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MOLECULAR PHYLOGENETICS OF *CYMBIDIUM* (ORCHIDACEAE: MAXILLARIEAE): SEQUENCE DATA FROM INTERNAL TRANSCRIBED SPACERS (ITS) OF NUCLEAR RIBOSOMAL DNA AND PLASTID *matK*[1]

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ABSTRACT: Nuclear ribosomal spacer (ITS) DNA and plastid *matK* sequence data were collected for 34 accessions of *Cymbidium* and six outgroups from Maxillarieae to evaluate infrageneric classification and phylogenetic relationships. The levels of sequence variation found were higher than in other Epidendroideae studied to date, and phylogenetic relationships obtained moderate to high bootstrap support. Both ITS and *matK* data sets were in overall agreement, and although two or three subgenera can potentially be defined within the genus, there was little molecular support for sectional delimitation because the level of variation was too low. Phylogenetic relationships indicate a southeastern Asian origin for the genus.

THE MOST RECENT, thorough taxonomic treatment of *Cymbidium* Sw. (Du Puy and Cribb, 1988) listed 44 species distributed from northern India throughout Asia to Japan and extending south to northeastern Australia. *Cymbidium* was placed initially in Vandeae by Lindley (1830–1840) based on the pollinarium structure. Dressler's (1993) system, after dismembering Vandoideae, retained *Cymbidium* in Cymbidieae as a member of Cyrtopodiinae together with 11 other genera: *Acrolophia* Pfitz., *Ansellia* Lindl., *Cymbidiella* Rolfe., *Cyrtopodium* R.Br., *Eulophiella* Rolfe., *Galeandra* Lindl., *Grammangis* Rchb.f, *Grammatophyllum* Bl., *Graphorkis* Thou., *Grobya* Lindl., and *Porphyroglottis* Ridl. The morphological cladistic analysis of Freudenstein and Rasmussen (1999) placed *Cymbidium* together with *Thecostele* (Thecostelinae) and *Acriopsis* (Acriopsidinae), and this group was in turn sister to *Catasetum* (Catasetinae). Cameron *et al.* (1999), in a study based on *rbcL* DNA sequences, indicated a placement of *Cymbidium* among Maxillarieae/Cymbidieae as sister to *Grammatophyllum*. Cymbidieae were paraphyletic to Maxillarieae in Cameron *et al.* (1999), and subsequent molecular work has continued to show this pattern; Chase, Freudenstein, and Cameron (2002) thus proposed to combine these two into one tribe, Maxillarieae (the circumscription used here).

Within the genus, four proposed classifications were summarized by Du Puy and Cribb (1988). Their own classification, which divided the genus in three subgenera and 15 sections, was an extended version of previous systems, mainly that of Seth and Cribb (1984). Recently, several studies in Orchidaceae have used DNA sequence data to resolve species phylogenetic relationships and infrageneric classification (Bateman, Pridgeon and Chase, 1997; Cox *et al.*, 1997; Ryan *et al.*, 2000; van den Berg *et al.*, 2000; Whitten, Williams, and Chase, 2000; Williams *et al.*, 2001). Most of these studies have found relatively little genetic variation within genera, and consequently phylogenetic reconstruction at that level was difficult. In this paper, we present sequence data from two DNA regions: the internal transcribed spacers in the nuclear ribosomal DNA (nrITS) and the plastid *matK* gene; we use these to assess monophyly and infrageneric classification of *Cymbidium* by providing a phylogenetic assessment of a majority of species in the genus.

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MATERIAL AND METHODS

A list of the species sampled, voucher numbers, and GenBank accession numbers are provided in Table 1. Although all subgenera and most sections were represented, we were unable to include in this study *C.* subg. *Cymbidium* sect. *Borneense* (monospecific, *C. borneense*), *C.* subg. *Cyperorchis* sect. *Parishiella* (monospecific, *C. tigrinum*), and *C.* subg. *Jensoa* sect. *Pachyrhizanthe* (monospecific, *C. macrorhizon*). For some species more than one accession was included (*C. madidum, C. erythrostylum, C. elegans, C. devonianum*) as a way to check for infraspecific variability and verify that at least all members of these species produced the identical DNA sequences (i.e., that the first plant sampled was not a hybrid with a sequence atypical for that species). Outgroups were defined based on a previous broad analyses that have included many genera of Maxillarieae (e.g. Cameron *et al.*, 1999) and another combined analysis that will be published elsewhere (Freudenstein, Chase, van den Berg, Pridgeon *et al.*, in prep.).

DNA was extracted from fresh leaves using a modified version of Doyle and Doyle (1987) CTAB protocol and purified through cesium chloride/ethidium bromide gradients (1.55g ml^{-1}). Amplification of ITS was performed with primers 17SE and 26SE of Sun *et al.* (1994). We also added 1M betaine to the amplification reactions to counteract the effects of secondary structure, which is frequent with ITS sequences. The amplification program consisted of 28 cycles: 94 C (1 min), 50 C (1 min), and 72 C (3 min) with a final extension of 72 C for 7 min. The *matK* region was amplified as a single piece with an initial 2 min 30 sec melt at 94 C, followed by 30 cycles: 94 C (1 min), 52 C (45 sec), and 72 C (initial 2 min 30 sec with an 8 sec auto extension per cycle) with a final extension of 72 C for 7 min. We used the primers −19F (CGT TCT GAC CAT ATT GCA CTA TG; Molvray *et al.*, 2000) and *trnK*2R (AAC TAG TCG GAT GGA GTA; Johnson and Soltis, 1994) for amplification. All PCR products were cleaned with Concert columns (Gibco BRL, Ltd) according to the manufacturer's protocols. ITS products were sequenced in both directions using 17SE, 26SE and sometimes ITS4 and ITS5 (White *et al.*, 1990). For *matK* we used −19F, 2R and the internal primers 731F (TCT GGA GTC TTT CTT GAG CGA) and 881R (TTM TCA TCA GAA TAA GAG T; both from Pridgeon *et al.*, 2001). Cycle sequencing reactions (26 cycles, 10 sec at 96°C, 5 sec at 50°C, 4 min at 60°C) with Big Dye™ Terminator Mix (Applied Biosystems, Inc.) were performed. Sequencing reactions were cleaned by ethanol precipitation in the presence of 3M sodium acetate (pH 4.6) and subsequently resuspended before running them on a 377 XL DNA sequencer (Applied Biosystems, Inc.).

