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Phylogenetic Position of *Titanotrichum oldhamii* (Gesneriaceae) Inferred From Four Different Gene Regions

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ABSTRACT. *Titanotrichum oldhamii* has been variously placed in Gesneriaceae or Scrophulariaceae, although most recent taxonomic treatments treat it as a monotypic tribe within Gesneriaceae. In this study, we reconstructed a broad-scale phylogeny containing *Titanotrichum* using gene sequences from four sequence regions (chloroplast *trnL-F* intron and spacer and *atpB-rbcL* spacer, nuclear 26S ribosomal DNA, and the low-copy developmental gene *CYCLOIDEA*, CYC). The phylogenies inferred from each individual data set and the combined data are congruent in placing *Titanotrichum* inside Gesneriaceae. The phylogenetic tree based on combined chloroplast and nuclear DNA sequences grouped *Titanotrichum* with subfamilies Gesnerioideae (New World) and Coronantheroideae (South Pacific and Chile). We have isolated *CYC*, from most of the species of Gesneriaceae and Scrophulariaceae represented in this study, and this gene phylogeny suggests the same placement of *Titanotrichum*. *CYC* was found to evolve three times faster than the *trnL-F* intron and spacer, 3.3 times faster than the *atpB-rbcL* spacer, and eight times faster than nuclear 26S rDNA. Although there is considerable phylogenetic information in this fast evolving gene, analysis is problematic because of high levels of homoplasy and paralogy. In addition to a duplication predating a split between New World and Old World taxa (*Gcyc1* vs. *Gcyc2*), there are several subsequent lineage-related duplications (mainly within *Gcyc1*).

The monotypic taxon, *Titanotrichum oldhamii* (Hemsl.) Soler., is of uncertain taxonomic affinity, being variously placed in Scrophulariaceae (sens. lat.; Wettstein 1891) and Gesneriaceae (Burtt 1962, 1977). The difficulty of classifying *Titanotrichum* arises because it shares several features with both Scrophulariaceae s.l. and Gesneriaceae. The species was first placed in *Rehmannia* (Scrophulariaceae s.l.) as its racemose inflorescence and showy bell-shaped flowers are reminiscent of Scrophulariaceae such as *Rehmannia* and *Digitalis* (Hemsley 1895). Later, in 1909, Solereder named it as a new genus *Titanotrichum* in Gesneriaceae based on the unilocular ovary (Solereder 1909). It was placed in the Old World subfamily Cyrtandroideae on account of its superior ovary and geographic distribution (Solereder 1909). Recent taxonomic treatments raised it to a monotypic tribe in the Cyrtandroideae because of its unique morphology (Wang and Pan 1992; Burtt and Wiehler 1995). A recent molecular phylogenetic study using chloroplast *ndhF* gene sequences addressed its position within Gesneriaceae; it was placed as sister to the rest of subfamily Cyrtandroideae but with little branch support (Smith et al. 1997a, 1997b). On the other hand, a chemotaxonomic study on phenolic acid compounds grouped *Titanotrichum*, *Cyrtandromoea*, and *Rehmannia* into Scrophulariaceae (Kvist and Pedersen 1986). Sealy (1949) allied *Titanotrichum* to New World Gesneriaceae genera, *Isoloma* (=*Kohleria*) and *Naegelia* (=*Smithiantha*), because they possess a similar habit and scaly rhizome. Anisocotyly is probably the most reliable character to separate Cyrtandroideae and Gesnerioideae (New World) (Burtt and Wiehler 1995). In Old World species the cotyledons become unequal in size soon after germination (anisocotyly) due to the extended activity of a meristem at the base of the cotyledon, while New World species of subfamily Gesnerioideae and Coronantheroideae are all isocotylous, lacking such persistent meristematic activity (Burtt 1962). Interestingly, *Titanotrichum*, although geographically Old World, is isocotylous (Wang and Cronk 2003). *Titanotrichum* also has some unique morphological characters, such as bulbil proliferation in inflorescences (Wang and Cronk 2003), not seen in any other Gesneriaceae species.

To resolve the placement of *Titanotrichum*, we use an approach combining molecular evidence from two chloroplast DNA (cpDNA) sequences, *trnL-F* intron and spacer and *atpB-rbcL* spacer, the 26S nuclear ribosomal DNA (nrDNA) and a nuclear developmental gene, *CYCLOIDEA* (*CYC*). For comparisons at the family level or above (as this gene is conservative), 26S data has proven to be phylogenetically useful, particularly for previously unresolved clades and taxa (e.g., *Circaeaster*, Oxelman and Lidén 1995; angiosperm phylogeny, Hershkovitz et al. 1999). The chloroplast *trnL-F* intron and spacer and *atpB-rbcL* spacer have been successfully used for inferring phylogenies at the generic and intrageneric level (Taberlet et al. 1991; Golenberg et al. 1993; Gielly and Taberlet 1994; Manen et al. 1994), and have also recently been used successfully on Gesneriaceae (Mayer et al. 2003).

CYCLOIDEA belongs to a multigene family, the TCP

family, which comprises axillary meristem identity genes in *Zea mays* (*TB1*), floral symmetry genes in *Antirrhinum majus* (*CYC*) and DNA- binding protein genes in *Oryza sativa* (*PCF*) (Cubas et al. 1999a, 1999b; Cubas 2002). The gene family encodes putative transcription factors (Doebley and Lukens 1998). The *TB1/CYC* subfamily is characterised by two conserved regions: a basic helix-loop-helix TCP domain and an arginine-rich R domain (Cubas 1999b). Möller et al. (1999) have isolated two putative paralogues in Gesneriaceae (*Gcyc1* vs. *Gcyc*2) from species with different flower symmetries, in an attempt to test their sequence divergence in relation to morphological changes (zygomorphy vs. actinomorphy). However, their results, together with a follow-up study (Citerne et al. 2000), did not suggest loss of functional genes in actinomorphic taxa. They found that *Gcyc* evolution was consistent with Gesneriaceae phylogenies at the generic and tribal levels (Möller et al. 1999; Citerne et al. 2000). Since *CYC* belongs to a multi-copy gene family, it is reasonable to expect that two or more homologues would be isolated in each taxon studied. Orthologues and paralogues may be identified by phylogenetic analysis (Baum 1998; Eisen 1998; Baum et al. 2002).

The aim of this study is to obtain chloroplast *trnL-F, atpB-rbcL,* nuclear 26S, and *CYCLOIDEA* gene sequences from selected taxa to investigate the phylogenetic position of *Titanotrichum oldhamii*, and to investigate how the *Gcyc* homologues evolved in these species with respect to gene duplication or extinction events. Because *Titanotrichum* has been placed in both Scrophulariaceae s.l. and Gesneriaceae, we sampled a large number of representative taxa from both families.

Materials and Methods

Plant Material. Plant material from selected Gesneriaceae, Scrophulariaceae, and Solanaceae species was collected either from living plants cultivated at the Royal Botanic Garden Edinburgh (E), the Institute of Botany, University of Vienna, or from field collections. For taxa cultivated at Edinburgh, voucher specimens were deposited in the herbarium (E). Details are given in Table 1. Seven taxa representing major clades of Scrophulariaceae s. l. were selected according to Olmstead et al. (2001), plus *Peltanthera floribunda* of Loganiaceae and 18 taxa of major tribes in Gesneriaceae following Burtt and Wiehler (1995). Solanales are sister to Lamiales (Albach et al. 2001) and so *Schizanthus* × *wisetonensis* and *Nicotiana tabacum* were chosen as outgroups.

DNA Extraction. DNA from fresh or silica-dried leaves was extracted following a modified CTAB procedure of Doyle and Doyle (1987). For DNA containing significant amounts of secondary metabolic compounds (e.g. *Epithema benthamii*), an additional phenol purification step was added (Sambrook et al. 1989).

