

Molecular Phylogeny and Character Evolution of *Cymbidium* (Orchidaceae)

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Abstract Molecular phylogenetic analyses using two data sets, derived from DNA sequences of *matK*, the maturase-encoding gene located in an intron of the chloroplast gene *trnK*, and ITS (internal transcribed spacer) region of the 18S–26S nuclear ribosomal DNA, were performed to examine relationships among 37 taxa in *Cymbidium*. Although each data set did not provide conclusive evidence in itself, the data sets combining the two regions yielded the following insights: (1) *Cymbidium* subgenus *Cyperorchis* and section *Pachyrhizanthé*, often treated as independent genera, were nested within the remaining members of *Cymbidium*. (2) All the three subgenera of *Cymbidium*, namely, *Cymbidium*, *Cyperorchis*, and *Jensoa*, turned to be para-/polyphyletic. (3) Among sections of *Cymbidium*, *Austrocymbidium*, *Cyperorchis*, *Iridorchis*, and *Maxillarianthe* did not show monophyly. (4) At specific level, our results indicated the polyphyly of *Cymbidium bicolor*, although bootstrap supports for the clades including *C. bicolor* were not robust.

Key words: *Cymbidium*, ITS, *matK*, molecular, Orchidaceae, phylogeny

Introduction

The genus *Cymbidium* Sw. comprises about 50 species. Geographic distribution extends from the northwestern Himalaya to Japan and south through Indochina and Malesia to northern and eastern Australia. This group exhibits distinctive ecological diversification and occurs as terrestrial and epiphytic life forms. Moreover, one section, *Pachyrhizanthé* Schltr. lacks foliage leaves and has a strongly mycoparasitic existence.

Swartz (1799) established the genus *Cymbidium* with a broad generic delimitation. Circumscription of the genus has been variously defined by later workers: Lindley (1833), Blume (1848, 1849, 1858), Reichenbach (1852, 1864), Hooker (1890), Schlechter (1924), Hunt (1970), Seth & Cribb (1984), and Du Puy & Cribb (1988). At

generic level, there have been inconsistencies on the delimitation. For example, Blume (1848, 1849, 1858), Reichenbach (1852), Hooker (1890), and Schlechter (1924) treated *Cyperorchis* as an independent genus. Another example is *Pachyrhizanthé*. As mentioned above, this group shows very distinctive vegetative morphology and nutritional habit, and Nakai (1931) thus elevated *Pachyrhizanthé* to generic rank.

The first major revision of *Cymbidium* was undertaken by Schlechter (1924) where he proposed sectional limits within the genus. His framework has been modified by Hunt (1970), and Seth & Cribb (1984). So far, the most extensive infrageneric classification of *Cymbidium* is that of Du Puy & Cribb (1988), because their system was based on the most recent information on morphological and anatomical characters.

However, satisfying conclusions have not been affirmed for the infrageneric relationships and circumscriptions of several species in *Cymbidium*.

Recently, DNA sequences of *matK*, the maturase-encoding gene located in an intron of the chloroplast gene *trnK*, and ITS (internal transcribed spacer region) of the 18S–26S nuclear ribosomal DNA have proven to be powerful tools for phylogenetic reconstruction within angiosperm families and genera (e.g., Baldwin, 1992; Johnson and Soltis, 1994; Steele and Vilgalys, 1994). In this study we compared and combined DNA sequences of *matK* and ITS to establish more concrete relationships in *Cymbidium*.

Material and Methods

In this study we selected 37 representative taxa from all the subgenera and sections in *Cymbidium*. Results of *matK* and ITS analyses for tribe Cymbidieae have indicated that the sister group of *Cymbidium* becomes the clade comprising *Acriopsis*, *Grammatophyllum*, and *Thecostele* (Yukawa, unpublished). Consequently, these genera were chosen as outgroup of this study. Materials used in the phylogenetic analyses are shown in Table 1. Voucher specimens are deposited at TNS.

Experimental methods were described in Yukawa *et al.* (1993, 1996). Sequences were determined by amplifying the *matK* (including parts of *trnK* introns) and ITS regions (including 5.8S rDNA and parts of 18S and 26S rDNA) via the polymerase chain reaction (PCR) from a total DNA extract. All of the primers were from Yukawa *et al.* (1999) for *matK* and Douzery *et al.* (1999) for ITS. DNA sequences were aligned manually. Gaps were treated as missing characters. The aligned data file is available from the first author upon request. Parsimony and distance analyses were conducted with PAUP* version 4.0b6 (Swofford, 2001). The heuristic option was used to perform Fitch parsimony analyses (Fitch, 1971). Distance trees were obtained using the

neighbor-joining (NJ) method (Saitou & Nei, 1987) with a Kimura two-parameter correction (Kimura, 1980). To assess the relative robustness for branches, the bootstrap method (Felsenstein, 1985) was used with 1000 replicates.