Eletropherograms were superimposed using Sequencher 3.1 (Genecodes Inc.); each position was individually examined for agreement of the two strands. We manually aligned all sequences following the recommendations of Kelchner (2000), which involves identification of the kind of indel (insertion/deletion) pattern prior to deciding how it should be aligned. We used PAUP 4.0 version 4.0b (Swofford, 1998) for all data analyses. Because there were 12 sequences missing for *matK*, we performed two combined analyses to check for potential discrepancies due to missing data: the first with all taxa including those missing *matK* and a second including only those taxa for which both regions were present. Because of putative incongruence between the two data sets, we performed two additional analyses: the first deleting *C. eburneum* and the second deleting both *C. eburneum* and *C. iridioides*. Heuristic searches included 1000 random taxon-addition replicates and tree-bisection reconnection (TBR) swapping algorithm with a limit of ten trees per replicate to reduce time spent in swapping on islands of longer trees. The trees produced by these replicates were then used as starting trees for an analysis with no tree limit, and all trees found were swapped to completion. For assessing internal support, we used 1000 replicates of character bootstrapping (Felsenstein, 1985) with simple taxon-addition and the sub-tree pruning re-grafting (SPR) algorithm, but saving only ten trees per replicate to prevent swapping on large islands of trees. Aligned data matrices are available from the authors upon request (CB, cassio@innocent.com, and MWC, m.chase@rbgkew.org.uk).

RESULTS

Plastid matK—We obtained *matK* sequence data for 24 accessions of *Cymbidium* and six outgroups. The aligned length of the matrix analyzed

TABLE 1. Plant material, voucher information and GenBank accession numbers in this study.

Species	Voucher (herbarium)	GenBank accession number: ITS/*matK*
Acriopsis javanica Reinw.	*Chase O-149* (K)	AF470492/AF470462
Ansellia africana Lindl.	*Chase O-147* (K)	AF470491/AF470461
Cymbidiella pardalina Rchb.f.	*Chase 10590* (K)	AF470489/AF470459
Cyrtopodium andersonii (Lamb.) R.Br.	*Chase O-341* (K)	AF470490/AF470460
Grammatophyllum speciosum Bl.	ITS from *Chase O-890* (K), *matK* from *Chase O-288* (K)	AF470488/AF470458
Grobya galeata Lindl.	*Chase O-295* (K)	AF470487/AF470457
Cymbidium Sw.		
Subgenus *cymbidum* Section *Austrocymbidium*		
Cymbidium chloranthum Lindl.	*Chase 1502* (K)	AF470499/no *matK*
Cymbidium madidum Lindl.	*Chase 1353* (K)	AF470493/no *matK*
Cymbidium madidum Lindl.	*Chase 1472* (K)	AF470507/AF470473
Subgenus *Cymbidium*, Section *Biggibarium*		
Cymbidium devonianum Paxton	*Chase 1500* (K)	AF470515/AF470479
Cymbidium devonianum Paxton	*Chase O-289* (K)	AF470519/no *matK*
Subgenus *Cymbidium*, Section *Cymbidium*		
Cymbidum aloifolium (L.) Sw.	*Chase 1464* (K)	AF470526/AF470485
Cymbidium atropurpureum (Lindl.) Rolfe	*Chase 1465* (K)	AF470497/AF470465
Cymbidium finlaysonianum Lindl.	*Chase 1480* (K)	AF470514/no *matK*
Cymbidium rectum Ridl.	*Chase 1460* (K)	AF470494/AF470463
Subgenus *Cymbidium*, Section *Floribundum*		
Cymbidium floribundum Lindl.	*Chase 1461* (K)	AF470500/AF470467
Cymbidium floribundum Lindl.	*Chase 1469* (K)	AF470506/no *matK*
Cymbidium suavisissimum Sander ex Curtis	*Chase 1467* (K)	AF470505/AF470472
Subgenus *Cymbidium*, Section *Himantophyllum*		
Cymbidium dayanum Rchb.f.	*Chase 1468* (K)	AF470498/AF470466
Subgenus *Cyperorchis*, Section *Annameae*		
Cymbidium erythrostylum Rolfe	*Chase 1471* (K)	AF470523/AF470483
Cymbidium erythrostylum Rolfe	*Chase 10588* (K)	AF470524/no *matK*
Subgenus *Cyperorchis*, Section *Cyperorchis*		
Cymbidium elegans Lindl.	*Chase 1479* (K)	AF470513/AF470478
Cymbidium elegans Lindl.	*Chase 1501* (K)	AF470516/no *matK*
Cymbidium whiteae King & Pantling	*Chase 1473* (K)	AF470508/AF470474
Subgenus *Cyperorchis*, Section *Eburnea*		
Cymbidium eburneum Lindl.	*Chase 1505* (K)	AF470503/AF470470
Cymbidium mastersii Griffith ex Lindl.	*Chase 1506* (K)	AF470518/AF470481
Subgenus *Cyperorchis*, Section *Iridorchis*		
Cymbidium erythraeum Lindl.	ITS from *Chase O-1463* (K), *matK* from *Chase O-1504* (K)	AF470502/AF470469
Cymbidium hookerianum Rchb.f.	*Chase 1466* (K)	AF470504/AF470471
Cymbidium insigne Rolfe	*Chase 1475* (K)	AF470510/AF470476
Cymbidium iridiodes D.Don	*Chase 1462* (K)	AF470501/AF470468
Cymbidium lowianum (Rchb.f.) Rchb.f.	*Chase 1476* (K)	AF470511/AF470477
Cymbidium sanderae Sander ex Rolfe	*Chase 1470* (K)	no ITS/AF470486
Cymbidium tracyanum L.Castle	*Chase 1482* (K)	AF470525/AF470484
Subgenus *Jensoa*, Section *Geocymbidium*		
Cymbidium lancifolium Hook.	*Chase 1474* (K)	AF470509/AF470475
Cymbidium lancifolium Hook.	*Chase O-293* (K)	AF470520/no *matK*
Subgenus *Jensoa*, Section *Maxillarianthe*		
Cymbidium goeringii (Rchb.f.) Rchb.f.	*Chase 1477* (K)	AF470521/AF470482
Cymbidium goeringii (Rchb.f.) Rchb.f.	*Chase 10587* (K)	AF470522/no *matK*
Subgenus *Jensoa*, Section *Jensoa*		
Cymbidium ensifolium (L.) Sw.	*Chase O-290* (K)	AF470496/AF470464
Cymbidium ensifolium (L.) Sw.	*Chase 1478* (K)	AF470512/no *matK*
Cymbidium kanran Mak.	*Chase 1499* (K)	AF470495/no *matK*
Cymbidium sinense (Jackson in Andr.) Willd.	*Chase 1503* (K)	AF470517/AF470480