PCR Conditions. The primers and PCR conditions used in this study were adopted from the original publications; i.e., chloroplast *trnL-F* intron and spacer (Taberlet et al. 1991), chloroplast *atpB-rbcL* spacer (Manen et al. 1994), partial 26S ribosomal nuclear DNA (Oxelman and Lidén 1995) and less stringent PCR conditions were utilized for extensive cloning of partial sequences of the nuclear developmental gene *CYCLOIDEA* (Citerne et al. 2000). Around 550 bp (~70%) of the down stream region of the *CYCLOIDEA* open reading frame (ORF) were amplified for our study using forward primer *GcycFS* and reverse primer *GcycR* (for the exact amplified *CYC* region see Möller et al. 1999). To amplify four different gene regions efficiently across distantly related species in our study, one new PCR primer was designed or existing ones modified from original publications. For the amplification of the complete *atpB-rbcL* spacer, a new forward primer 'ABF' (5′-GGA AAC CCC AGA ACC AGA AG-3′) was designed and combined with the reverse primer 'JF5′ from Manen et al. (1994). To obtain the complete *trnL-F* intron and spacer region, primers 'c' and 'f' from Taberlet et al. (1991) were chosen. To obtain the upper part of the 26S ribosomal DNA region, forward primer 'ITS-3P' located in 5.8S (Möller and Cronk 1997) and reverse primer '28S2R' were used (Oxelman and Lidén 1995). Circa 1,200 base pairs (bp) (35%) from the 5′end of the 26S gene were amplified. 50–100 ng template DNA was incorporated in 50μl reactions, containing 1μM primer, 100μM each dNTPs (Roche, USA), 2.5mM $MgCl_2$, and 0.5U *Taq* polymerase (Bioline, UK) and 1X *Taq* buffer (16mM $(NH_4)_2SO_4$, 67mM Tris-HCl (pH 8.8), 0.01% Tween20).

Except for the *CYCLOIDEA* region (PCR conditions described in Citerne et al. 2000), a universal PCR profile for *trnL-F, atpB-rbcL,* and 26S was used as follows: 3 min at 95°C, then five cycles of 1 min at 95°C, 1 min at 57°C, 2 min at 72°C, followed by 30 cycles of 45 s at 94°C, 45 s at 57°C, 2 min at 72°C, with a final extension step at 72°C for 7 minutes. Amplification products were checked on 1% agarose gels in 1X TBE buffer and visualized under UV after ethidium bromide staining (0.1μg/ml). PCR products were purified using Qiagen purification columns (Qiagen Ltd, Dorking, Surrey, UK) according to the manufacturer's protocols.

Sequencing. Direct cycle sequencing was carried out using the big-dye terminator ready reaction mix (Perkin Elmer Applied Biosystems division, Warrington, UK) following standard protocols. Sequencing products were analysed on an ABI PRISM 3100 automatic DNA sequencer (Applied Biosystems, Warrington, UK). The PCR amplifying primers of all gene regions were also used as sequencing primers. Several internal primers were designed for our samples to allow complementary sequence confirmation. These were for the *atpB-rbcL* spacer region (atpB-VMF forward: 5′-GAA TTC CGC CTW TTT TCA CAT CTA-3′; VM-R reverse: 5′-TAG ATG TGA AAA TAG GCG GAA T-3′), for 26S (26S-Q1F forward: 5′-CAT TCG ACC CGT CTT GAA AC-3′; 26S-Q1R reverse: 5′-TTT CAA GAC GGG TCG AAT GG-3′), and for *CYCLOIDEA* (Cyc-NF forward: 5′-GCR AGG GCB AGR GAA AGA AC-3′; Cyc-NR reverse: 5′-GCA CAT TTT CTC YYT YGT TCT TTC-3′).

Cloning of* CYCLOIDEA *Homologues. To ensure a high degree of recovery of the *CYCLOIDEA* homologues present in our selected taxa, including rarer copies, more than 20 clones were examined for each taxon. PCR fragments were cloned into the pCR4-TOPO vector, and the recombinants were transformed to TOP10 Chemically Competent *E. coli* following the instructions of the TOPO TA Cloning kit (Invitrogen, Paisley, UK). Plasmid DNA containing cloned products were extracted and purified using the Qiagen Spin Miniprep kit (Qiagen Ltd, Dorking, Surrey, UK), prior to sequencing.

Sequence Analysis. Nucleotide sequences of the three different gene regions (*trnL-F, atpB-rbcL,* and 26S) from 30 taxa were first aligned using default settings in CLUSTALX (Thompson et al. 1997), then adjusted manually. Since *CYC* evolved faster than the other three gene regions, alignment difficulties arose when very divergent sequences were present (i.e., from Solanaceae outgroups to Scrophulariaceae s.l. and Gesneriaceae). *CYC* nucleotide sequences were first translated into amino acid sequences and then aligned in CLUSTALX to aid manual alignment afterward. To align common motifs that CLUSTALX failed to detect, alignments were adjusted manually on the basis of shared motifs and related amino acid groups (similar chemical structure) and finally re-converted into nucleotide sequences. Aligned matrices were deposited in TreeBASE (study acc. no.= S965). Sequence characteristics (sequence divergence, G/C contents, etc., Tables 2, 3) of each gene region were obtained with PAUP*, version 4.0b8 (Swofford 2001) and features such as transition and transversion ratios calculated using MacClade version 3.01 (Maddison and Maddison 1992). The

TABLE 1. Selected species used in the phylogeny reconstruction in this study, with specimen number, origin and GenBank numbers for the individual genes used. The specimen no. is also used as the voucher number. Voucher specimens are deposited in the respective herbaria E, G, or MO.

Solanaceae. ***Schizanthus × wisetonensis***: Cultivar UoE (E), cultivated; *trnL-F* AY423121, *atpB-rbcL* AY423103, 26S AY423076, CYCLOIDEA Chadwick (1997). ***Nicotiana tabacum*** Linn.: Cultivar UoE (E), cultivated; *trnL-F* Z00044, *atpB-rbcL* Z00044, 26S AF479172, CYCLOIDEA Chadwick (1997).

Scrophulariaceae s.l. ***Paulownia tomentosa*** (Thunb.) Steud.: 19892925 (E), Japan; *trnL-F* AY423122, *atpB-rbcL* AY423104, 26S AY423079, CYCLOIDEA AY423141 (Pcyc1); AY423140 (Pcyc2). ***Scrophularia canina*** Linn.: Perret S1.119 (G), Europe; *trnL-F* AY423123, *atpB-rbcL* AY423105, 26S AY423080, CYCLOIDEA AY423138 (Scyc1); AY423139 (Scyc2). ***Rehmannia glutinosa*** Steud.: MMO 0152B (E), China; *trnL-F* AY423124, *atpB-rbcL* AY423106, 26S AY423081, CYCLOIDEA —. ***Antirrhinum majus*** L.: Cultivar UoE (E), cultivated; *trnL-F* AJ492270, *atpB-rbcL* AJ490883, 26S AY423077, CYCLOIDEA AF208341 (cyc); AF208494 (dich). ***Tetranema mexicanum*** Benth.: 19697819 (E), Mexico; *trnL-F* AJ492272, *atpB-rbcL* AJ490884, 26S AY423078, CYCLOIDEA AY423142 (Tcyc2). ***Calceolaria arachnoidea*** Graham: 19912379 (E), Chile; *trnL-F* AY423126, *atpB-rbcL* AY423108, 26S AY423083, CYCLOIDEA AY423143 (Ccyc1); AY423144 (Ccyc2). ***Jovellana punctata*** Ruiz & Par.: 19980599 (E), Chile; *trnL-F* AY423127, *atpB-rbcL* AY423109, 26S AY423084, CYCLOIDEA AY423145 (Ccyc2).