Results

Phylogenetic analyses

Figure 1 shows a strict consensus of 28,800 most parsimonious (MP) trees derived from the *matK* sequences. The tree had a consistency index (CI) of 0.7726 (0.6115 excluding uninformative characters) and a retention index (RI) of 0.8033. The NJ topology with more than 50% bootstrap support was identical (Fig. 1). A strict consensus of 325 MP trees on the basis of ITS data set is shown in Fig. 2. CI was 0.6848 (0.5606 excluding uninformative characters) and RI was 0.7790. In this data set the NJ topology with more than 50% bootstrap support was identical (Fig. 2). On the other hand, analyses of *matK* sequences provided results substantially concordant with those achieved via analyses of ITS sequences except for positions of *Cymbidium erythrostylum* and *Cymbidium sanderae*. In such cases, analyses of combined data sets excluding problematic taxa provide more resolution and internal support for relationships than do the individual data sets (e.g., Olmstead and Sweere, 1995). We thus conducted a combined analysis of the information derived from *matK* and ITS sequences. In this analysis *C. erythrostylum* and *C. sanderae* were excluded. The length of aligned sequence was 2829 base pairs. Figure 3 shows a strict consensus of 168 MP trees from the combined data set. CI was 0.7283 (0.5931 excluding uninformative characters) and RI was 0.7788. In this data set the clades revealed by NJ analyses with more than 50% bootstrap support was also identical (Fig. 3).

In the combined analysis, the following insights were provided (Fig. 3): (1) *Cymbidium* subgenus *Cyperorchis* and section *Pachyrrhizanthae*, often treated as independent genera, were nested within the remaining members of *Cymbid-*

Table 1. Taxa of *Cymbidium* and outgroups used for sequencing.

Species	Voucher
Subgenus <i>Cymbidium</i>	
Section <i>Cymbidium</i>	
<i>Cymbidium aloifolium</i> (L.) Sw.	TBG118253*
<i>C. bicolor</i> Lindl. subsp. <i>obtusum</i> Du Puy & P. J. Cribb	TBG124328
<i>C. bicolor</i> Lindl. subsp. <i>pubescens</i> (Lindl.) Du Puy & P. J. Cribb	TBG118509
<i>C. finlaysonianum</i> Lindl.	TBG122826
<i>C. rectum</i> Ridl.	Leiden Bot. Gard. 913298
Section <i>Hymantophyllum</i> Schltr.	
<i>C. dayanum</i> Rchb. f.	TBG127899
Section <i>Borneense</i> Du Puy & P. J. Cribb	
<i>C. borneense</i> J. J. Wood	Suzuki s.n.
Section <i>Austrocymbidium</i> Schltr.	
<i>C. canaliculatum</i> R. Br.	TBG135991
<i>C. chloranthum</i> Lindl.	TBG127438
<i>C. hartinahianum</i> J. B. Comber & Nasution	Cibodas Bot. Gard. s.n.
<i>C. madidum</i> Lindl.	TBG119076
Section <i>Floribundum</i> Seth & P. J. Cribb	
<i>C. floribundum</i> Lindl.	TBG127357
<i>C. suavissimum</i> Sander ex C. Curtis	TBG128817
Section <i>Bigibbarium</i> Schltr.	
<i>C. devonianum</i> Paxton	TBG119073
Subgenus <i>Cyperorchis</i> (Blume) Seth & P. J. Cribb	
Section <i>Iridorchis</i> (Blume) P. Hunt	
<i>C. erythraeum</i> Lindl.	TBG135399
<i>C. hookerianum</i> Rchb. f.	TBG133783
<i>C. insigne</i> Rolfe	TBG135526
<i>C. iridioides</i> D. Don	TBG118251
<i>C. lowianum</i> (Rchb. f.) Rchb. f.	TBG54943
<i>C. sanderae</i> (Rolfe) Du Puy & P. J. Cribb	Hiroshima Bot. Gard. 5138
<i>C. tracyanum</i> L. Castle	TBG124406
Section <i>Eburnea</i> Seth & P. J. Cribb	
<i>C. eburneum</i> Lindl.	Suzuki s.n.
<i>C. roseum</i> J. J. Sm.	TBG133724
Section <i>Annamaea</i> (Schltr.) P. Hunt	
<i>C. erythrostylum</i> Rolfe	TBG126650
Section <i>Cyperorchis</i> (Blume) P. Hunt	
<i>C. elegans</i> Lindl.	Karasawa s.n.
<i>C. cochleare</i> Lindl.	Ohba s.n.
Section <i>Parishiella</i> (Schltr.) P. Hunt	
<i>C. tigrinum</i> Parish ex Hook.	TBG133781
Subgenus <i>Jensoa</i> (Raf.) Seth & P. J. Cribb	
Section <i>Jensoa</i> (Raf.) Schltr.	
<i>C. ensifolium</i> (L.) Sw.	TBG118255
<i>C. kanran</i> Makino	TBG133782
<i>C. sinense</i> (Jackson in Andr.) Willd.	Iwagawa s.n.
Section <i>Maxillarianthe</i> Schltr.	
<i>C. aliciae</i> Quisumb.	TBG119071
<i>C. faberi</i> Rolfe	TBG133593
<i>C. goeringii</i> (Rchb. f.) Rchb. f.	TBG115919
<i>C. tortisepalum</i> (Fukuyama) Y. S. Wu & S. C. Chen	TBG133595
Section <i>Geocymbidium</i> Schltr.	
<i>C. lancifolium</i> Hook.	TBG56111
Section <i>Pachyrhizanthus</i> Schltr.	
<i>C. aberrans</i> (Finet) Schltr.	Yagame s.n.
<i>C. macrorrhizon</i> Lindl.	Yagame s.n.
<i>Acriopsis liliifolia</i> (Koen.) Ormerod	Kyoto Bot. Gard. s.n.
<i>Grammatophyllum scriptum</i> (L.) Blume	TBG118874
<i>G. speciosum</i> Blume	Shinjukugyoen s.n.
<i>G. stapeliiflorum</i> (Teijsm. & Binn.) J. J. Sm.	TBG124396
<i>Thecostele alata</i> (Roxb.) Par. & Rchb. f.	TBG118540