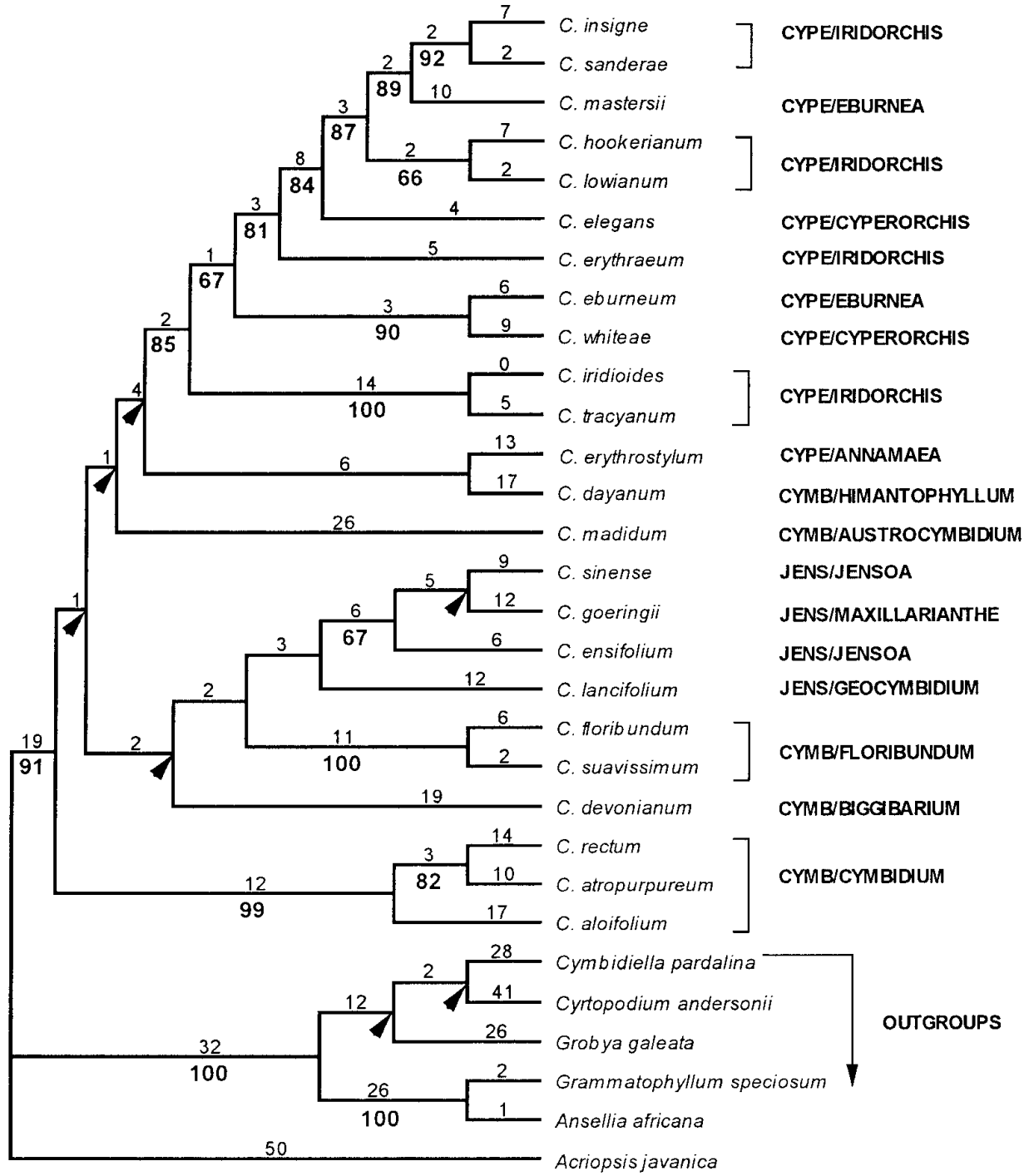

Fig. 1. One of the 32 most parsimonious trees from the *matK* analysis of *Cymbidium*: L = 1276, CI = 0.73, RI = 0.64. Numbers above branches are branch length estimates (ACCTRAN optimization); bootstrap percentages greater than 50% are given below each branch (if no percentage is indicated, then this group received less than 50%). Arrows indicate clades collapsing in the strict consensus tree.

was 1941 base pairs (bp), of which 408 (21%) were variable and 143 (7%) potentially parsimony informative. From the total aligned length, 600 bp were from the spacers and 1341 bp from the exon. One of the 32 most-parsimonious trees produced is shown in Fig. 1: length (L) of 1276 steps, consistency index (CI) of 0.73, and retention index (RI) of 0.64 (Table 2). Clades not present in the strict consensus tree are noted with an arrowhead.

Cymbidium was monophyletic in relation to the outgroups included with 91 bootstrap percentage (BP). One group with all species of *C.* subg. *Cyperorchis* except *C. erythrostylum* had 85 BP, and *Cymbidium* subg. *Cymbidium* sect. *Cymbidium* had 100 BP. The spine of the tree was either unresolved or weakly supported. Some pairs of species received high BP, such as *C. insigne/sanderae* (92 BP), *C. eburneum/whiteae* (90 BP), *C. iridioides/tracyanum* (100 BP), and *C. floribundum/suavissimum* (100 BP). Although there was good

TABLE 2. Data set size, number of informative characters, tree lengths, number of changes, consistency index (CI), retention index (RI) and transition/transversion (ts/tv) ratios across the different DNA regions included in this study, optimized (ACCTRAN) on one of the trees from the combined analysis.

DNA region	Aligned length	Number of inform. char.	Tree length	Number of changes per variable site	CI	RI
ITS whole region	894	196 (21.92%)	756	—	0.67	0.63
ITS1	283	72 (25.44%)	279	3.9	0.72	0.61
ITS2	281	93 (33.10%)	320	3.4	0.63	0.71
matK	1941	143	576	—	0.79	0.74
1st positions	447	34 (7.61%)	116	3.4	0.78	0.82
2nd positions	447	30 (6.71%)	122	4.1	0.73	0.63