Loganiaceae. ***Peltanthera floribunda*** Benth.: Hammel 20144 (MO), Peru; *trnL-F* AY423125, *atpB-rbcL* AY423107, 26S AY423082, CYCLOIDEA —.

Gesneriaceae (tribes):

(Titanotricheae) ***Titanotrichum oldhamii*** (Hemsl.) Solereder: 19973433 (E), Taiwan; *trnL-F* AY423129, *atpB-rbcL* AY423111, 26S AY423085, CYCLOIDEA AY423150 (Gcyc1).

(Beslerieae) ***Besleria labiosa*** Hanst.: 19822666 (E), Venezuela; *trnL-F* AY423128, *atpB-rbcL* AY423110, 26S AY423086, CYCLOIDEA AY423148 (Gcyc1).

(Napeantheae) ***Napeanthus reitzii*** B.L. Burtt ex Leeuwenb.: Perret 156 (G), Brasil; *trnL-F* AJ492321, *atpB-rbcL* AJ493036, 26S AY423087, CYCLOIDEA AY423149 (Gcyc1).

(Coronanthereae) ***Fieldia australis*** A. Cunn.: 19696862 (E), Australia; *trnL-F* AY423130, *atpB-rbcL* AY423112, 26S AY423088, CYCLOIDEA AY423151 (Gcyc1E); AY423152 (Gcyc1F). ***Mitraria coccinea*** Cav.: 19792696 (E), Chile; *trnL-F* AY423131, *atpB-rbcL* AY423113, 26S AY423089, CYCLOIDEA AY423153 (Gcyc1).

(Episcieae) ***Chrysothemis pulchella*** Dece.: 19802568 (E), cultivated; *trnL-F* AJ492312, *atpB-rbcL* AY423115, 26S AY423091, CYCLOIDEA AY423154 (Gcyc1).

(Gloxinieae) ***Sinningia schiffneri*** Fritsch: 19781514 (E), Brasil; *trnL-F* AJ439745, *atpB-rbcL* AJ439900, 26S AY423092, CYCLOIDEA AF208327 (Gcyc1). ***Kohleria eriantha*** (Benth.) Hanst.: 19821486 (E), Ecuador; *trnL-F* AY423132, *atpB-rbcL* AY423114, 26S AY423090, CYCLOIDEA AY423155 (Gcyc1).

(Gesnerieae) ***Gesneria humilis*** Linn.: Chautems 1179 (G), Cultivated; *trnL-F* AJ439821, *atpB-rbcL* AJ439976, 26S AY423093, CYCLOIDEA AY423156 (Gcyc1).

(Epithemateae) ***Rhynchoglossum hologlossum*** Hayata: Wang 1207 (E), Taiwan; *trnL-F* AY423133, *atpB-rbcL* AJ490899, 26S AY423094, CYCLOIDEA —. ***Epithema taiwanense*** (C. B. Clark) Li & Kao: Wang 1208 (E), Taiwan; *trnL-F* AJ492276, *atpB-rbcL* AY423117, 26S AY423096, CYCLOIDEA —. ***Epithema benthamii*** C. B. Clark: 19972563 (E), Phillipines; *trnL-F* AY423135, *atpB-rbcL* AY423118, 26S AY423097, CYCLOIDEA AY423157 (Gcyc1); AY423146 (Gcyc2). ***Whytockia sasakii*** (Hayata) B. L. Burtt: 19991504 (E), Taiwan; *trnL-F* AY423134, *atpB-rbcL* AY423116, 26S AY423095, CYCLOIDEA —.

(Didymocarpeae) ***Streptocarpus holstii*** Engl.: 19592272 (E), Tanzania; *trnL-F* AJ492304, *atpB-rbcL* AJ490917, 26S AY423099, CYCLOIDEA AF208338 (Gcyc1A); AF208334 (Gcyc1B). ***Ramonda myconi*** (L.) Rchb.: 19821564 (E), Macedonia; *trnL-F* AJ492301, *atpB-rbcL* AJ490914, 26S AY423098, CYCLOIDEA AF208323 (Gcyc1); AF208318 (Gcyc2). ***Didymocarpus citrinus*** Ridl.: 19830510 (E), Malaysia; *trnL-F* AJ492293, *atpB-rbcL* AJ490906, 26S AY423100, CYCLOIDEA AY423158 (Gcyc1C); AY423159 (Gcyc1D).

(Cyrtandreae) ***Cyrtandra apiculata*** C. B. Clake: Cronk & Percy T91 (E), Tahiti; *trnL-F* AY423136; *atpB-rbcL* AY423119, 26S AY423101, CYCLOIDEA AY423160 (Gcyc1); AY423147 (Gcyc2).

(Trichosporeae) ***Loxostigma*** sp.: 19962309 (E), China; *trnL-F* AY423137, *atpB-rbcL* AY423120, 26S AY423102, CYCLOIDEA AY423161 (Gcyc1C); AY423162 (Gcyc1D).

TABLE 2. Sequence characteristics of the chloroplast *trnL-F* intron & spacer, *atpB-rbcL* spacer, nuclear 26S gene and *CYCLOIDEA* gene region.

Characteristic parameters	*trnL-F*	*atpB-rbcL*	26S	CYCLOIDEA nucleotide	CYCLOIDEA amino acid
Length range (total), bp or a.a.	614–842	717–1278	1190–1194	462–726	154–234
Length range, bp or a.a. (Gesneriaceae)	614–827	717–799	1191–1193	531–702	185–234
Length mean (total), bp or a.a.	801.3	745.1	1191.91	608.8	202.9
Length mean, bp or a.a. (Gesneriaceae)	793.5	743.3	1191.6	635.9	212.0
Sequence divergence (%) (Gesneriaceae)	1.6–9.5	0.8–9.1	0.2–8.0	2.4–26.5	5.5–39.9
Overall sequence divergence (%)	1.6–17.0	0.8–16.6	0.2–11.2	2.4–49.0	5.5–64.1
G + C content mean %	34.6	30.81	58.3	41.1	—

TABLE 3. Sequence characteristics relevant for the phylogenetic analysis. CI = Consistency Index; RI = Retention Index; RC = Rescaled Consistency Index.

Statistical description	*trnL-F*	*atpB-rbcL*	26S	combined 3-gene data	CYCLOIDEA nucleotide	CYCLOIDEA amino acid
Aligned length, bp or a.a.	989	946	1200	3133	1020	340
Number of excluded sites	63	41	0	102	178	12
Size of indels	1–35	1–28	1–2	1–35	3–114	1–34
No. of indels	37	31	10	78	43	38
Percentage of constant sites	61.4	62.7	69.1	65.0	30.6	23.8
Percentage of variable sites	38.6	37.3	30.9	35.0	69.4	76.2
Percentage of uninformative sites	16.8	20.9	13.9	17.0	21.4	25.3
Percentage of informative sites	21.7	16.5	17.0	18.0	48.0	50.9
Transitions/transversions	0.70	0.89	2.87	—	1.06	—
Average number of steps per character	0.63	0.58	0.69	0.65	2.23	3.32
Number of MP trees	12	149	198	19	12	324
Length of MP trees	584	521	827	1962	1879	1089
CI	0.781	0.793	0.567	0.682	0.572	0.729
RI	0.756	0.729	0.514	0.624	0.542	0.684
RC	0.590	0.578	0.291	0.425	0.310	0.458

comparison of evolutionary distance between the gene regions was analysed by calculating pair-wise sequence divergences among taxa using the DISTANCE option (average pairwise distance) in PAUP*, based on unambiguously alignable regions without gaps.