*TBG series indicate accession numbers in living collection database at Tsukuba Botanical Garden.

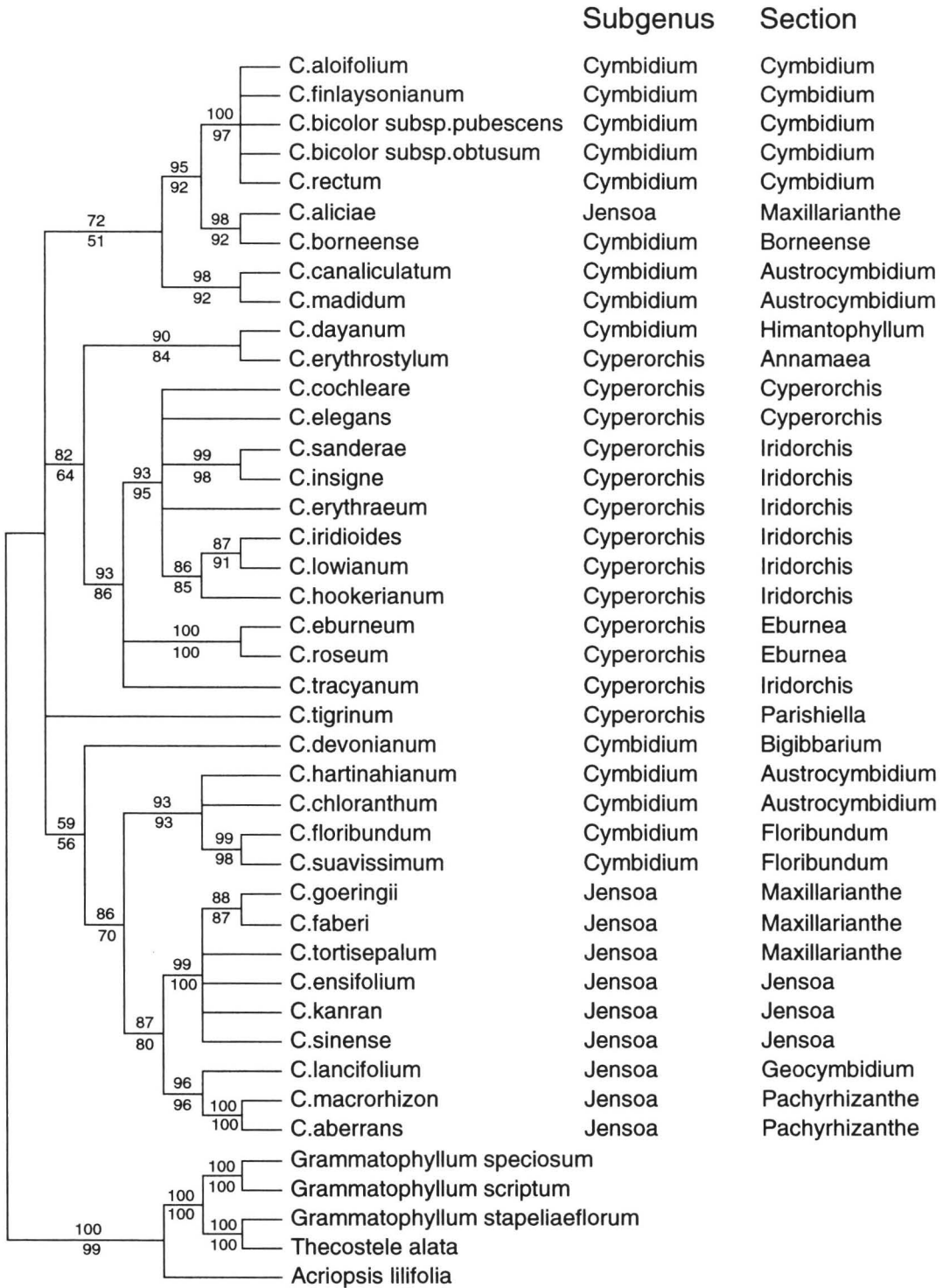


Fig. 1. Strict consensus of 28,800 most-parsimonious Fitch trees based upon *matK* sequences: length=475, consistency index=0.7726 (0.6115 excluding uninformative characters), retention index of 0.8033. Numbers above internodes indicate bootstrap values from 1,000 replicates of Fitch parsimony analysis (maxtree limit of 200 per replicate). Numbers below internodes indicate bootstrap values from 1,000 replicates of neighbor-joining distance analysis.