3rd positions	447	48 (10.74%)	165	3.4	0.73	0.67

resolution within *C.* subg. *Cyperorchis,* the remaining relationships were weakly supported.

Nuclear ribosomal ITS—34 accessions of *Cymbidium* and six outgroups were included in the analysis. The length of the matrix was 894 bp, of which 384 (43%) were variable and 196 (22%) potentially parsimony informative. One of the 702 most parsimonious trees is shown in Fig. 2: L = 1380 steps, CI = 0.69, RI = 0.62 (Table 2).

Cymbidium was again monophyletic in relation to the outgroups included (98 BP). Although there was low bootstrap support along the spine of the tree, all nodes were resolved in the strict consensus tree. *Cymbidium devonianum* was placed with low support (53 BP) as sister to *C.* subg. *Cyperorchis.* The remaining species of this subgenus were a well-supported group (89 BP). *Cymbidium* subg. *Jensoa* was weakly supported (65 BP) but present in all trees. *Cymbidium* subg. *Cymbidium* was composed of two unrelated clades. The first included *C.* sect. *Floribundum* plus *C. chloranthum* (*C.* sect. *Austrocymbidium*; 94 BP), and the second (69 BP) was composed of *C.* sect. *Cymbidium* as sister to two accessions of *C. madidum* (*C.* sect. *Austrocymbidium*). Within subgenera, support was generally weak, mostly due to the low levels of sequence divergence.

Although there is an overall agreement about groups and their interrelationships among the individual ITS and *matK* phylogenetic trees, small discrepancies should be pointed out. In the ITS tree, *C. dayanum* is embedded in *C.* subg. *Cyperorchis* by the position of *C. hookerianum* and *C. erythrostylum.* However, the nodes in this part of both trees had weak support except the one that included *C.* subg. *Cyperorchis* (89 BP). On the other hand, *matK* placed *C. dayanum* as sister to *C. erythrostylum* but with less than 50 BP. Also, *matK* placed *C. eburneumas* sister to *C. whiteae* with 90 BP, whereas they were distant in the ITS tree, in which *C. eburneum* was sister to *C. mastersii* with 79 BP.

