000). Maximum parsimony (MP) analyses were conducted for each data set using the search strategy of Olmstead and Palmer (1994) where five searches are performed using 1000 random stepwise addition replicates with nearest neighbor interchange and MulTrees off saving all shortest trees. The trees from each of these searches were then used as starting trees for a search using the tree-bisection reconnection (TBR) branch swapping algorithm and MulTrees on. The shortest trees from all searches were used to generate a strict consensus. Bootstrap support (BS) for nodes (Felsenstein, 1985) was estimated with 100 heuristic simple taxon addition searches using TBR and MulTrees on except for analyses of G*CYC* using OG options 1 and 2 which used "fast" stepwise addition.

Maximum likelihood trees were generated only for outgroup option three (rooted using Coronantherae) using the Hasegawa et al. (1985) model (HKY) allowing for rates to vary among sites according to a discrete approximation to a gamma distribution (Γ) with eight rate categories and the shape parameter (α) estimated from the data (Yang, 1994a,b). Parameters were estimated on an MP tree and were then fixed prior to beginning a simple addition sequence, TBR search with MulTrees on.

Bayesian analyses were conducted using MRBAYES version 3.0B4 (Huelsenbeck and Ronquist, 2001) for OG options 1 and 3. Analyses used four linked chains, heated sequentially with a heat of 0.2, run for two mil-

lion generations and the HKY model with a gamma distribution. All analyses were repeated a second time to confirm adequate mixing.

The partition homogeneity test (PHT; Farris et al., 1994) as implemented in PAUP* was performed on all data sets in this study (outgroup option 3 only). The test was carried out using 1000 random partitions with G*CYC* indels rescored as multi-state characters. Searches used parsimony with simple taxon addition, TBR branch swapping and MulTrees on.

Analyses of *GCYC* indicated the positions of *Diastema* and *Bellonia* to be different from previous hypotheses of their phylogenetic placement (Smith and Atkinson, 1998), therefore analyses of *GCYC* also were conducted independently constraining *Diastema* and *Bellonia* within Gloxinieae and these trees were compared to analyses without constraining its placement using the KH (Kishino and Hasegawa, 1989) and Wilcoxon signed-rank (Larson, 1994; Mason-Gamer and Kellogg, 1996; Templeton, 1983) tests. The PHT test also was performed excluding *Diastema* to determine if this species may alter the level of incongruence among data sets.

One means of assessing differential rates of sequence divergence across lineages is to determine if a molecular clock can be enforced without significantly reducing the likelihood of the data. We used the MP combined tree under OG option 3 as a fixed topology and estimated its likelihood using the program codeml in PAML 3.0 (Yang, 2000) under an HKY + Γ model of molecular evolution. This was repeated with and without a molecular clock enforced and with the imposition of a "local clock," wherein radially and bilaterally symmetric taxa were permitted different rates. Hierarchical likelihood ratio tests were used to see which of these three models is most compatible with these data.

To resolve if positive selection is correlated with morphological shifts away from bilateral symmetry, the ratio of non-synonymous to synonymous substitutions at the codon level was examined (d*N*/d*S* or ω; Goldman and Yang, 1994; Muse and Gaut, 1994). This ratio was estimated for G*CYC* using the codeml program of PAML version 3.0 (Yang, 2000) using two trees: the MP tree derived from G*CYC* sequences alone, and the tree obtained from a combined analysis of G*CYC* and the other four genes. Three models allowing for lineage specific differences in ω were tested, (1) a different ratio for each branch, (2) a two ratio model that allowed one ratio for bilateral taxa and another for radial genera (*Bellonia*, *Niphaea*, *Phinaea*, *Napeanthus apodemus*), and (3) a one ratio model that kept ratios constant over the entire tree (Yang, 1998). The multiple ratio and two ratio scores were compared to the one ratio model scores using a likelihood ratio test (Huelsenbeck and Rannala, 1997; Yang et al., 1995) with $2n - 4$ and 1 degree of freedom, respectively. The above models only allow ω to vary among lineages, not among codon sites, but shifts in gene function may be due to selection at a few amino acid sites along specific lineages. Therefore, we tested for directional selection at codon sites along lineages leading to radially symmetric taxa using the branch-sites tests (Yang and Nielsen, 2002). For each lineage leading away from bilateral symmetry, model 3 and model B were implemented. Model 3 estimates two codon site classes from the data (ω_0 and ω_1). Model B estimates an additional class (ω_2) along predefined lineages, in this case lineages leading to radially symmetric taxa. As with ω_0 and ω_1, ω_2 is free to vary and therefore may be greater than 1. The likelihood scores under models 3 and B were compared using a likelihood ratio test with 2 degrees of freedom.

3. Results

Amplifications resulted in products 2055, ~400,~500, ~400, and ~600 bp for *ndh*F, ITS, *trn*L intron, *trn*L–*trn*F spacer, and G*CYC*, respectively (Table 2). Sequences using both the primers of Möller et al. (1999) for G*CYC* and G*CYC*FS1 were identical indicating that the new forward primer did not select a different paralogue. Cloned sequences of G*CYC* from a given species were identical with the exception of members of

Table 2
Nucleotide sequence characteristics of ITS, *trn*L intron, *trn*L–*trn*F spacer, *ndh*F, and G*CYC* for 22 species of Gesnerioideae

Parameter	ITS	*trn*L–*trn*F	G*CYC*	*ndh*F	*trn*L intron
Length range, bp	377–413	383–400	546–627	2055	493–509
Length mean, bp	405.1	390.7	575.8	2055	499
Aligned length, bp	497	446	750	2055	529
G/C content range (%)	46.1–56.4	29.9–40.5	38.4–43.1	32.5–33.8	32.8–41.7
G/C content mean (%)	52.0	37.5	40.3	33.1	35.8
Number of indels	123	31	21	0	23
Size of indels, bp	1–12	1–7	3–51	NA	1–6
% constant sites	11.8	44.8	47	62.9	66.2
% autapomorphic sites	25.5	34.5	24.8	19.6	28.3
% informative sites	62.7	20.7	28.2	17.5	5.5
% sequence divergence	8.8–46.7	0.9–29.6	2.3–29.2	3.4–12.0	0.0–17.6

Site data with gaps excluded.