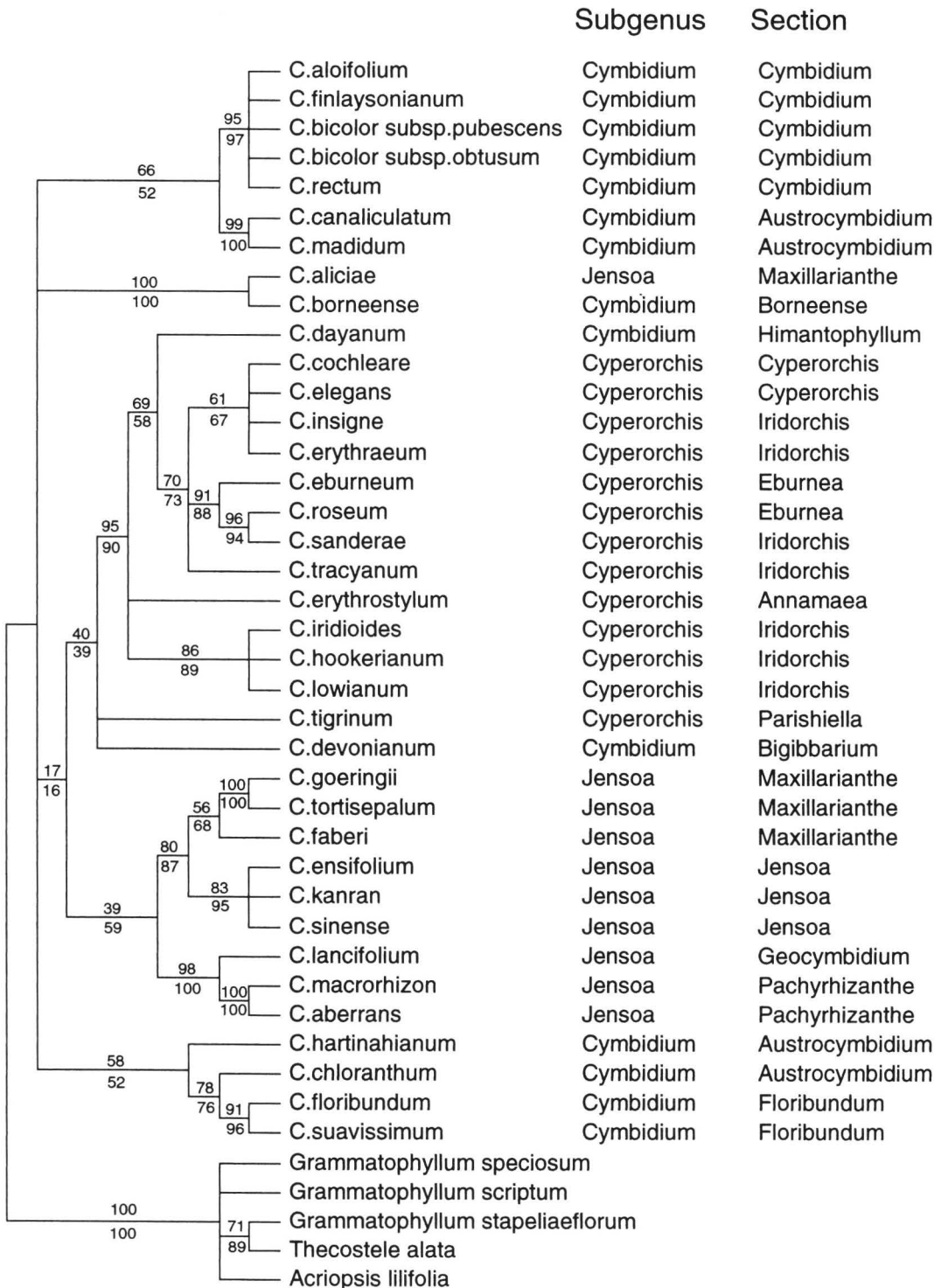


Fig. 2. Strict consensus of 325 most-parsimonious Fitch trees based upon ITS sequences: length=368, consistency index=0.6848 (0.5606 excluding uninformative characters), retention index of 0.7790. Numbers above internodes indicate bootstrap values from 1,000 replicates of Fitch parsimony analysis (maxtree limit of 200 per replicate). Numbers below internodes indicate bootstrap values from 1,000 replicates of neighbor-joining distance analysis.