Combined analysis—Because of some apparent incongruence found between the ITS and *matK* datasets within *C.* subg. *Cyperorchis,* we performed four different analyses. We did not use any of the available "tests" for combinability because these have been shown to be unreliable in detecting incongruence (Yoder, Irwin, and Payseur, 2001; Reeves *et al.,* 2001); these studies demonstrated that although the tests indicated the presence of incongruence, the combined analyses demonstrated data congruence. We preferred in our case to look for groups that receive lower support or become unresolved in the combined analysis relative to the individual analyses. As pointed out by Wiens (1998), node by node inspection is the only way to assess which phenomena might be causing incongruence. In the case of these two DNA sequence regions, incongruence was present only in the position of *C. eburneum* and *C. iridioides* within *C.* subgenus *Cyperorchis,* and only the former case with some bootstrap support. From the analyses excluding only *C. eburneum* and then excluding both taxa, we present only the affected differing portions of the trees (Fig. 4A, B; see discussion for a comparison with the analysis including all taxa).

The analyses excluding and including species that lack *matK* did not present topological discrepancies or lower levels of bootstrap support, so we present and discuss that including all taxa.

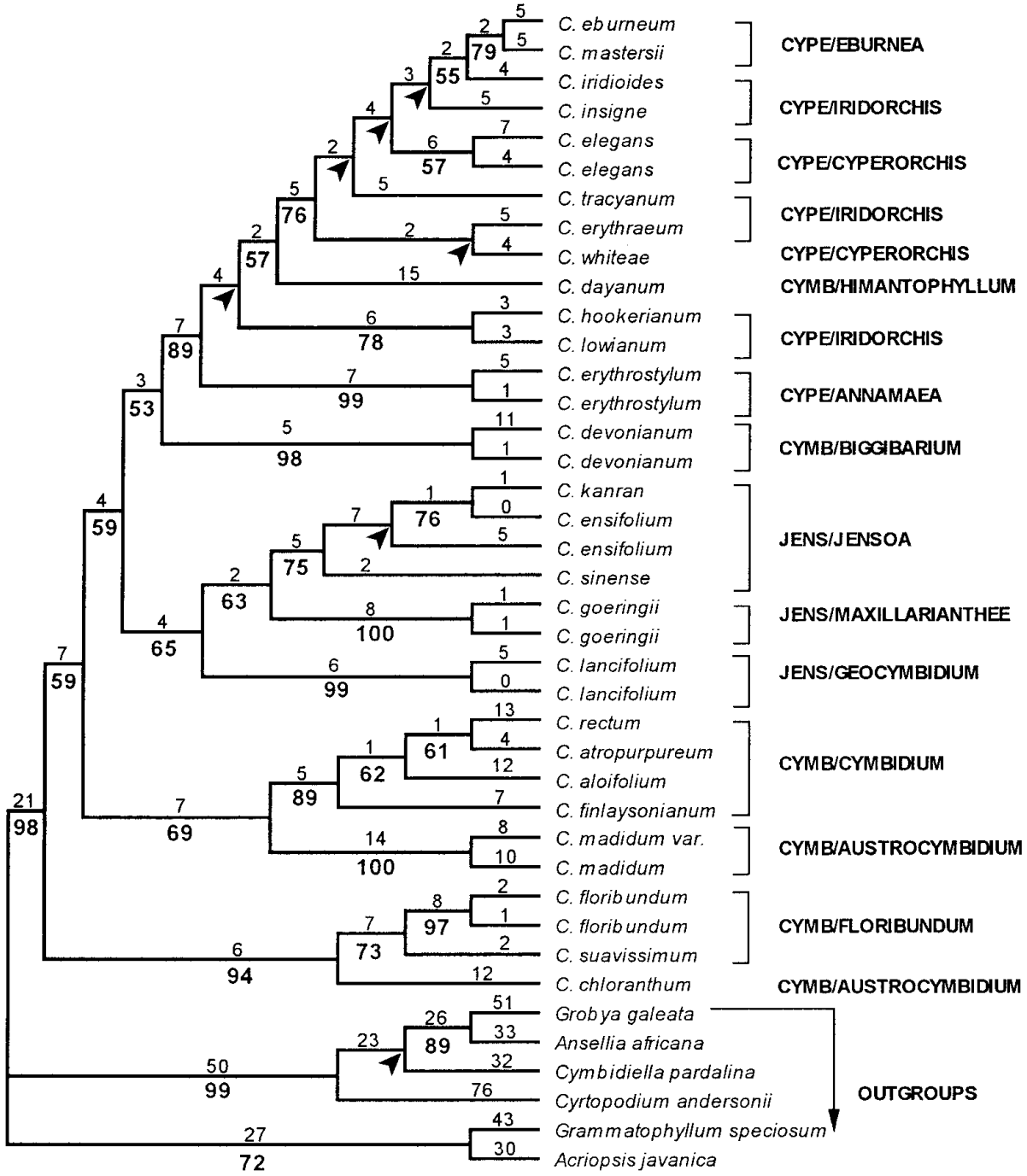

Fig. 2. One of the 702 most parsimonious trees from the ITS analysis of *Cymbidium:* L = 1380, CI = 0.69, RI = 0.63. Numbers above branches are branch length estimates (ACCTRAN optimization); bootstrap percentages greater than 50% are given below each branch (if no percentage is indicated, then this group received less than 50%). Arrows indicate clades collapsing in the strict consensus tree.