Coronanthereae, where different alignable sequences were found.

3.1. Phylogenetic analysis

Parsimony analyses of the full G*CYC* data set (OG option 1) with indels rescored as multi-state characters resulted in 54 trees of 1543 steps each, consistency index, excluding uninformative characters (CI) = 0.64, retention index (RI) = 0.82. One of these trees is presented in Fig. 1. Analyses that excluded indels (trees not shown) did not run to completion and the strict consensus of 112,700 trees of 619 steps each (CI = 0.53, RI = 0.79) had poor resolution among Gesnerioideae. The Bayesian analysis produced identical trees for both runs which differed from the MP trees (Fig. 1) in that the Cyrtandroideae G*CYC*2 clade was sister to all other Gesneriaceae, the Cyrtandroideae G*CYC*1 clade was in a trichotomy with Beslerieae and the remainder of Gesnerioideae and Napeantheae, Coronanthereae 1 and Coronanthereae 2 were sequential sister groups to the remainder of Gesnerioideae. All other relationships were the same with the exception of *Nautilocalyx* which was sister to the Gesnerieae/Gloxinieae clade rather than part of Episcieae.

Outgroup option 2, with indels scored as multi-state characters resulted in 54 trees of 1360 steps each, CI = 0.65, RI = 0.85. The strict consensus of these trees is identical to the portion of taxa that were in common with the analysis using OG option 1 (Fig. 1). With indels deleted, 54 trees of 1286 steps each (CI = 0.65, RI = 0.85) were obtained. The strict consensus of these trees (not shown) were less resolved within Sinningieae and Gesnerieae than Fig. 1, but were otherwise identical.

The MP analysis of G*CYC* sequences with indels rescored as multi-state characters and using OG option 3 resulted in five trees of 463 steps each (CI = 0.83, RI = 0.86). One of these three trees is presented in Fig. 2. With indels excluded, four trees of 375 steps each were obtained (CI = 0.79, RI = 0.83). The strict consensus of these trees had less resolution among genera of Gesnerieae than the analysis with indels scored, and supported a sister group relationship between Episcieae and Sinningieae (Fig. 2). The ML tree for the same data set (with indels excluded) produced a single tree with a score of 3632.27083 that was similar to the trees derived from the two MP analyses (Fig. 2). The only noteworthy difference is that whereas MP trees placed *Nematanthus* as sister to a *Columnea*/*Alloplectus* clade, the ML tree placed it as sister to *Columnea*. A local clock model (allowing different rates for radially symmetric taxa) did not explain the data significantly better than a global clock ($p = 0.12$). However, both a global clock and a local clock explained the data significantly worse than a non-clock model ($p = 0.00$), suggesting extensive lineage-to-lineage rate heterogeneity. The tree obtained from Bayesian analysis was identical in topology to the MP tree except that Episcieae was sister to Sinningieae/Gesnerieae/Gloxinieae and Sinningieae was sister to Gesnerieae/Gloxinieae.

Maximum parsimony analysis of *ndh*F, *trn*L intron, *trn*L–*trn*F spacer, and ITS analyzed separately yielded three trees of 1647 steps (CI = 0.40, RI = 0.34), 62 trees of 289 steps (CI = 0.73, RI = 0.78), 1955 trees of 298 steps (CI = 0.58, RI = 56), and six trees of 545 steps (CI = 0.52, RI = 0.56), respectively. Trees are not shown for each of these analyses, but overall topology was similar for each. A summary of informative sites, GC content, indels, etc. for all loci in this study is presented in Table 2.

The PHT indicated no significant incongruence among the four data sets excluding G*CYC* ($p = 0.093$). Combined, these four genes resulted in four trees of 2900 steps (CI = 0.45, RI = 0.41) with indels excluded (Fig. 3). The ML analysis resulted in a single tree that was consistent with the MP trees except that Sinningieae is sister to Episcieae/Gesnerieae/Gloxinieae and weakly supported branches within Gloxinieae were somewhat rearranged. The Bayesian analysis produced a tree identical to the MP tree. The four-gene tree differed from the G*CYC* tree in two regards (Figs. 2 and 3). (1) *Diastema* is placed within Gloxinieae, whereas it is within Gesnerieae according to G*CYC*. The former position accords better with traditional classifications (Burtt and Wiehler, 1995) and previous phylogenetic studies (Smith, 1996, 2000a; Smith and Atkinson, 1998; Smith et al., 1997; Zimmer et al., 2002). (2) Napeantheae is placed sister to Beslerieae, whereas it is sister to the remainder of Gesnerioideae excluding Coronanthereae according to G*CYC*. Despite these topological conflicts, a PHT test detected no significant incongruence between a partition of the four data sets and G*CYC* ($p = 0.083$), especially once *Diastema* was excluded ($p = 0.49$). The MP analyses of G*CYC* that constrained *Diastema* within Gloxinieae and included indels as multi-state characters resulted in six trees of 465 steps, only two steps longer than without the constraint, which is not significant as judged by KH and Templeton tests ($p = 0.13$ and 0.29, respectively).