Phylogenetic Analysis. Phylogenetic trees were reconstructed using PAUP* 4.0b8. Each gene region (*trnL-F, atpB-rbcL,* 26S, and *CYC*) was first analyzed independently by heuristic tree searches, in the attempt to find the most parsimonious (MP) trees. To increase the chances of including all islands of most parsimonious trees, 10,000 replicates of RANDOM ADDITION SEQUENCE were first performed without swapping, saving all shortest trees. This was followed by TBR swapping on all the resulting trees with MULTREES, STEEPEST DESCENT, branch COLLAPSE (max.) on and ACCTRAN optimization (Möller and Cronk 1997). All most parsimonious trees were summarized in strict consensus trees for each dataset. Characters and substitution rate congruence between different gene regions was tested using the partition homogeneity test (incongruence length differences test, ILD) (Farris et al., 1995) in PAUP*. A parsimony analysis on combined data from *trnL-F, atpB-rbcL,* and 26S sequences was also conducted as described above and used to reconstruct a species tree for the GeneTree analysis (see below).

Branch support for maximum parsimony trees (MP-BS) was tested using 10,000 replicates of bootstrap analyses (Felsenstein 1985) in PAUP*, set to HEURISTIC search and SIMPLE ADDITION SEQUENCE, with TBR swapping but without MULTREE and STEEPEST DESCENT. Bremer support indices (DI) (Bremer 1994) were also calculated using Autodecay version 4.0 (Eriksson 1998) to estimate the additional steps for collapsing individual clades. Descriptive statistics for the measures of character fit in parsimony analysis, such as the consistency index (CI) (Kluge and Farris 1969), retention index (RI) (Farris 1989), and rescaled consistency index (RC) were also calculated. For the 26S nrDNA region, reweighting to correct for transition/transversion bias was also evaluated but the results gave no increase in resolution and further analyses were performed unweighted.

To explore our data further, Maximum Likelihood (ML) and Bayesian analysis (BA) were also performed. For nucleotide ML analysis, the best-fitted model of substitution was selected using ModelTest version 3.06 (Posada and Crandall 1998). The model parameters recommended in ModelTest were then implemented in PAUP* to perform heuristic searches as above. The 3-gene (combined *trnL-F, atpB-rbcL,* and partial 26S sequence data) ML analysis was performed using a GTR+I+G model (general time reversible with estimates of proportion of invariable sites and gamma distribution) selected by AIC (Akaike Information Criterion) in ModelTest. The best model for *CYCLOIDEA* ML analysis selected by AIC in Modeltest was TVM+I+G (transversion model with estimates of the proportion of invariable sites and gamma distribution).

For branch support of Maximum Likelihood trees (ML-BS), 500 replicates of ML bootstrap analyses were performed. For *CYCLOIDEA* data alone a protein ML analysis was performed using PHYLIP 3.6 (Felsenstein 2002) with a Jones et al. (JTT) model of amino acid substitution (Jones et al. 1992), allowing one invariant rate plus 4 categories of gamma distributed rate heterogeneities (the parameters were estimated in TREEPUZZLE version 5.0, Schmidt et al. 2002). Branch support was obtained from TREEPUZZLE (using the same parameters as above). Bayesian analysis also applies model parameters from ModelTest and was carried out using MrBayes version 2.01 (Huelsenbeck and Ronquist 2001). The analysis was run for 1,000,000 generations with trees sampled every 100 generations. Four exchangeable Markov chains were specified allowing tree construction to explore the tree space. The first 1,000 "burn-in" phase trees were discarded. The resulting trees were summarised in a 50% majority-rule consensus tree for the calculation of Bayesian branch support (BA-BS) under PAUP*.

CYCLOIDEA ***Gene Evolution.*** In order to investigate *CYCLOIDEA* duplications in relation to the species evolution, the *CYCLOIDEA* gene phylogeny was reconciled with the species tree inferred from the 3-gene data set (*trnL-F, atpB-rbcL,* and 26S) using GeneTree (Page and Cotton 2000). The 3-gene data set was reduced by exclusion of those taxa for which a *CYCLOIDEA* sequence could not be obtained. The reduced data matrix was then reanalysed as above. The most likely *CYC* gene tree was selected in GeneTree by minimising the cost of the optimality criteria such as deep coalescence, duplication and loss events between each gene tree and species tree combination.

RESULTS

Sequence Analysis of* trnL-F, atpB-rbcL, *and 26S. The sequence characteristics of all sequence matrices are summarized in Tables 2 and 3. For the chloroplast *trnL-F* intron and spacer region, Gesneriaceae species showed a slightly higher length variation compared to Solanaceae and Scrophulariaceae s.l. species. But the mean length and sequence divergence of Scrophulariaceae species was higher than for Gesneriaceae species. The *atpB-rbcL* spacer contained a 479 bp AT-rich insertion (position 424 to 903) unique in *Epithema benthamii*. The length range of the entire *atpB-rbcL* spacer was

somewhat smaller (717–799 bp, excluding the insertion in *Epithema benthamii*). Both chloroplast data sets possessed similar GC contents (G + C= 34.6% and 30.8%, Table 2). For alignment, more than 30 indels (insertion/deletion events) were recognized for each cpDNA region (Table 3). Despite a similar length, the *trnL-F* matrix contained a higher proportion of informative sites (21.7%) compared to the *atpB-rbcL* matrix (16.5%), but a similar proportion of variable sites (~38%). This difference was due to a higher proportion of autapomorphic sites in the latter.

Approximately 1,192 bp of 26S were amplified for each species. Generally the length was more conserved across all taxa studied compared to the cpDNA fragments. A few, one to two bp, indels were found in the expansion segment. About a quarter longer than *atpB-rbcL,* 26S included a similar proportion of informative sites (17.0%). During PCR amplification, different copies of the 26S gene from *Antirrhinum majus* and *Titanotrichum oldhamii* were obtained with different internal sequencing primers. Though these putative pseudogenes were still length conserved, their GC contents were significantly lower than potentially functional ones (40.2% vs. 58.3%). Thus they were excluded from analysis. The characteristics of the nuclear 26S gene were quite different from the chloroplast intron and spacer regions, the former possessed a higher GC content (58.3%), and transition/transversion ratio (2.87) and a low proportion of variable sites (30.9%).

26S Evolution. In the 26S rDNA analysis, we found several sites within the expansion segments (Bult et al. 1995) that contained homoplastic substitutions. Transitional substitutions are extremely frequent among these regions (e.g., C-T changes have been observed 12 times more often than other base changes). On the other hand, nucleotide changes were rare in the conserved core region. The distinctive nature of 26S evolution may be responsible for the low CI (0.567) and RI (0.514) in our 26S MP analysis (Table 3). However, this problem mainly affects relationships above family level, and the strict consensus topology was congruent with our other data sets. Thus we included 26S data in our combined analysis to increase the number of informative sites.

CYCLOIDEA *Clones.* The amplified length of the *CYCLOIDEA* homologues was between 462 and 726 bp, indicating a significant length redundancy of functional *CYCLOIDEA* copies (but see below). Only putatively functional copies were included in the matrix (possessing no premature stop codon or frame shifts). The aligned length of the *CYCLOIDEA* matrix was 1,020 bp, indicative of the numerous indels necessary for alignment.

Different *CYC* homologues, up to two copies per species, were obtained after extensive cloning. In *Titanotrichum oldhamii*, one copy was isolated from all clones of all PCR products, although other copies may not be able to be picked up with non-redundant primers. In all New World Gesneriaceae species of subfamily Gesnerioideae analyzed to date only one copy has been isolated. In the other two subfamilies (Coronantheroideae and Cyrtandroideae), two homologues were found in each species except for *Mitraria coccinea* where only one copy was isolated. Some of these within-species homologues were very divergent from each other (i.e., the uncorrected pairwise distance between *Epithema benthamii Gcyc2* and *Gcyc1* was 34%, between *Cyrtandra apiculata Gcyc2* and *Gcyc1* 23%). Other pairs were closer to each other (i.e., the uncorrected pairwise distance between *Didymocarpus citrinus Gcyc1C* and *Gcyc1D* was 11%, between *Fieldia australis Gcyc1E* and *Gcyc1F* 8%). Maximum divergence within Gesneriaceae homologues was 39.9%. Up to two *CYC* homologues were also isolated from Calceolariaceae and Scrophulariaceae species with the exception of *Tetranema mexicanum* and *Jovellana punctata*. Two *Peltanthera floribunda CYC* homologues were also isolated. However, inclusion of these copies resulted in a high level of homoplasy (CI of the resulting most parsimonious trees was reduced from 0.57 to 0.41). They were therefore excluded from the phylogenetic analysis. We have been unable to amplify *CYC* homologues from *Rhynchoglossum hologlossum*, *Whytockia sasakii*, *Epithema taiwanense*, and *Rehmannia glutinosa*, perhaps because the primer sites are not conserved.