This analysis produced 42 trees: L = 1332 steps, CI = 0.72 and RI = 0.6 (Table 2). *Cymbidium* was again monophyletic in relation to the outgroups (100 BP). Four main groups appeared in the tree in addition to *C. devonianum,* for which the position was not clearly resolved. The spine of the tree consisted of nodes with low support (52–56 BP). From the bottom of Fig. 3 upwards, the first clade contained *C.* subg. *Cymbidium* sect. *Floribundum* plus *C. chloranthum* (*C.* sect. *Austrocymbidium*; 93 BP). A second clade contained (79 BP) *C.* subg. *Cymbidium* sect. *Cymbidium* as sister to two accessions of *C. madidum* (*C.* sect. *Austrocymbidium*). A third clade included all species of *C.* subg. *Jensoa* (81 BP), and a fourth group contained all *C.* subg. *Cyperorchis* plus *C. dayanum* (*C.* subg. *Cymbidium* sect. *Himantophyllum*). There was more overall support for

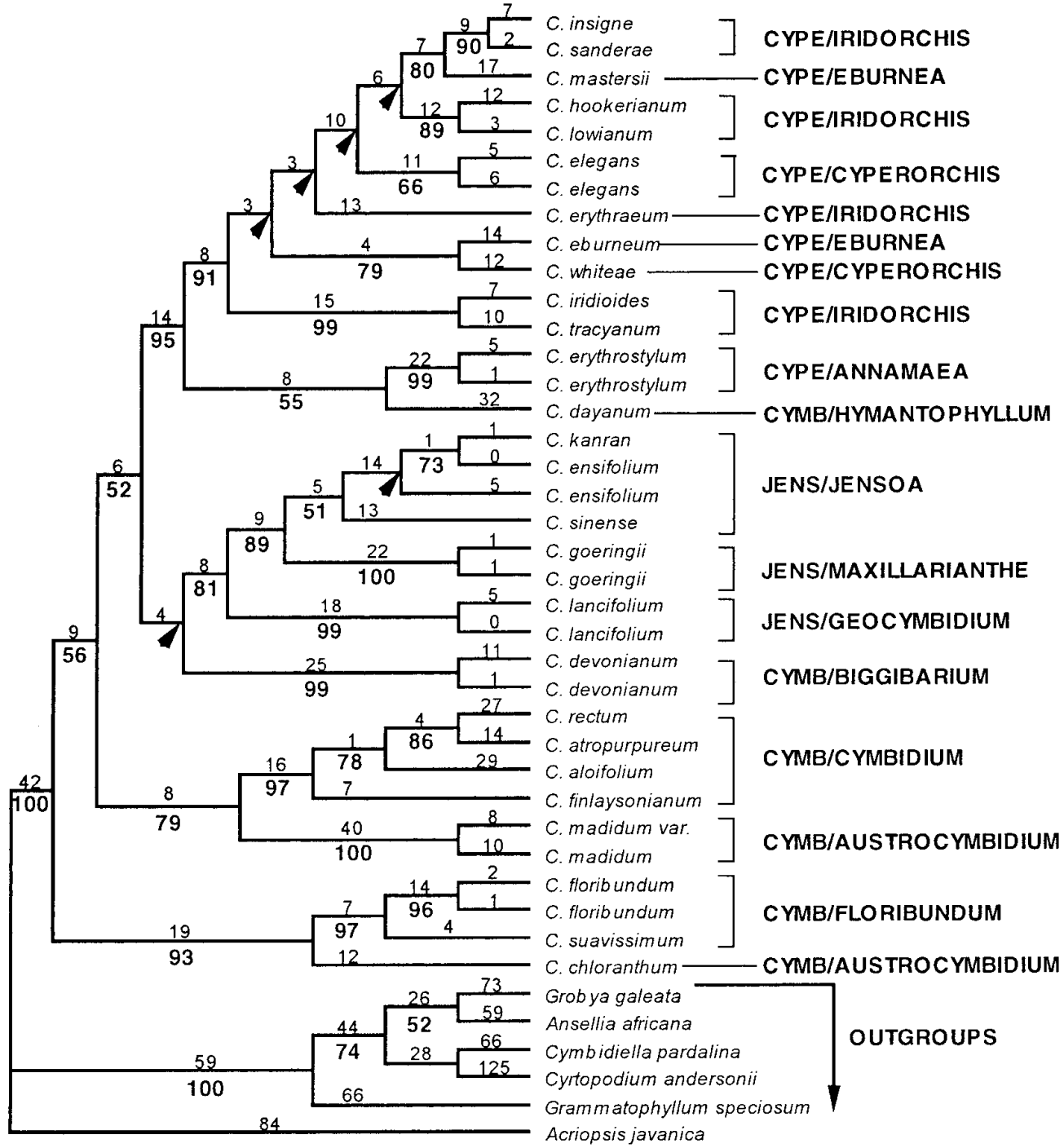

Fig. 3. One of the 42 most parsimonious trees from the combined ITS and *matK* analysis of *Cymbidium* including all taxa (even though one region may be missing): L = 1332, CI = 0.72, RI = 0.67. Numbers above branches are branch length estimates (ACCTRAN optimization); bootstrap percentages greater than 50% are given below each branch (if no percentage is indicated, then this group received less than 50%). Arrows indicate clades collapsing in the strict consensus tree.

clades in the combined analysis than in individual datasets; however, a larger number of clades collapsed within *C.* subg. *Cyperorchis.*

DISCUSSION

Molecular evolution—The levels of genetic variation found in *Cymbidium* are somewhat higher than other published studies involving epiphytic tropical orchids (*Lycaste, Cattleya, Sophronitis s. l., Catasetum*; Pridgeon *et al.*, 1998; Ryan *et al.*, 2000; van den Berg *et al.*, 2000). This is also apparent in the comparatively high number of well-supported groups in the ITS phylogeny. The percentage informative sites in the ITS region is around three-fold that in the *matK* exon, if we do not consider the coding regions, 18S, 5.8S, and 26S, included in the ITS matrix; these have a much lower percentage of variable sites. However, because *matK* is much longer, the total number of characters in each region is similar. As in

other studies (Whitten, Whitten, and Chase, 2000; Williams et al., 2001), CI and RI are higher in the *trnK* region (including *matK*) than in ITS, which reflects mainly the greater numbers of indels and potentially more ambiguous alignment for ITS rather than being directly correlated to the smaller number of variable sites.