A combined analysis of all five genes with indels rescored as multi-state characters for G*CYC* and excluded for other regions resulted in five trees of 3380 steps each, CI = 0.50, RI = 0.48. One of these four is presented in Fig. 4. This tree has less resolution within Gloxinieae than either of the partitions analyzed separately, but otherwise combines aspects of both topologies: *Diastema* is placed within Gloxinieae as found with the four data set analysis and Napeantheae is sister to all remaining Gesnerioideae excluding Coronanthereae as was found with G*CYC*. Excluding all indels produced five trees (not shown) of 3258 steps each, CI = 0.48, RI = 0.46. The strict consensus of these trees is identical

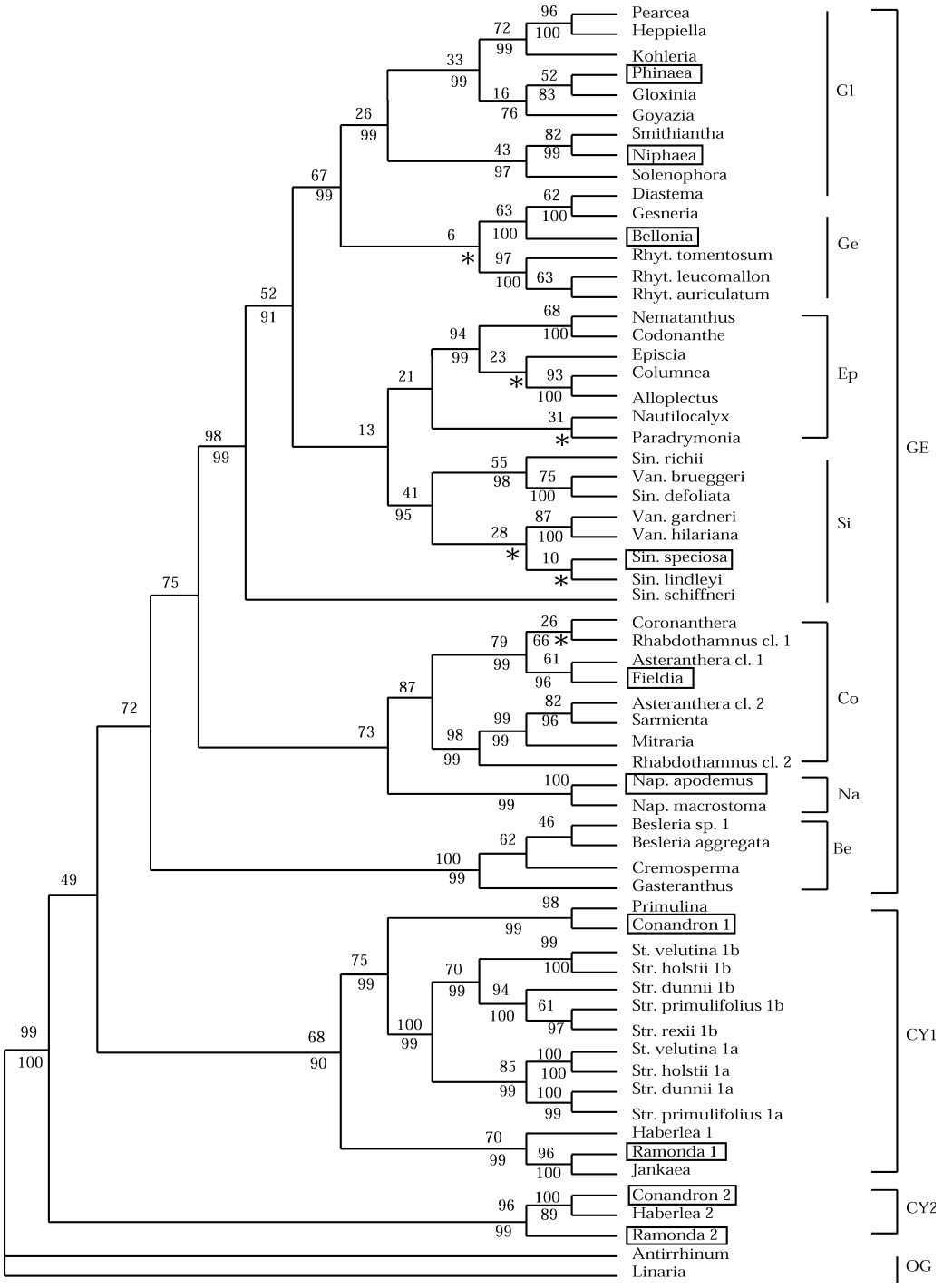

Fig. 1. One of MP trees of 1543 steps of G*CYC* sequences with indels rescored as multi-state characters (CI = 0.64, RI = 0.82). Asterisks mark clades that collapse in the strict consensus. The topology of this tree remains the same if *Antirrhinum* and *Linaria* are removed and Cyrtandroideae are used as the outgroup (54 trees of 1360 steps, CI = 0.65, RI = 0.85). Numbers above the clades are BS values, numbers below clades are Bayesian inference values. Boxes around taxon names represent species with radially symmetric flowers. Abbreviations to right of tree are: Be, Beslerieae; Co, Coronanthereae; CY, Cyrtandroideae (1 and 2 used to represent paralogues of G*CYC*); Ep, Episcieae; GE, Gesnerioideae; Ge, Gesnerieae; Gl, Gloxinieae; Na, Napeantheae; Nap., *Napeanthus*; OG, outgroup; Rhyt., *Rhytidophyllum*; Si, Sinningieae; Sin., *Sinningia*; St., *Saintpaulia*; and Str., *Streptocarpus*; Van., *Vanhouttea*. Paralogues of G*CYC* detected in the *Saintpaulia*/*Streptocarpus* clade are designated as A and B. Different cloned sequences are indicated by the abbreviation cl. Only tribes that are monophyletic in this analysis are marked with brackets, all other tribes are indicated with a vertical line.