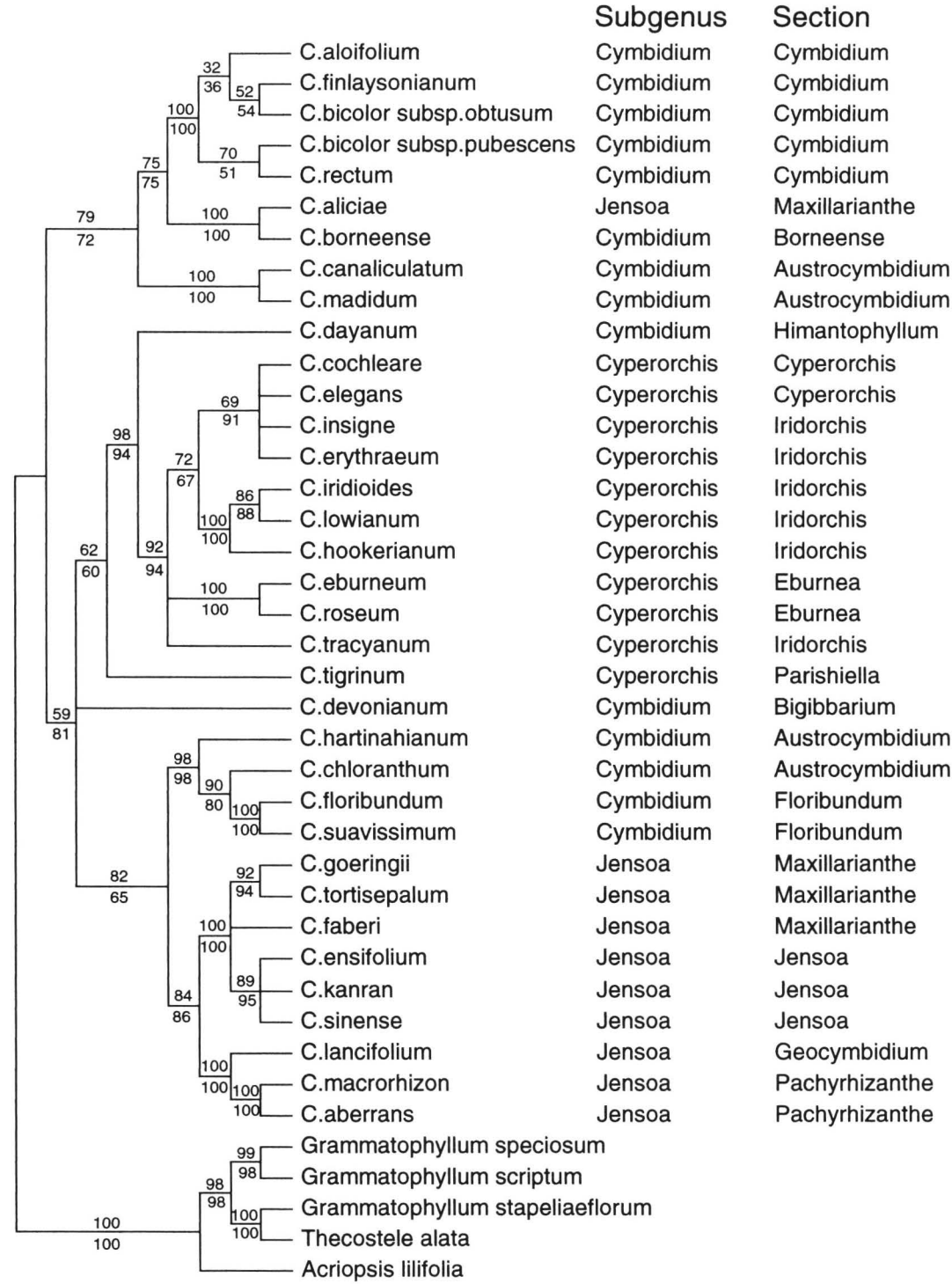


Fig. 3. Strict consensus of 168 most-parsimonious Fitch trees based upon *matK* and ITS sequences: length=828, consistency index=0.7283 (0.5731 excluding uninformative characters), retention index of 0.7788. Numbers above internodes indicate bootstrap values from 1,000 replicates of Fitch parsimony analysis (maxtree limit of 1,000 per replicate). Numbers below internodes indicate bootstrap values from 1,000 replicates of neighbor-joining distance analysis.

ium. (2) All the three subgenera of *Cymbidium*, namely, *Cymbidium*, *Cyperorchis*, and *Jensoa*, turned to be para-/polyphyletic. (3) Among sections of *Cymbidium*, *Austrocymbidium*, *Cyperorchis*, *Iridorchis*, and *Maxillarianthe* did not show monophyly. (4) At specific level, our results indicated the polyphyly of *Cymbidium bicolor*, although bootstrap supports for the clades including *C. bicolor* were not robust.