Phylogenetic relationships—It is clear that *C. dayanum* is placed with the members of *C.* subg. *Cyperorchis,* but more data will be necessary to determine its exact position within this subgenus. This species has some peculiar morphological characters, and for this reason it was placed alone in its own section (*Himantophyllum*) in all classifications subsequent to that of Schlechter (1924). The position of *C. devonianum* also poses problems. ITS data indicated that it is sister to *C.* subg. *Cyperorchis* (only 53 BP), whereas in the *matK* tree it is unresolved along the spine of the tree. This unresolved position was retained in the combined analysis, and it is impossible to be sure of its affinity relative to the three subgenera. Some morphological characters, nevertheless, indicate a closer relationship to *C.* subg. *Cyperorchis,* with which it shares narrow, acuminate leaf margins in transverse section. Another shared character for *C. devonianum* and *C.* subg. *Cyperorchis* is a seed type that is shorter than in the other subgenera. However, *C. canaliculatum* (not included in this study) also has short seeds.

Many of the other clades were present in both analyses, and the combined tree resembles more closely that of ITS. For example, a well-supported clade with *C.* subg. *Cymbidium* sect. *Cymbidium* (*C. aloifolium/atropurpureum/rectum/finlaysonianum*) appeared in all analyses (*matK* failed to amplify for *C. finlaysonianum*). With *matK* only, this group was placed in an unresolved position along the spine of the tree, whereas with ITS and in the combined analysis it was sister to the two accessions of *C. madidum* (*C.* sect. *Austrocymbidium*). This relationships had 69 BP with only ITS, but it received increased support (79 BP) in the combined analysis. This would indicate that *C.* sect. *Austrocymbidium* could be polyphyletic because the other member, *C. chloranthum,* was placed with high BP in the ITS and combined analyses as sister to *C.* subg. *Cymbidium* sect. *Floribundum* (*C. floribundum/suavissimum*). Unfortunately, we did not have *matK* available to confirm placement of this species, and *matK* also placed *C. madidum* in an unresolved position.

The clade of *C.* sect. *Cymbidium* presents several typical anatomical characters, such as stomata with an elliptical cover and slit-shape pores and a complete layer of subepidermal schlerenchyma cells (all morphological characters reviewed in Du Puy and Cribb, 1988). None of these is present in *C. madidum* (*C.* sect. *Austrocymbidium*), but the first two are found in *C. borneense* (*C.* sect. *Borneense*), which was not included in this study. *Cymbidium* subg. *Jensoa* was monophyletic in all analyses, with less support in ITS alone (65 BP), but increasing to 81 BP in the combined analysis. The species of this section also possess some defining anatomical characters in the leaves, such as a stomatal cover raised above the surrounding epidermis with a circular-shaped pore and a papillose epidermal-cell surface. Within *C.* subg. *Jensoa,* relationships appeared clear, with *C.* sects. *Maxillarianthe* and *Geocymbidium* as successive sisters to *C.* sect. *Jensoa.*

If it were not for the position of *C. dayanum, C.* subg. *Cyperorchis* would be monophyletic. On the other hand, sectional delimitation within *C.* subg. *Cyperorchis* appears to be in conflict between the ITS and *matK* trees. There was little support in the remaining clades within *C.* subg. *Cyperorchis* in the ITS tree, and good support for these with *matK.* Consequently, the topology of the combined tree followed that of *matK* closely. The discrepancies between these datasets might explain why most of the internal branches within *C.* subg. *Cyperorchis* collapse in the strict consensus of the combined analysis. The main difference is in the position of *C. eburneum.* The sequence of *matK* for this species places it close to *C. whiteae* (15 substitutions differ between them), but ITS places it close to *C. mastersii* (they differ by only ten substitutions, whereas for ITS *C. whiteae* and *C. eburneum* differ by 29), which belongs to the same section in Du Puy and Cribb's (1988) classification. The combined analysis followed *matK.* Forcing this taxon to be sister to *C. mastersii* in the combined tree requires seven additional steps. Its removal from the analysis results in reduction of 18 steps but does not alter resolution of the relationships within *C.* subg. *Cyperorchis* (Fig. 4A). Several other species in this group (e.g. *C. iridioides*) are also potentially dis-