***Pseudo Copies and Allelic Variation of* CYCLOIDEA.** In addition to functional homologues, pseudogenes and allelic variants were also amplified using the same primer set. Some pseudocopies contained stop codons inside the ORF (i.e., *Calceolaria arachnoidea* clone 21 and *Gesneria humilis* clone 7, data not shown). A single base mutation resulted in a premature stop codon in *Titanotrichum oldhamii* clone 26 (data not shown), but a PCR artefact from non-proofreading *Taq* polymerase cannot be ruled out. All apparent pseudogenes were thus excluded from further analyses. Allelic variation could be discriminated from locus variation by comparing the pairwise sequence differences. Putatively allelic clones had up to four nucleotide changes or three amino acid changes, while differences between putative loci (classified based on their position in phylogenetic trees) included at least 17 bp changes or nine amino acid changes. The pairwise distances between likely alleles was less than 0.6% but the distance between the nearest homologues was 2.4% (i.e., *Gesneria humilis Gcyc1* and *Kohleria eriantha Gcyc1*). Allelic variants were reduced to one for simplicity. In exploratory analyses all allelic variants from a single taxon always formed sister relationships.

***Phylogenetic Position of* Titanotrichum oldhamii.** The phylogeny inferred from *trnL-F, atpB-rbcL,* and 26S rDNA data sets, individually and combined, resulted

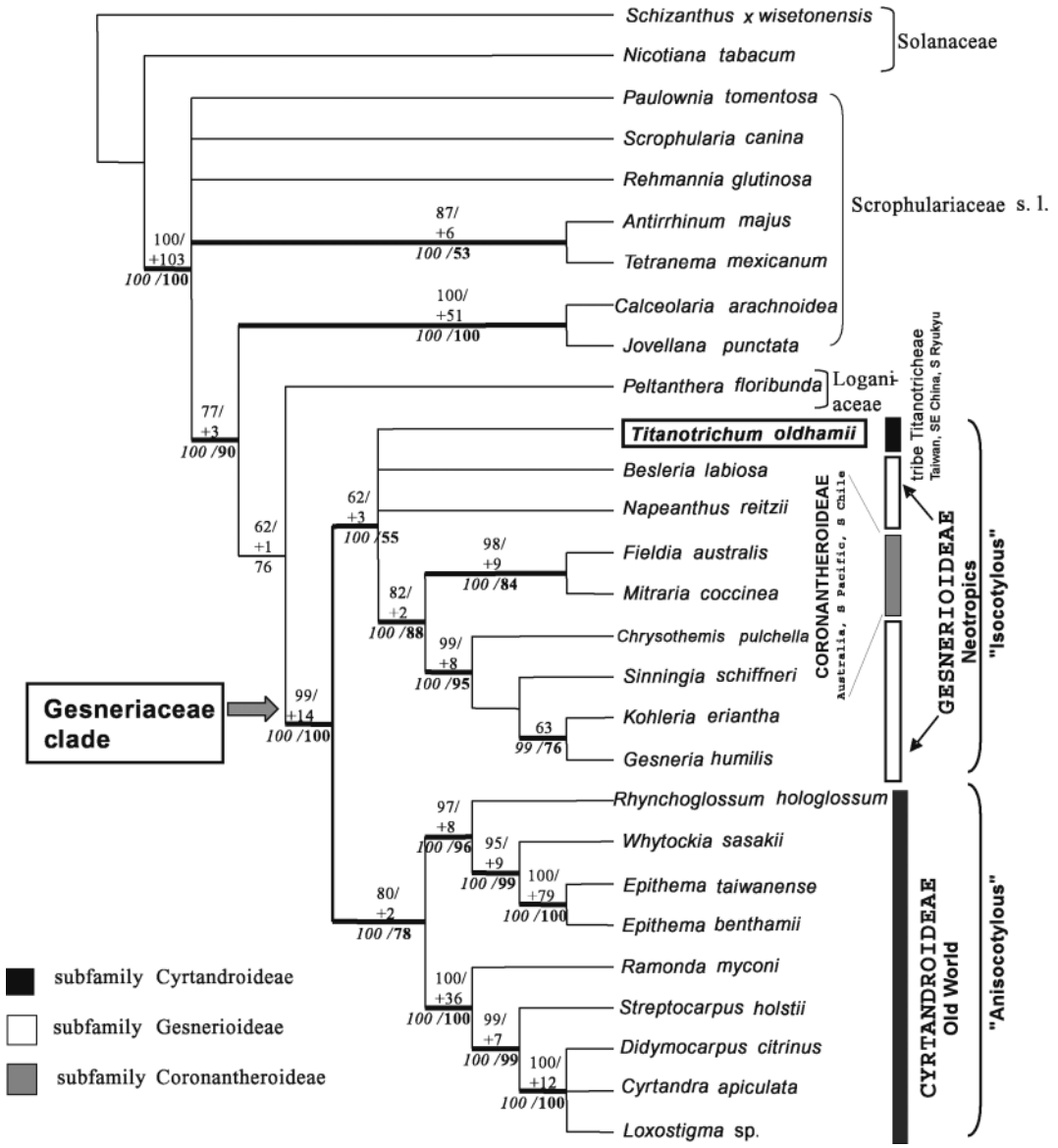

FIG. 1. Strict consensus tree of 19 most parsimonious trees based on combined *trnL-F, atpB-rbcL* and partial 26S sequence data. Numbers above the branches are MP bootstrap values and decay indices. Numbers below the branches are Bayesian 50% majority consensus and ML bootstrap values (bold). Branches in bold indicate the branches persisting in the MP analysis of individual datasets. The subfamily relationship and cotyledon type are indicated

in similar topologies with respect to *Titanotrichum*. All analyses indicated that the genus belongs to Gesneriaceae, and has a position within the ''New World and south Pacific Rim clade'' (subfamilies Gesnerioideae and Coronantheroideae).

Although partition homogeneity tests (ILD) for the three gene regions (*trnL-F, atpB-rbcL,* and 26S) rejected total congruence (P= 0.214), the resulting tree topology was similar in each individual data set. Thus, we combined data from three genes to reconstruct a species phylogeny. Parsimony analysis of the combined 3-gene data set resulted in 19 MP trees. The tree length was 1,962 steps, when 102 ambiguous aligned sites out of 3,133 sites were excluded, with a CI of 0.682, a RI of 0.624, and a RC of 0.425 (Table 3). In the strict consensus topology, Gesneriaceae species formed a well-supported monophyletic group including *Titanotrichum* (MP-BS=99%, DI=14 steps, BA=100%, ML-BS=100%, Fig. 1). The Gesneriaceae can further be divided into two distinct clades with high branch

support, basically corresponding to geographic distribution (e.g., Old World species [MP-BS=80%, DI=2 steps, BA=100%, ML-BS=78%] versus New World plus South Pacific species [MP-BS=62%, DI=3 steps, BA=100%, ML-BS=55%]), except for the Asiatic *Titanotrichum*, which unexpectedly grouped with the New World and South Pacific Rim clade. Within each clade, the tribal relationships were partly resolved. Tribe Epithemateae (MP-BS=97%, DI=8 steps, BA=100%, ML-BS=96%) was grouped as sister to the rest of the Old World species (MP-BS=100%, DI=36 steps, BA=100%, ML-BS=100%), which included the tribes Didymocarpeae, Trichosporeae, and Cyrtandreae. *Didymocarpus* (Didymocarpeae) was included in a polytomy with representatives of tribe Trichosporeae and Cyrtandreae. In the New World clade, *Titanotrichum* and tribes Beslerieae and Napeantheae formed a basal polytomy in New World and South Pacific Rim clade. *Calceolaria*, *Jovellana*, and *Peltanthera floribunda* (Loganiaceae) appeared to be the closest sister groups to Gesneriaceae (MP-BS=77%, DI=3 steps, BA=100%, ML-BS=90%). Other Scrophulariaceae species were sister to all the above groups.