Discussion

Classification

Generic circumscription of *Cymbidium* by modern taxonomists was supported in this study. *Cyperorchis*, sometimes treated as an independent genus (e.g. Blume, 1848, 1849, 1858; Reichenbach, 1852; Hooker, 1890; and Schlechter, 1924) was nested within other clades of *Cymbidium*. *Pachyrhizanthus*, another problematic group with reduced vegetative morphology and strongly mycoparasitic nutritional existence, was elevated to a new genus by Nakai (1931). The results of this study showed that *Pachyrhizanthus* makes a sister group relationship with section *Geocymbidium* and that it is reasonable to treat this group as a section of *Cymbidium*. Moreover, these two sections have the following synapomorphies: (1) underground rhizomes that survive throughout their life history, (2) whitish perianth lobes with purple striations and markings.

We clarified that all the three subgenera of *Cymbidium* proposed by Seth & Cribb (1984) represent para-/polyphyly. Subgenus *Cymbidium* comprises four clades and these clades become polyphyletic assemblage. It is apparent that the subgenus was defined by plesiomorphic characters such as a lip without any fusing structures and two pollinia. The monophyly of subgenus *Cyperorchis* was broken by a nested position of subgenus *Cymbidium* section *Himantophyllum*. Transfer of section *Himantophyllum* to a member of subgenus *Cyperorchis* results in the loss of a prominent synapomorphic character of subgenus *Cyperorchis*, that is, a fused basal part of the lip with the column. Subgenus *Jensoa* will become a

monophyletic group, if *Cymbidium aliciae* is removed. The four pollinia is a principal synapomorphic character of subgenus *Jensoa*, but *C. aliciae* and *Cymbidium borneense*, distantly related to subgenus *Jensoa*, also share this character. Taking the phylogenetic relationships and the morphological characters into consideration, subdivisions of *Cymbidium* at subgeneric level are not useful. In other words, we did not find any stable synapomorphic characters that feature each major clade clarified in this study.

Our results further indicated that sectional delimitation of *Cymbidium* also has several problems. Among members of subgenus *Cymbidium*, section *Austrocymbidium* exhibits paraphyly and comprises the following three clades: (1) *Cymbidium canaliculatum* and *Cymbidium madidum*; (2) *Cymbidium hartinahianum*; (3) *Cymbidium chloranthum*. The first clade includes the type of this section (*C. canaliculatum*) and corresponds to a revised circumscription of section *Austrocymbidium*. Since the remaining two clades further form another major clade together with section *Floribundum* and morphological characters of the two clades are consistent with those of section *Floribundum*, we extend the limits of this section to include members of the two clades, namely, *C. hartinahianum* and *C. chloranthum*.

In subgenus *Cyperorchis*, the monophyly of the clade including sections *Cyperorchis*, *Eburnea*, and *Iridorchis* was supported by high bootstrap values. However, support on several relationships among members of sections *Cyperorchis* and *Iridorchis* was weak due to few synapomorphic nucleotide substitutions for these groups. It is likely that section *Iridorchis* was established on the basis of plesiomorphic characters such as widely-opened perianth lobes and an operculum without an obvious backwards-pointing beak, but explicit relationships on the basis of more molecular signals are needed to rearrange the classification for these taxa.

As mentioned above, *Cymbidium aliciae* broke the monophyly of subgenus *Jensoa*, more specifically section *Maxillarianthe*. Du Puy & Cribb (1988) placed *C. aliciae* into subgenus *Jensoa*

section *Maxillarianthe* as a synonym of *Cymbidium cyperifolium* Wall. ex Lindl. Our results did not support this placement and suggested a sister group relationship of *C. aliciae* to *Cymbidium borneense*, a sole representative of subgenus *Cymbidium* section *Borneense*. Observations of morphological and anatomical characters (Yukawa & Stern, 2002; Yukawa, unpublished) of *C. aliciae* also supported the affinity of these two species and a distant relationship of section *Maxillarianthe* to them. For example, *C. aliciae* and *C. borneense* have hypodermal fiber strands both in abaxial and adaxial surfaces, but members of section *Maxillarianthe* lack this character at least in abaxial surface. Du Puy & Cribb (1988) established section *Borneense*, because *C. borneense* shows mixed diagnostic characters of subgenus *Cymbidium* section *Cymbidium* and subgenus *Jensoa*. The present results corroborated the soundness of section *Borneense* and the two species, *C. borneense* and *C. aliciae*, constitute the section.