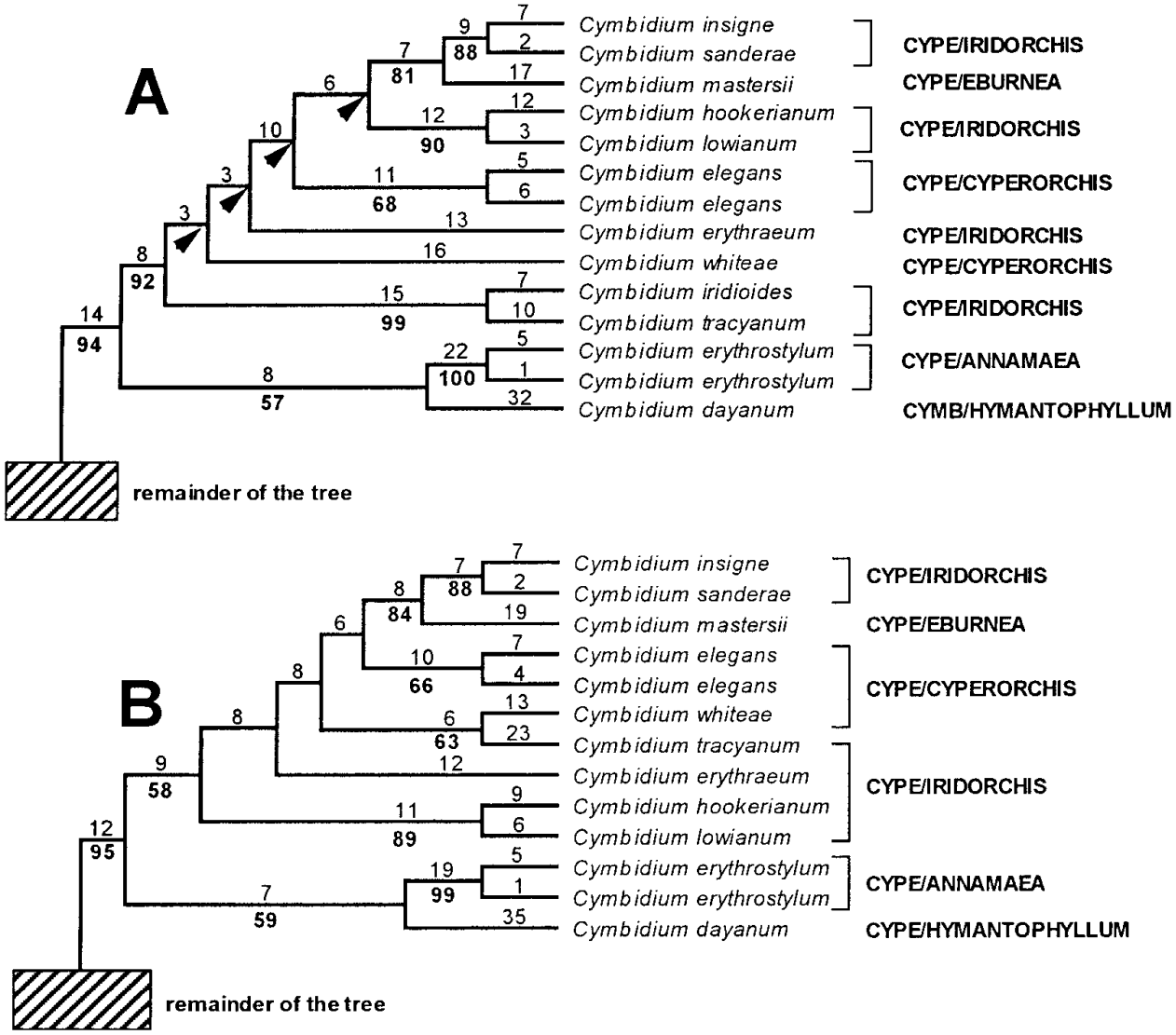

Fig. 4. A. Portion of the tree including *C.* subgen. *Cyperorchis* from one of the 18 shortest trees in the combined analysis excluding *C. eburneum*: L = 1314, CI = 0.72, RI = 0.68. B. Portion of the tree including *C.* subgen. *Cyperorchis* from one of the six shortest trees in the combined analysis excluding both *C. eburneum* and *C. iridoides*: L = 1303, CI = 0.73, RI = 0.67. Numbers above branches are branch length estimates (ACCTRAN optimization); bootstrap percentages greater than 50% are given below each branch (if no percentage is indicated, then this group received less than 50%). Arrows indicate clades collapsing in the strict consensus tree.

cordantly placed in the two analyses, so there appears to be a general pattern of incongruence within this clade. With the exclusion of both *C. eburneum* and *C. iridioides* (Fig. 4B), there were no collapsed branches within *C.* subg. *Cyperorchis,* but these relationships did not receive higher BP. It is noteworthy that the *matK* tree (Fig. 1) contained several groups with support not recovered in the combined analysis, even excluding these two taxa with a clearer pattern of incongruence. The most plausible explanation for this contrast would be several events of hybridization and possible introgression in this group, which would explain not only the conflicting positions of *C. eburneum* and *C. iridioides* but also a more diffuse and subtler pattern of incongruence that persists even when these two species are excluded. More data, morphological and molecular, are needed for this group.

The geographical origin of *Cymbidium* indicated by the relationships from the combined analysis would likely be southeastern Asian. The clade that falls as sister to all the rest comprises three species, two of which are Indo-Chinese, the other being found in Sumatra and Java. The only Australian species of *C.* sect. *Austrocymbidium* included in the analyses, *C. madidum,* lies in the next clade that is successively sister to the rest and is sister to species of *C.* sect. *Cymbidium* from southeastern Asia and the eastern Malay Archipelago. Thus, it appears likely that *Cymbidium* was an early and perhaps repeated colonizer of

Australia from southeastern Asia via the western Malay Archipelago. An analysis of Cyrtopodiinae (C. van den Berg, in prep.) placed *Acriopsis* as the genus closest to *Cymbidium* (which is also the case here, although without a non-Maxillarieae outgroup the root cannot be determined), and the distribution of this genus, which is also predominantly southeastern Asian (with one species extending into Australasia), also supports this pattern. Future studies should aim at sampling the remaining species and adding more DNA regions to increase the levels of internal support.

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