to the strict consensus with indels rescored as multi-state characters. The ML analysis produced a single tree (not shown) that differs from the MP trees only by resolving relationships of genera within Gesnerieae and Gloxinieae. The Bayesian analysis produced a tree identical to the MP tree with the exception that the two runs

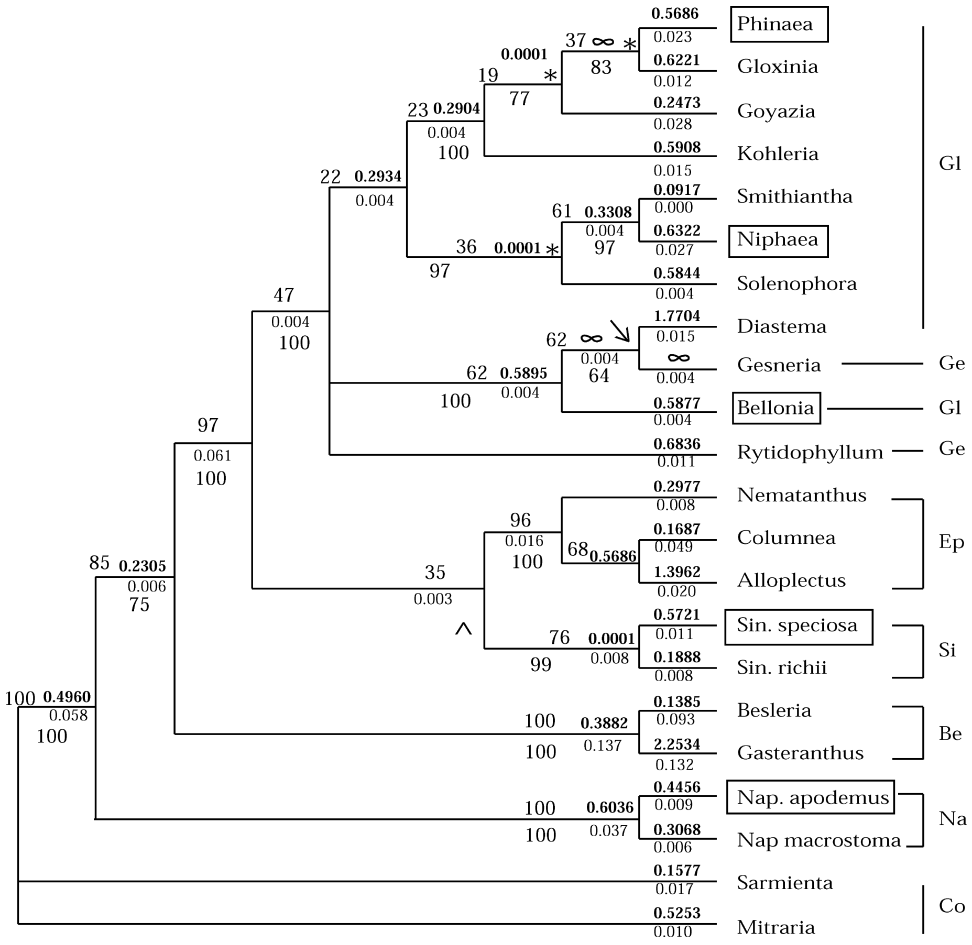

Fig. 2. One of five MP trees of 463 steps of GCYC sequences each using representatives of the tribes of Gesnerioideae, rooting the tree with Coronanthereae, and indels rescored as multi-state characters (CI = 0.83, RI = 0.86). A carat marks where the clades collapse in the strict consensus of these trees. Asterisks mark clades that collapse in a ML analysis that excluded indels, and an arrow marks the clade that collapses in the strict consensus of four trees of 375 steps, (CI = 0.79, RI = 0.83) with indels excluded. Numbers above the clades are BS values, whole numbers below clades are Bayesian inference values. Numbers less than one below branches are branch lengths from the ML tree excluding indels. Boxes around taxon names represent species with radially symmetric flowers. d*N*/d*S* (ω) values are above branches in bold although it should be noted there is no statistical support for the multiple ratios displayed here from a single ratio of 0.3918 for all branches. Values of ω that that are unresolved due to the absence of synonymous substitutions for that branch are represented by an infinity symbol. Abbreviations to right of tree are: Be, Beslerieae; Co, Coronanthereae; Ep, Episcieae; Ge, Gesnerieae; Gl, Gloxinieae; Na, Napeantheae; Nap., *Napeanthus*; Si, Sinningieae; and Sin., *Sinningia*. Only tribes that are monophyletic in this analysis are marked with brackets, all others are indicated with a vertical line.

differed in their relationships among genera in Gloxinieae and differed from those presented here (Fig. 4). A strict consensus of all analyses would result in no resolution among these genera.

According to the combined analysis, *Bellonia* is placed within Gesnerieae contrary to previous classifications of this genus (Burtt and Wiehler, 1995; Wiehler, 1983). Unlike *Diastema*, the placement of *Bellonia* is consistent for all genes. Constraining *Bellonia* within Gloxinieae using G*CYC* sequences resulted in six trees of 466 steps, three steps longer than without the constraint, which is not significant as judged by KH ($p = 0.07$) and Templeton tests ($p = 0.09$). Using the five gene data set and constraining *Bellonia* to Gloxinieae resulted in a single tree of 3389 steps, nine steps longer than without the constraint. This tree is significantly longer than the tree without the constraint as judged by the KH ($p = 0.02$) and Templeton ($p = 0.04$) tests.