Relationships at species level also may include problems. Our results indicated the polyphyly of *Cymbidium bicolor*, although bootstrap supports for the clades including *C. bicolor* were not robust. On the other hand, a species complex including *Cymbidium ensifolium*, *Cymbidium kanran*, and *Cymbidium sinense* has low resolution. Accumulation of more molecular signals is needed to clarify these relationships.

On the other hand, we found discrepant positions for *Cymbidium erythrostylum* and *Cymbidium sanderiae* between *matK* and ITS trees. Since maternal inheritance of plastid DNA was confirmed in Orchidaceae (Corriveau & Coleman, 1988; Sears, 1980), inconsistent phylogenetic positions for these two species in *matK* trees can be caused by introgressions. Interestingly, collection records of *C. erythrostylum* and *C. sanderiae* are very few, and this fact also may account for their hybrid origin. Moreover, Du Puy and Cribb (1988) also indicated a hybrid nature of *C. sanderiae*. Further studies by use of more markers both from nuclear and plastid genomes can confirm this hypothesis.

Pollinia number

The most controversial morphological character of *Cymbidium* is different numbers of pollinia (two or four) in the genus. Most members of *Cymbidium* have two pollinia, but subgenus *Jensoa* and subgenus *Cymbidium* section *Borneense* exhibit four pollinia. On the other hand, the sister group of *Cymbidium* (*Acriopsis*, *Grammatophyllum*, and *Thecostele*) and the sister group to these taxa (*Bromheadia*; Yukawa, unpublished) share two pollinia. Since the pollinia number of Orchidaceae is usually considered to be stable and has been treated as a cardinal character among taxonomists, variations in this genus have provoked much discussion (e.g. Du Puy & Cribb, 1988). The results of this study showed that the four pollinia are an apomorphic character in *Cymbidium* and likely evolved twice on the lineages leading to subgenus *Jensoa* and subgenus *Cymbidium* section *Borneense* (both in ACCTRAN and DELTRAN optimizations). Our results also indicated that the conservative nature of pollinia number is not a rule, but it is a kind of “dogma” in orchid classification. Actually, variations of pollinia numbers are found in other small- to middle-sized monophyletic groups such as *Thunia* (e.g. Seidenfaden, 1986) and *Phalaenopsis* (e.g. Christenson, 2001).

Life form

As mentioned before, *Cymbidium* exhibits a great variation of life forms. In this study we define epiphytes in a broad sense, that is, a group of species inhabiting trees and/or rocks. In *Cymbidium*, many species grow on trees and some of them also have potentials to live on rocks. However, accurate figures on habitats of each species are not known due to the lack of observations in situ. Besides, there are no *Cymbidium* species that grow exclusively on rocks. We thus use a broad concept of epiphyte.

The sister group taxa of *Cymbidium* (*Acriopsis*, *Grammatophyllum*, and *Thecostele*) are epiphytes and the sister group to these taxa (*Bromheadia*; Yukawa, unpublished) comprises both epiphytic and terrestrial species. The most