The topology of the ML tree was identical to the single MP tree (data not shown). This phylogram also showed that most species from the Neotropics and South Pacific, including *Titanotrichum*, had relatively short branch lengths compared to Old World species, particularly in tribe Epithemateae.

CYCLOIDEA ***Gene Phylogeny.*** It appears that third codon positions in the TCP + R domain had four times more changes (2.06) than the first and second codon positions (0.51 and 0.43, respectively) (Fig. 2A). This ratio was twice as high per character as the ratio for intervening regions (first codon position: second codon position: third codon position = 0.67: 0.60: 1.05) (Fig. 2B). Thus, the third codon positions of the TCP and R domain were likely to be saturated and therefore were excluded. The reconstructed *CYCLOIDEA* gene phylogeny was highly congruent with our combined 3-gene analysis, despite a negative result in the ILD test (P=0.312). The MP analysis of nucleotide data resulted in 12 most parsimonious trees of 1,879 steps (CI= 0.572, RI=0.542 and RC=0.310, Table 3). The Gesneriaceae homologues formed a monophyletic group (BA=80%, DI=4 steps, Fig. 3). *Epithema Gcyc2*, *Ramonda Gcyc2*, and *Cyrtandra Gcyc2* formed a sister group to the rest of the *Gcyc* sequences (*Gcyc1*) albeit with low branch support (DI= 4 steps, BA=51%). *Gcyc1* homologues could be further divided into two well-supported clades reflecting their geographical distribution; Old World taxa (MP-BS=51%, DI=3 steps, BA=89%) and New World/South Pacific taxa (BA=86%, DI=2 steps). The only exception was the *Titanotrichum Gcyc1* homologue, an Old World taxon that was placed within the latter clade. The *Gcyc1* phylogeny also revealed that *Epithema Gcyc1* was basal to the rest of the Old World *Gcyc1* homologues while *Besleria Gcyc1* occupied this position among the Neotropical/South Pacific *Gcyc1* homologues. Consistent with the topology inferred from the 3-gene analysis, *Calceolaria* and *Jovellana CYCLOIDEA* homologues were the closest to all Gesneriaceae homologues (ML-BS=74%, BA=99%, DI=5 steps). *Antirrhinum CYC* and *DICH* together with *Tetranema Tcyc2* formed a distinctive clade (MP-BS=74%, ML-BS=65%, BA=100%, DI=6 steps) sister to the previous clade. Similar results were obtained in the ML analysis (data not shown).

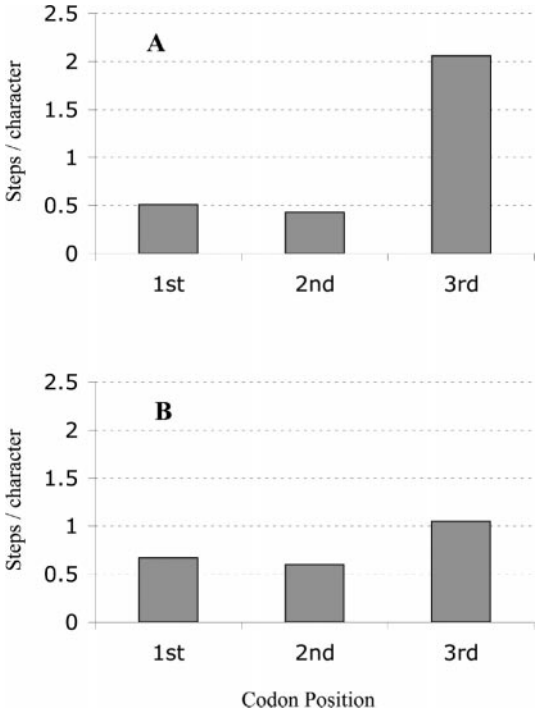

FIG. 2. Frequency of character changes (steps/characters) of *CYCLOIDEA* nucleotides between different codon positions from one of the MP trees illustrating the differences of third codon changes between TCP + R domain (A), and the intervening regions (B).

The topology of the nucleotide phylogeny was generally also recovered by the amino acid analysis, which resulted in 324 MP trees of 1,089 steps (CI= 0.729, RI= 0.684 and RC=0.458). The protein strict consensus tree only differed from the nucleotide topology in that *Napeanthus Gcyc1* instead of *Besleria Gcyc1* was basal to the New World/South Pacific Rim *Gcyc1* clade, and the grouping of *Schizanthus cyc2* and *Nicotiana cyc1* collapsed. In general, the bootstrap support values for the protein tree were lower than those for the nucleotide phylogeny. The protein ML analysis resulted in similar relationships compared to the protein MP analysis. ML branch support from TREEPUZZLE was generally