3.2. Molecular evolution of GCYC

In order to align Gesnerioideae G*CYC* sequences, 21 indels ranging from 3 to 51 base pairs were required, all in multiples of three. The only apparent stop-codons were in the two *Napeanthus* species at the aligned position 766, which would be expected to delete approximately the last third of the protein. No other copies of G*CYC* were detected for these two species, but we cannot rule out there being a functional gene that failed to amplify.

A single copy of G*CYC* was found in all Gesnerioideae as had previously been reported with a lesser

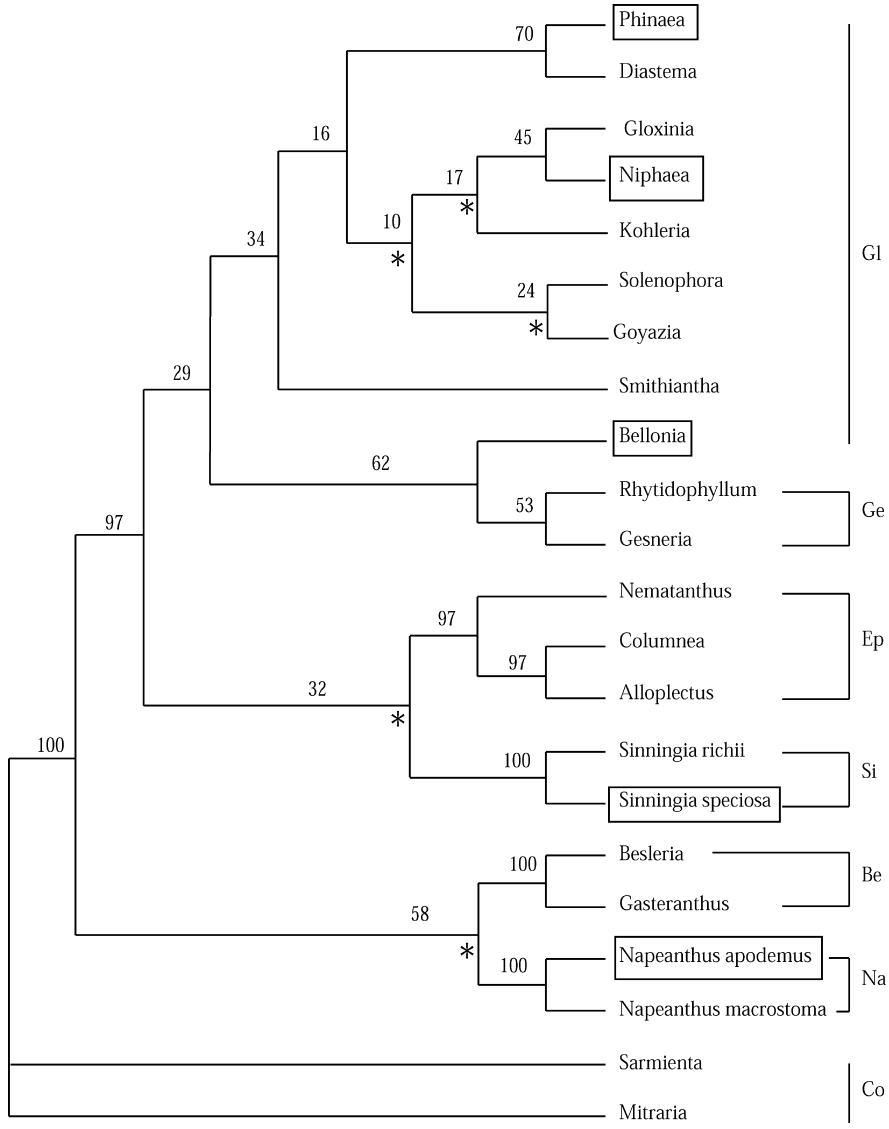

Fig. 3. One of four MP trees of 2900 steps each from the combined four data sets excluding G*CYC* sequences and all indels for representatives of Gesnerioideae (CI = 0.45, RI = 0.41). Asterisks mark clades that collapse in the strict consensus. Numbers above the clades are BS values. Boxes around taxon names represent species with radially symmetric flowers. Abbreviations to right of tree are tribes: Be, Beslerieae; Co, Coronanthereae (used to root this tree); Ep, Episcieae; Ge, Gesnerieae; Gl, Gloxinieae; Na, Napeantheae; and Si, Sinningieae. Only tribes that are monophyletic in this analysis are marked with brackets, all others are indicated with a vertical line.

degree of sampling (Citerne et al., 2000; Möller et al., 1999). The exception is two genera of Coronanthereae, *Rhabdothamnus* and *Asteranthera*, which each had two distinct sequences. The sequences from both copies were readily aligned with other sequences and were identical in length for both *Rhabdothamnus* clones. *Asteranthera* clone 2 has a 12 bp insertion relative to clone 1, which is shared with *Mitraria* and *Sarmienta*. It is likely that alignable duplicates of *GCYC* exist in the remaining species of Coronanthereae, but that these were not represented among the clones that were sequenced. In addition to these alignable sequences, there were some G*CYC* sequences from Coronanthereae that were only alignable in part (fragments of ~30 to 50 base pairs, generally at the beginning or end of the sequence) to the other G*CYC* sequences. These may represent pseudogenes, or PCR recombinants with other regions that are amplified with these primers.