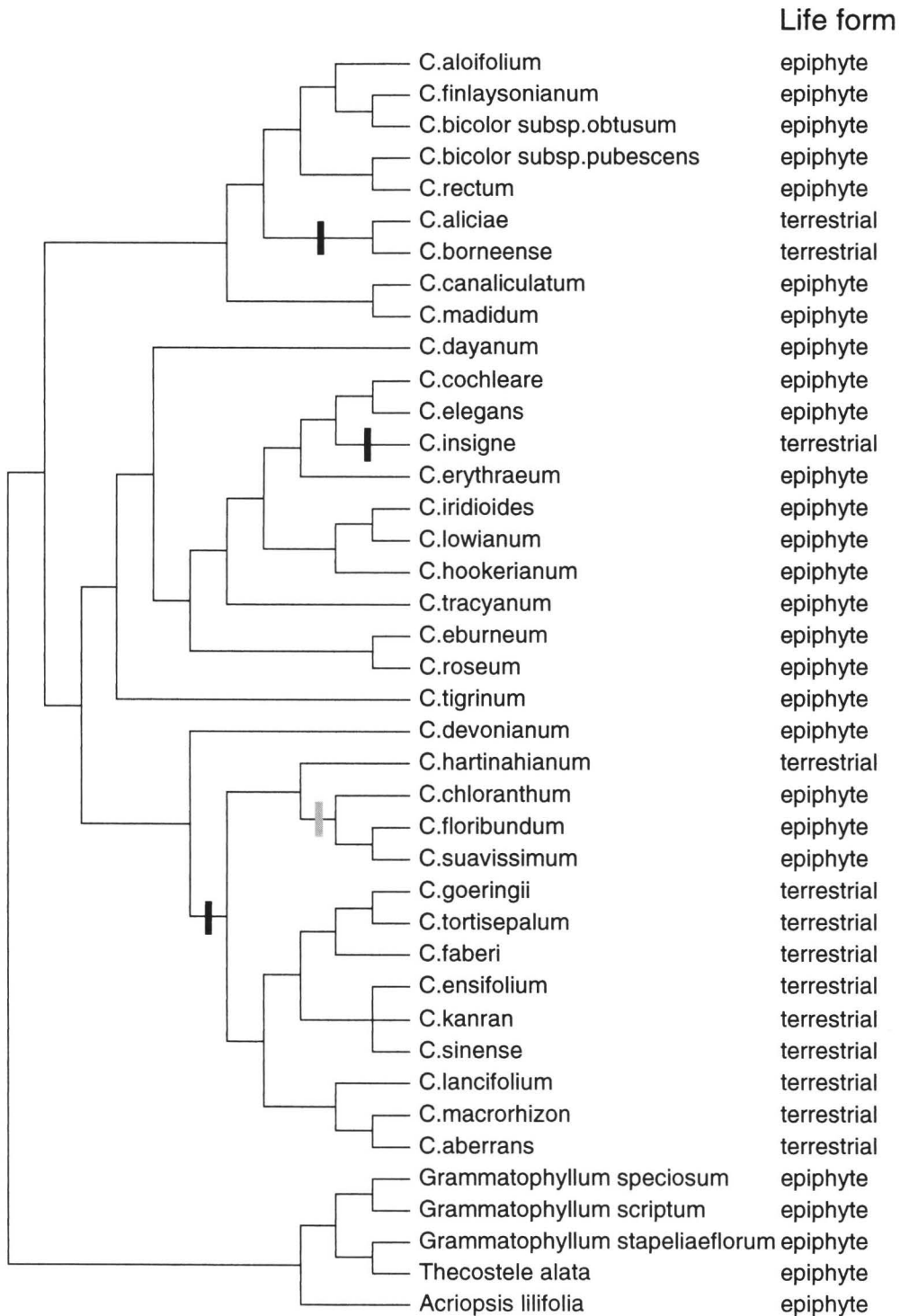


Fig. 4. Reconstruction of diversification in life form in *Cymbidium* under ACCTRAN optimization. The tree is one of 168 most-parsimonious Fitch trees based upon *matK* and ITS sequences. Black bars=change of states from epiphytic to terrestrial habitat; grey bars=change of states from terrestrial to epiphytic habitat.

parsimonious reconstruction of character evolution on life forms indicated changes of character states at internodes shown by bars (Fig. 4). The ancestor of *Cymbidium* was likely an epiphyte. Under ACCTRAN optimization, the epiphytic *Cymbidium* evolved terrestrial plants three times; moreover, a single reversal from terrestrial to epiphytic condition likely occurred in *Cymbidium*. Under DELTRAN optimization, the epiphytic ancestor of *Cymbidium* evolved into terrestrial plants four times, that is, (1) in the common ancestor of *Cymbidium* section *Borneense*, (2) in the ancestor of *Cymbidium insigne*, (3) in the ancestor of *Cymbidium hartinahianum*, (4) in the ancestor of *Cymbidium* subgenus *Jensoa* (results not shown in figures).

Recent phylogenetic analyses clarified that Orchidaceae had a terrestrial ancestry (Neyland and Urbatsch, 1995; Cameron *et al.*, 1999). Therefore, evolution from epiphytic to terrestrial habits in *Cymbidium* represents reversal in Orchidaceae as a whole. These results indicated that the changes of habitats appeared repeatedly in the lineage of *Cymbidium* and are not rare evolutionary events in Orchidaceae.

Nutritional habit

Cymbidium section *Pachyrhizanth* Schltr. keeps strong mycotrophy throughout life history and lacks foliage leaves as well as roots. Albeit Nakai (1931)'s suggestion on its separate taxonomic status from *Cymbidium*, we found that this group belongs to *Cymbidium*. The present results also showed that the state, mycotrophy throughout life history, evolved only once within a clade of terrestrial *Cymbidium* species. All the terrestrial species of *Cymbidium* have obligate mycotrophy in their juvenile stage of life history. During this stage, the rhizome only represents the body and functions as the home of symbiotic fungi. Undoubtedly, the existence of obligate mycotrophy during juvenile stage in terrestrial species of *Cymbidium* acted as preadaptation of complete mycotrophy throughout life history in section *Pachyrhizanth*. Furthermore, the growth habit of section *Pachyrhizanth* only composed

of rhizomes and inflorescences represents a case of heterochrony.

Our molecular data further showed low genetic divergence between the mycotrophic clade and its autotrophic sister clade, namely, *Cymbidium lancifolium* (section *Geocymbidium*). The number of base differences and sequence divergences within the ITS region was as follows: *Cymbidium macrorhizon*-*C. lancifolium*: 9, 0.0118; *Cymbidium aberrans*-*C. lancifolium*: 9, 0.0106. This fact indicated that these apparently drastic shifts of nutritional habit and morphological characters were established within relatively short period of time.

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