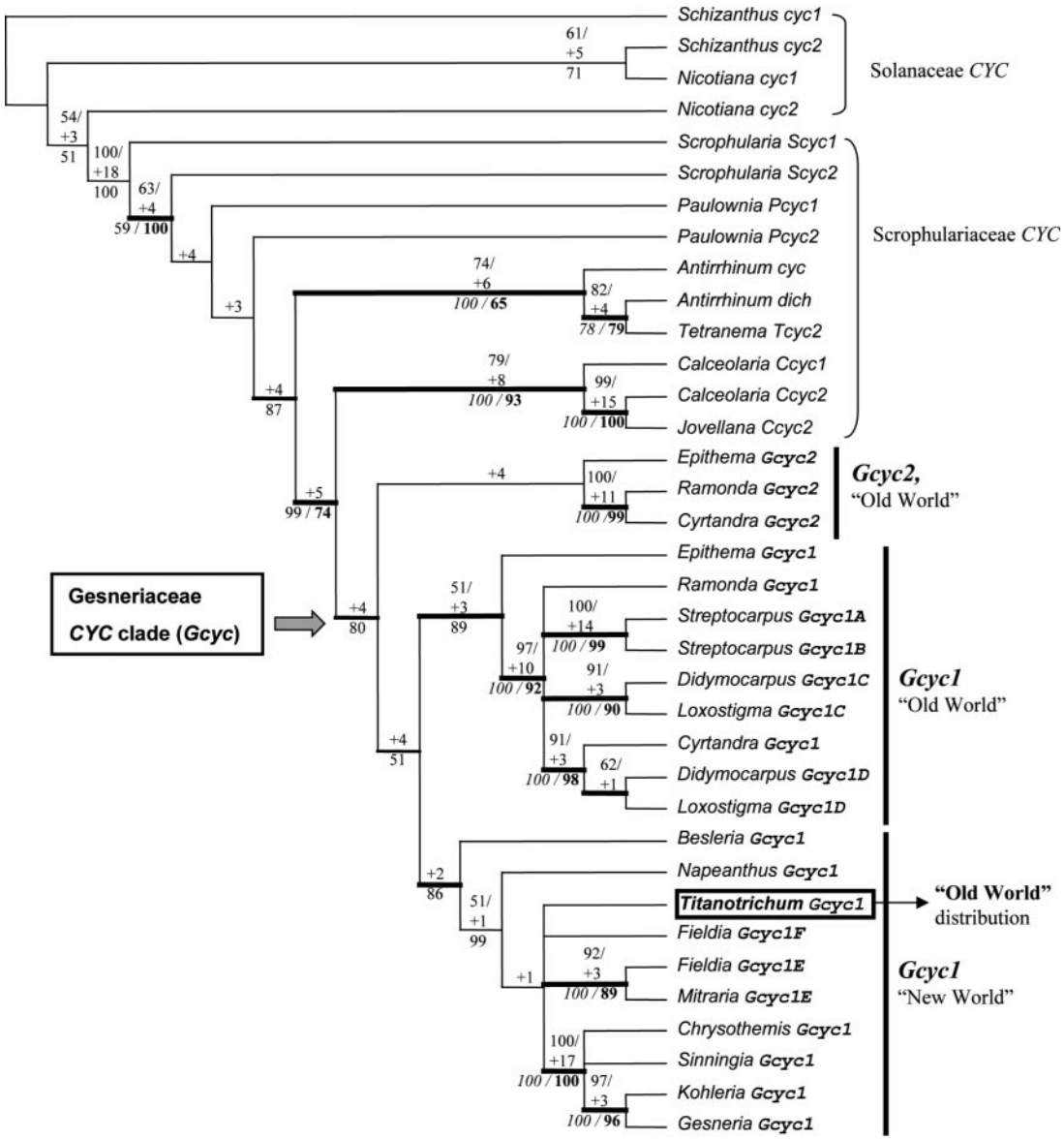

FIG. 3. Strict consensus of 12 most parsimonious trees based on *CYCLOIDEA* nucleotide sequence data. The numbers above the branches are bootstrap values and decay indices. Numbers below the branches are Bayesian 50% majority consensus and ML bootstrap values (bold). Branches in bold indicate those persisting in the nucleotide and protein analysis. The gene abbreviations of *CYC* homologues are accordingly as in Table 1.

low but the major clades were still supported (data not shown). The branches that appeared in both protein and nucleotide strict consensus trees are in bold (Fig. 3).

In pairwise sequence comparisons (Fig. 4), *CYCLOIDEA* apparently evolves ca. three times faster than the chloroplast *trnL-F* intron and spacer region, ca. 3.3 times faster than the chloroplast *atpB-rbcL* spacer, and eight times faster than the nuclear 26S rDNA region used here. Although our results indicate that *CYCLOIDEA* has an eight times higher rate of evolution compared to 26S rDNA, the ratio varied between different *CYC* regions. For instance, when only TCP and R domain sequences were included, the rate of evolution of *CYCLOIDEA* was about the same as for the conserved core region in 26S rDNA.

In our results, *CYCLOIDEA* had the highest average number of steps per character across trees compared to the other gene regions (2.23 for nucleotide data, Table 3). The tanglegram reconciling the most likely *CYC* gene evolution tree and the corresponding species phy-

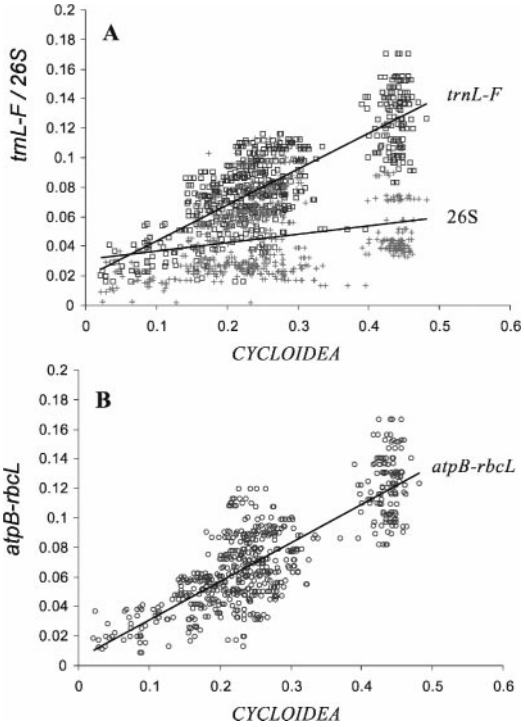

FIG. 4. Comparative rate of *CYCLOIDEA* evolution. Each point represents a pairwise distance (gaps excluded) between every two sequences of *CYCLOIDEA* (x axis) against the pairwise distance between any two sequences of *trnL-F,* 26S or *atpB-rbcL* (y axis). [a]. *trnL-F* (□) vs. *CYC* and 26S rDNA (+) vs. *CYC* ; [b]. *atpB-rbcL* (◦) vs. *CYC.*

logeny is shown in Fig. 5. Ten gene duplication events and twelve losses can be reconstructed.

DISCUSSION

***Phylogenetic Position of* Titanotrichum.** From our combined 3-gene analysis and the *CYCLOIDEA* phylogeny it becomes clear that 'Gesneriaceae including *Titanotrichum*' is a well-supported monophyletic group (Figs. 1, 3). An inconclusive result was obtained in a previous study based on more conserved gene regions, notably chloroplast *atpB* and *rbcL* genes and the nuclear 18S rDNA gene (Soltis et al. 2000). Although Soltis et al. (2000) found strong branch support for a monophyletic Gesneriaceae (jackknife support = 96%), very few Gesneriaceae taxa were sampled (*Rhynchoglossum*, *Cyrtandra*, and *Streptocarpus*), and *Titanotrichum* was placed as the sister group to the rest of the family. The same conclusion was drawn in a study of the phylogeny of the asterids (Albach et al. 2001). They added a further chloroplast gene region (*ndhF*) to perform a 4-gene analysis, which placed *Titanotrichum* as sister to the remaining Gesneriaceae with high branch support (bootstrap value = 85%, on p.164, Fig. 1B). However, because only a fraction of the diversity of this family was sampled in either study, without any New World or South Pacific species included, the conclusions must be viewed as tentative. This shortcoming was specifically addressed in our analysis. Our combined 3-gene study and the *CYCLOIDEA* phylogeny is based on a more extensive sampling, including all tribes of Gesneriaceae and a representative range of taxa from Scrophulariales s. l. Our results show unambiguously that *Titanotrichum* lies within Gesneriaceae and has strong affinities to the subfamilies Gesnerioideae and Coronantheroideae rather than subfamily Cyrtandroideae (Bayesian support values BA-BS=100% in 3-gene tree and 99% in *CYCLOIDEA* tree, Figs. 1, 3).

Unlike Gesnerioideae and Coronantheroideae, *Titanotrichum* is of Old World distribution (Taiwan, Southern Ryukyu and SE China). All other Old World Gesneriaceae show anisocotyly involving the persistent growth of a macro-cotyledon (Jong 1973; Jong and Burtt 1975), but *Titanotrichum* is isocotylous (Wang and Cronk 2003). A recent report of anisocotyly in *Titanotrichum* (Wang et al. 2002) appears to refer to cotyledon asymmetry upon germination rather than a persistent meristematic activity. The isocotyly of *Titanotrichum* is consistent with our placement of the genus with the isocotylous New World Gesneriaceae. *Titanotrichum* possesses scaly rhizomes, unusual among Old World species, which led Sealy (1949) to relate the plant to the New World Gesneriaceae genera *Isoloma* (=*Kohleria*) and *Naegelia* (=*Smithiantha*) of tribe Gloxinieae.

Titanotrichum has a high chromosome number (2n=40) (Ratter 1963), in common with all South Pacific and Australian taxa of subfamily Coronantheroideae analysed to date (2n=~74, ~80, ~90 with 2n=74 most common) (Ratter 1963; Ratter and Prentice 1967). Of the closest allies of *Titanotrichum* in our analysis, *Besleria* has 2n=32 chromosomes (*Napeanthus* has not been investigated). In subfamily Gesnerioideae, the majority have 2n=18, 22, 26, 28 (Burtt and Wiehler 1995). For Old World taxa 2n=18, 30, 32, 34 are most common numbers.