The log-likelihood score for the multiple ω model constrained to the G*CYC* topology (Fig. 2) was −3274 and with the one ratio model was −3297 ($\omega = 0.3918$). This difference was not significant using 40 degrees of freedom ($p = 0.25$), suggesting that the one-ratio model is preferred. Constraining the analysis to the combined data tree (Fig. 4) suggested a similar ω (0.3869) and again favored the one-ratio model ($p = 0.34$). Likewise, on both trees, allowing different ratios for radial and bilateral lineages did not significantly improve the fit of

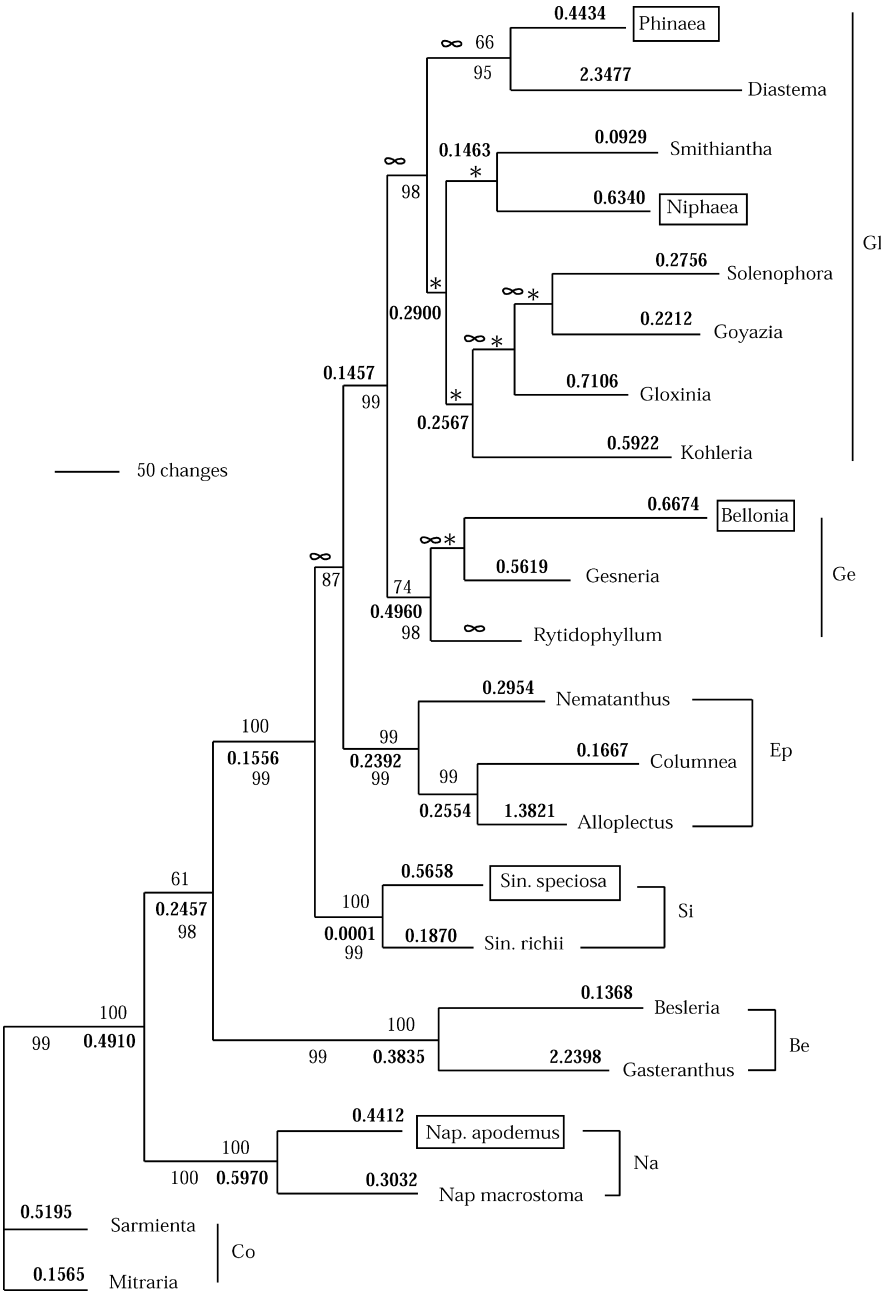

Fig. 4. A phylogram of one of five MP trees of 3380 steps (CI = 0.50 RI = 0.48) from all data combined with indels rescored as multi-state characters for G*CYC* and deleted for other regions. Asterisks indicate clades that collapse in the strict consensus. Numbers above the clades are BS values, whole numbers below clades are Bayesian inference values. d*N*/d*S* (ω) values are above or below branches in bold although it should be noted there is no statistical support for the multiple ratios displayed here from a single ratio of 0.3869 for all branches.. Values of ω that that are unresolved due to the absence of synonymous substitutions for that branch are represented by an infinity symbol. Boxes around taxon names represent species with radially symmetric flowers. Abbreviations to right of tree are: Be, Beslerieae; Co, Coronanthereae (used to root this tree); Ep, Episcieae; Ge, Gesnerieae; Gl, Gloxinieae; Na, Napeantheae; Nap., *Napeanthus*; Si, Sinningieae; and Sin., *Sinningia*. Only tribes that are monophyletic in this analysis are marked with brackets others are indicated with a vertical line.

the data over the one ratio model ($p = 0.93$ and 0.96), although in each case the higher ratio was estimated for the radial lineages (0.3752 vs. 0.5637 and 0.3730 vs. 0.5136).

The branch-sites test (Yang and Nielsen, 2002) was implemented independently for all lineages leading to radially symmetric taxa to look for sites that might have been subject to directional selection. In all but one case the data did not significantly support model B and thus directional selection is not indicated. The only branch-sites test that accepted model B as a better explanation of the data than model 3 was when ω_2 was estimated on the lineage leading to *Napeanthus apodemus* using the G*CYC* tree ($p = 0.006$). However, in this case, ω_2 was

estimated to be > 500, an unreasonably large value for ω, and the result disappeared when the analysis was conducted on the combined tree ($p = 0.79$). These results suggest that the data do not provide adequate signal for parameter optimization, and thus fail to support positive selection in radially symmetric taxa.

4. Discussion

4.1. Radial floral symmetry and GCYC evolution

Despite three species in this analysis with radial corollas (*Niphaea*, *Phinaea*, and *Napeanthus apodemus*), one with full radial floral symmetry (*Bellonia*), and the cultivated peloric form of *Sinningia speciosa*, there appears to be no obvious disruption in G*CYC* at least at the DNA level, except for an apparent frameshift mutation in *Napeanthus apodemus* which would be expected to result in a stop-codon and thus delete approximately the last third of the protein. The latter is however unlikely to explain the radial floral symmetry of *Napeanthus apodemus* because a related bilateral species (*Napeanthus macrostoma*) also has the same mutation. This result could be explained if *Napeanthus* has other functional G*CYC* genes besides the one sampled, or if G*CYC* has lost a role in floral symmetry determination in these taxa.

A lack of candidate mutations in the coding region does not rule out changes in the regulation of G*CYC* having been important in the evolution of radial symmetry. In that case, one might expect relaxed purifying selection and, hence more rapid molecular evolution in species with radial floral symmetry. Within Gesnerioideae a molecular clock and a local clock could both be rejected for G*CYC*, implying that some branches are evolving at rates different than others.

Comparing species with radial floral symmetry, both *Niphaea* and *Phinaea* have branches that are at least twice the length of their bilaterally symmetric flowered sister genera. However, *Bellonia*, *Sinningia speciosa*, and *Napeanthus apodemus* have branch lengths that are nearly equal to those of their sister species. These results are particularly revealing in that *Niphaea*, *Phinaea* and *Napeanthus apodemus* do not have full radial symmetry in that each of these taxa has only four stamens. If G*CYC* also can be implicated in stamen abortion as *CYC* is in other taxa (Hileman et al., 2003), then it would still be expected to function in taxa with four stamens and only to be fully lost in *Bellonia* and *Sinningia speciosa* where the flowers are fully radially symmetric. Thus, the most likely explanation for the longer branch lengths in G*CYC* is stochastic variation among taxa, perhaps compounded by taxon sampling issues and phylogenetic misplacement of some taxa (due to poor resolution within Gloxineae).

Another signature of relaxed purifying selection is an elevated value of ω. Although, a two-rate model suggested a higher ω for radially symmetric taxa, there was no statistical support for a two-rate model over the simpler one-rate model. Thus, there is no evidence of different patterns of selection on G*CYC* sequences between genera with bilateral or radially symmetric flowers. Additionally, branch-sites tests did not detect specific codons under directional selection on lineages leading to radially symmetric taxa.

Studies in model species often turn up strong mutant effects that involve single non-conservative amino-acid substitutions. Therefore, it is always possible that a particular amino acid substitution could have disrupted one or another function of G*CYC* in the radially symmetric taxa but that there has not been time for this changed function to feedback into widespread and statistically detectable differences in molecular evolution. In this regard it is noteworthy that all bilateral taxa except *Phinaea* have at least one non-conservative amino-acid substitutions (defined as a non-conservative amino acid substitution in a position that is otherwise fully conserved or only with conservative amino acid changes). Equally, *Phinaea* has a seven amino acid deletion in a moderately conserved region near the end of the sequenced fragment. Thus, there is certainly the potential for cryptic loss-of-function mutations in any of these G*CYC* genes. However, since many of the bilateral taxa also have non-conservative changes it is impossible to know how much weight to place on these changes without genetic studies.

Investigations of G*CYC* sequences in radially symmetric genera of Cyrtandroideae similarly found no mutations at the DNA level that would obviously disrupt G*CYC* function (Citerne et al., 2000). Citerne et al. (2000) also examined the floral development of *Ramonda*, a genus with radially symmetric flowers, to determine if bilateral symmetry was important at some stage in development. Their results indicated that no bilateral symmetry was detected at any stage in floral development in *Ramonda*, implying that G*CYC* may have another function besides determining floral symmetry in at least some Gesneriaceae (Citerne et al., 2000). The lack of any disruptive mutations and limited sequence divergence among radially symmetric Gesnerioideae also implies that G*CYC* may have a function beyond determining floral symmetry in these taxa as well.

Recent work by Cubas et al. (2001) sheds some light on one possible explanation for the conserved sequence of G*CYC* in taxa with radially symmetric flowers. *CYCLOIDEA* (*CYC*) is a member of the TCP family of DNA-binding proteins that have been identified in the *Arabidopsis* genome (Cubas et al., 1999). *TCP1* in *Arabidopsis* is most similar to *CYC*, therefore as a means of observing what appears to be a *CYC* homolog in a plant with radially symmetric flowers, Cubas et al. (2001)

studied expression patterns of *TCP1* in *Arabidopsis* flowers. They found that similar to *CYC*, *TCP1* is expressed early in floral development, in only the dorsal part of the flower meristems. However, unlike *CYC*, the gene was not expressed in later stages. *TCP1* also was expressed in the dorsal region of all axillary shoot meristems, which suggests that *TCP1* may serve as a marker for axillary vs. apical meristems. Since *TCP1* has function in radially symmetric flowers it is not surprising to find a conserved DNA sequence for G*CYC* in genera with radially symmetric flowers such as those found in this study and others (Citerne et al., 2000).