***Evolution of* CYCLOIDEA.** The evolution of *CYCLOIDEA* homologues appeared to be well correlated with the species phylogeny, if duplication events are taken into account (Fig. 5). Reconciling the 3-gene and the *CYC* tree, ten gene duplication events are necessary (Figs. 3, 5). In Gesneriaceae, there is one early duplication event leading to the split between *Gcyc1* and *Gcyc2*. The *Gcyc* homologues (*Gcyc1*) seem to be derived from one lineage of this ancestral duplication event (*Gcyc1*), whereas *Gcyc2* apparently has been lost in all lineages but Epithemateae and Cyrtandreae (Figs. 3, 5) or has not been found yet. Within *Gcyc1,* several lineage-related independent duplication events were also observed. One of these duplications seems to have happened within subfamily Coronantheroideae (e.g., *Fieldia Gcyc1F* vs. *Fieldia Gcyc1E*), which

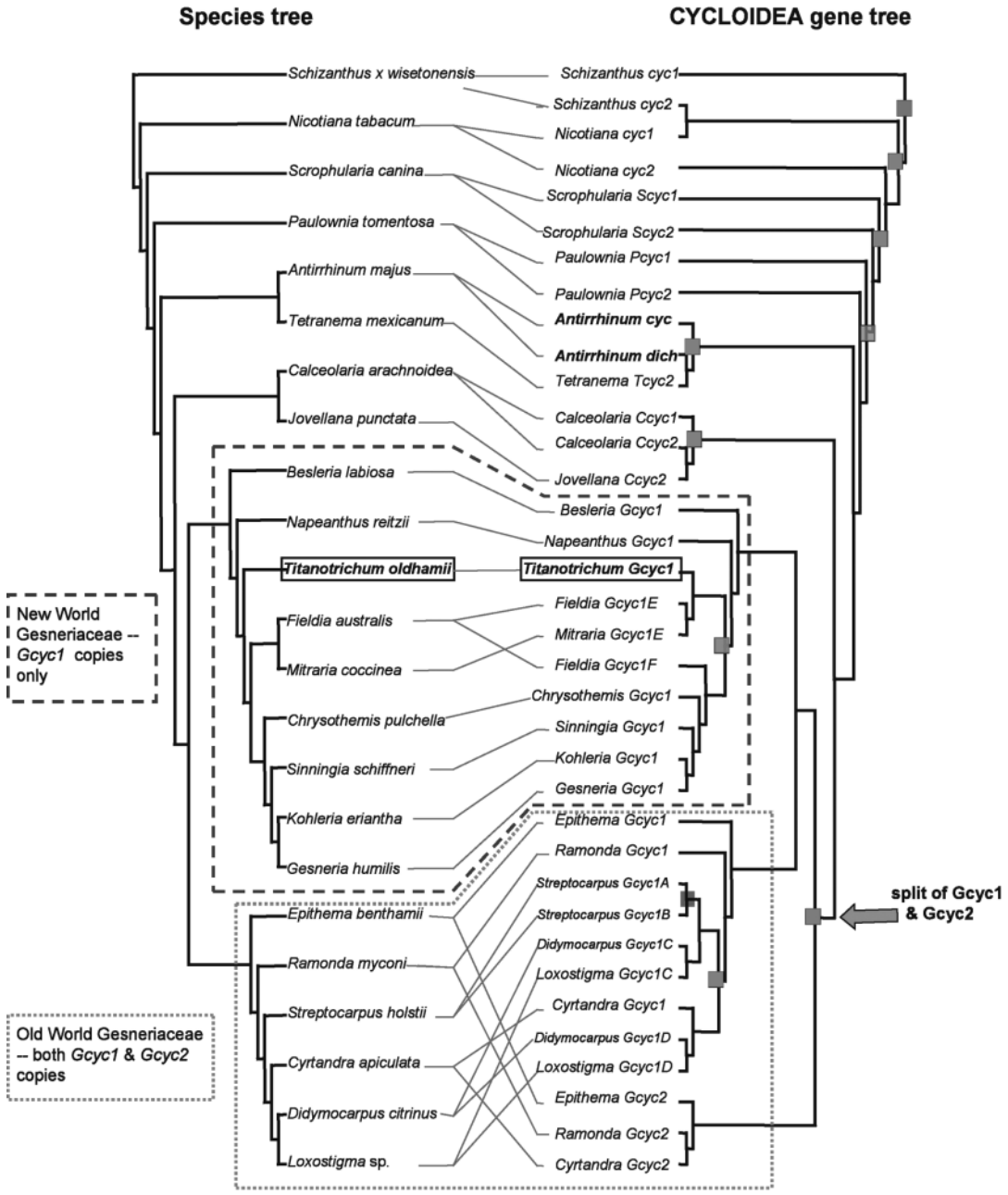

FIG. 5. Tanglegram of reconciled MP trees based on combined 3-gene data and *CYCLOIDEA* data, representing the best combination between 30 *CYCLOIDEA* trees and 32 3-gene (reduced taxa) species tree selected by GENETREE criteria counting the fewest gene duplication and loss events. Square blocks indicate nodes where the duplication events occurred.

may be related to the high polyploidy of this group. A second duplication event happened prior to the divergence of *Loxostigma*, and *Didymocarpus* (*Loxostigma* and *Didymocarpus Gcyc1C* vs. *Loxostigma* and *Didymocarpus Gcyc1D*). A third duplication arose apparently very recently within the genus *Streptocarpus* (*Streptocarpus Gcyc1A* vs. *Streptocarpus Gcyc1B*), previously reported in Citerne et al. (2000).

Therefore the duplication pattern of *Gcyc* in Gesneriaceae falls into two categories: lineage related duplication (associated with cladogenesis events) and the ancient duplication predating the split between New World and Old World taxa. Lineage-related duplications show on average ca. 18.5 % amino acid divergence but the ancient duplication is associated with ca. 39% divergence.

The amino acid sequence of the TCP and R domain was apparently conserved for all taxa we studied but an elevated substitution rate at the third codon position was found in these conserved regions (Fig. 2). The reconstructed *CYC* nucleotide phylogeny was congruent with the amino acid phylogeny only when the third codon positions of the TCP and R domain were excluded. Coding regions beyond these domains were highly variable, both in length and amino acid sequence. They could easily be aligned across closely related taxa but were sometimes ambiguous for taxa from different families. In addition, Möller et al. (1999) found that *Gcyc* evolved in a clock-like manner in their smaller data set from within Gesneriaceae but this has not been tested in our larger data set. Judging from our experience here, the utility of *Gcyc* in phylogenetic studies will be best at the genus level or below. Pico et al. (2002) have even investigated *Gcyc* sequence variation at the population level in *Ramonda myconi*. The use of *Gcyc* for phylogenetic purposes at a higher than generic level is problematic because of alignment difficulties and gene duplication events.

Functional Implications from the* CYCLOIDEA *Homologues. Although only approximately 70% of the ORF of *CYCLOIDEA* was isolated in our analysis, several features indicate that these homologues used in our analysis were functional. All isolated nucleotide sequences could be translated into amino acid sequences with no premature stop codons or frame shifts, and all contained the characteristic TCP and R domain (Cubas et al. 1999b). The few isolated sequences that contained stop codons and frame shifts were believed to be pseudogenes as they lacked the R domain. In addition, all functional homologues clearly had a higher substitution rate at their third codon positions compared to the first and second position, implying that the gene is under tight functional constraints, especially in the TCP + R domains.

Our analysis clearly places *Titanotrichum* within Gesneriaceae and therein with subfamily Gesnerioideae. However, further work is required to establish unambiguously the relationships between *Titanotrichum* and its closest allies in tribes Beslerieae and Napeantheae. Further molecular and cytological data may prove useful in elucidating the precise relationships. A fully resolved phylogeny would also allow a long overdue discussion of the geographic history of the whole family.

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