4.2. Multiple copies of GCYC in Coronanthereae

Multiple copies of G*CYC* were recovered by Citerne et al. (2000) in Cyrtandroideae and were attributed to two duplication events based on phylogenetic analyses of the sequences. One duplication appeared to predate the divergence of the two subfamilies and a second duplication occurred within the *Saintpaulia*/*Streptocarpus* clade (Fig. 1). In Gesnerioideae, however, we only found duplicate genes among members of Coronanthereae. Complete duplicate sequences were only obtained from *Rhabdothamnus* and *Asteranthera*. *Mitraria*, *Fieldia*, and *Sarmienta* had two genes but the second copy was only alignable to G*CYC* for part of the sequence. Each species had a different sequence for the other part that showed no strong similarity to any sequences in GenBank. The duplications in *Rhabdothamnus* and *Asteranthera* appear to be consistent with a hypothesis of duplication prior to the divergence of genera in the tribe. This likely involved polyploidy given the high chromosome counts for this tribe relative to other Gesnerioideae (Kiehn and Weber, 1997; Kiehn et al., 1997; Skog, 1984). The unalignable copies may therefore be pseudogenes that underwent a genome rearrangement (deletion, transposition, or inversion) that fused part of the coding sequence to another, unrelated piece of noncoding DNA. Alternatively the unalignable sequences could represent a novel intron that was introduced into the gene. These possibilities could be resolved by screening genomic libraries and characterization of transcribed G*CYC* genes using RT-PCR.

4.3. Placement of Diastema and Bellonia with GCYC

The placement of *Diastema* as sister to *Gesneria* (Figs. 1 and 2) and *Bellonia* within Gesnerieae rather than Gloxinieae (Figs. 1–4) are discrepant from previous phylogenetic analyses of Gesnerioideae (Smith et al., 1997; Zimmer et al., 2002) and classification systems (Burtt and Wiehler, 1995), although the latter relationship has been seen previously (Smith and Atkinson, 1998). Multiple amplifications and clones of G*CYC* for *Diastema* and *Gesneria* were sequenced to verify these results and different DNA extractions were tested to determine if contamination were a possibility. All analyses resulted in monophyly of all sequences, respectively, for each of these taxa, implying that the discrepancy is not a simple contamination problem.

The analyses of the four combined data sets excluding G*CYC* produced a tree that is mostly well-supported based on BS values, at least at the tribal level. *Diastema* is within Gloxinieae (Fig. 3; BS = 70 for sister relationship to *Phinaea*). Furthermore, constrained analyses showed that the G*CYC* data could not statistically reject the inclusion of *Diastema* within Gloxinieae. By examining the G*CYC* data that place *Diastema* in Gesnerieae and apart from Gloxinieae there are four base substitutions (one synapomorphic to Gloxinieae, the other three either synapomorphic to the Gesnerieae clade, or are synapomorphies within the clade) and two indels (one synapomorphic to Gloxinieae the other synapomorphic to *Diastema* and *Gesneria*). The most likely explanation for these synapomorphies placing *Diastema* in Gesnerieae is homoplasy due to saturation of substitutions in G*CYC*.

Traditional classification systems have placed *Bellonia* in Gloxinieae (Burtt and Wiehler, 1995; Wiehler, 1983) where it shares a radially symmetric corolla with two other genera, *Phinaea* and *Niphaea*. These three genera have been considered closely related due to shared radial corolla symmetry (Zhaoran and Skog, 1990). However, different chromosome numbers between *Phinaea*, *Bellonia*, ($x = 13$; Skog, 1984) and *Niphaea* ($x = 11$; Skog, 1984), five stamens in *Bellonia* (four in the other two genera), and analyses of *ndh*F sequences (Smith and Atkinson, 1998) all have argued that these genera are not sister to each other. None of the data analyses presented here argues for a close relationship among these genera either (Figs. 1–4). The combined data analysis clearly places the genera apart from each other and also places *Bellonia* in Gesnerieae. The latter result was observed previously using only *ndh*F sequences (Smith and Atkinson, 1998), but because the result was only weakly supported with BS values, was assumed to be potentially spurious. Constraining *Bellonia* within Gloxinieae with the G*CYC* sequences resulted in a tree that was three steps longer than without the constraint regardless of how indels were scored. This topology was not significantly longer based on KH and Templeton tests, implying that *Bellonia*, like *Diastema* may be placed in Gloxinieae based on G*CYC* data alone. However the five data set analysis presented here clearly places *Bellonia* in Gesnerieae where it is well supported by a BS of 74 (Fig. 4). Each of the genes separately contribute to this placement although to a greater or lesser degree (ITS contributes 13 synapomorphic substitutions plus an additional five synapomorphic substitutions with a reversal in either *Gesneria*

or *Rhytidophyllum* ; the *trn*L intron contributes a single synapomorphic substitution). Each data set analyzed separately placed *Bellonia* within Gesnerieae. The relatively strong (BS of 74; Fig. 4) and broad (all genes) support for the placement of *Bellonia* in Gesnerieae suggest that its placement there is more accurate than earlier classifications. This result has also been confirmed in additional studies of this genus at the Smithsonian Institution (E. Roalson, pers. comm.).

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