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Research paper

Phylogenetic incongruence in *Cymbidium* orchids

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ABSTRACT

Cymbidium, which includes approximately 80 species, is one of the most ornamental and cultivated orchid genera. However, a lack of markers and sparse sampling have posed great challenges to resolving the phylogenetic relationships within the genus. In the present study, we reconstructed the phylogenetic relationships by utilizing one nuclear DNA (nrITS) and seven plastid genes (*rbcL*, *trnS*, *trnG*, *matK*, *trnL*, *psbA*, and *atpI*) from 70 species (varieties) in *Cymbidium*. We also examined the occurrence of phylogenetic conflict between nuclear (nrITS) and plastid loci and investigated how phylogenetic conflict bears on taxonomic classification within the genus. We found that phylogenetic conflict and low support values may be explained by hybridization and a lack of informative characteristics. Our results do not support previous classification of the subgenera and sections within *Cymbidium*. Discordance between gene trees and network analysis indicate that reticulate evolution occurred in the genus *Cymbidium*. Overall, our study indicates that *Cymbidium* has undergone a complex evolution.

1. Introduction

The genus *Cymbidium* SW., with approximately 80 species, is primarily distributed throughout the subtropics and tropical areas of Asia and northern Australia (Du Puy and Cribb, 2007; Chen et al., 2009; Pridgeon et al., 2009). In China, more than 50 species are found according to the most recent revision and recently published new species (Liu and Chen, 1998, 2002, 2004, 2005; Long et al., 2003; Liu et al., 2006; Chen et al., 2009; Lan et al., 2018; Zhang et al., 2018). *Cymbidium* is one of the earliest orchid groups to be cultivated, making excellent potted plants and cut flowers due to its extremely high ornamental and economic value. Commercially important hybrids have been cultivated for a long time in China and adjacent regions (Liu et al., 2006). Despite its acknowledged importance, limited and often ambiguous morphological differences pose challenges to understanding intergeneric relationships within *Cymbidium*. The intergeneric relationships of *Cymbidium* remain an open question due to limited and often ambiguous morphological differences.

Since the establishment of *Cymbidium* by Swartz in 1799, various generic delimitations and infrageneric systems have been proposed based on morphological characters. Dressler (1981) places *Cymbidium* in Cymbidieae of Vandioideae, which contains all of the sympodial vandoid orchids, mostly with two pollinia. Schlechter (1924) proposes an infrageneric system of *Cymbidium* with eight sections, which is the basis of the modern infrageneric classification of *Cymbidium*, and most sections are still being recognized more or less in their original form. Hunt (1970) included *Cyperorchis* within *Cymbidium* and maintained Schlechter's sectional divisions. Seth and Cribb (1984) initially divided *Cymbidium* into three subgenera based on the number of pollinia and the state of fusion between lip and column: subgenus *Cymbidium* with two pollinia and free lip, subgenus *Cyperorchis*

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with two pollinia and fusion of the lip and column base, and subgenus *Jensoa* with four pollinia and free lip. Puy and Cribb (1988) slightly modified this treatment and added section *Borneensia* for the recently described *Cymbidium borneense* Wood. Liu et al. (2006) followed the treatment of Puy and Cribb (1988) with some modifications and additions, added sections *Nanula* and *Axillaria*, transferred the section *Borneensia* from subgenus *Cymbidium* into the subgenus *Jensoa*, and reduced the section *Maxillarianthe* to synonymy of the section *Jensoa.*

Recently, the results of molecular analyses have shed new light on the taxonomy of *Cymbidium* (Cameron et al., 1999; Berg, 2002; Yukawa and Stern, 2002; Zhang et al., 2002; Sharma et al., 2012; Yang et al., 2013; Lan et al., 2018). The phylogeny of *Cymbidium* reconstructed by Zhang et al. (2002) based on the analyses of nrITS sequences in 30 taxa belonging to three subgenera showed that the genus was split into several clades and intermixed with the main subgenera, suggesting that the previous division among three subgenera should be evaluated with more data. Berg (2002) performed a molecular phylogenetic analysis of *Cymbidium* using one cpDNA marker (*matK*) and nrITS and indicated that two or three subgenera can potentially be defined within the genus and affirmed a southeast Asian origin for the genus. Yukawa and Stern. (2002) obtained the same result in their strict consensus tree based on nrITS and *matK* sequences. Assessment of phylogenetic interrelationships in the genus *Cymbidium* from northeast India based on nrITS showed that this genus was divided into three subgenera (Sharma et al., 2012). Du Puy and Cribb (2007) according to the DNA studies by Berg (2002) and Yukawa and Stern (2002), concluded that the subgenera of *Cymbidium* were not monophyletic, and they retained the sections but dispensed with subgenera.

All of the pre-DNA era classifications of *Cymbidium* were based on a relatively small set of morphological aspects and features, especially on the lip and pollinium numbers, which have led to considerable taxonomic uncertainty and debates (Dressler, 1993; Freudenstein and Rasmussen, 1999). As previous results of molecular systematics were largely based on sparse sampling across *Cymbidium* or mainly utilized either a single DNA marker (especially nrITS) or two markers (nrITS and *matK*), some conclusions and results were weakly supported or even without statistical support (Cameron et al., 1999; Yukawa and Stern, 2002; Zhang et al., 2002; Yang et al., 2013; Lan et al., 2018). Therefore, it is necessary to understand the relationships within *Cymbidium* and the delimitation of the infrageneric taxa to base the analyses on multiple DNA markers and a denser sampling across *Cymbidium*.

One additional challenge to accurately reconstructing phylogenetic relationships is the possibility of phylogenetic incongruence between cpDNA and nuclear sequence data (Tu et al., 2008; Pelser et al., 2010; Guo et al., 2015; Tang et al., 2015). Phylogenetic incongruence may be a result of stochastic errors, systematic errors, incomplete lineage sorting (ILS), introgressive hybridization (IH), paralogous gene sampling, or horizontal gene transfer (HGT) (Geuten et al., 2004; Richardson and Palmer, 2007; Russell et al., 2010; Yang et al., 2012; Francine et al., 2017).

In this study, we reconstructed the phylogeny of 70 representatives in three subgenera of *Cymbidium* using both plastid DNA (*rbcL*, *trnS*, *trnG*, *matK*, *trnL*, *psbA*, and *atpI*) and nrITS sequences. Our goals were to (1) establish a phylogeny based on the seven plastid DNA and one nuclear gene for the *Cymbidium* with significantly increased taxa sampling with an emphasis on Chinese species, (2) identify relationships that were inconsistent between nuclear and plastid trees, and (3) explore possible causes of the incongruence.

2. Material and methods

2.1. Taxon sampling

To assess phylogenetic relationships within *Cymbidium*, we used seven plastids (*rbcL*, *trnS*, *trnG*, *matK*, *trnL*, *psbA* and *atpI*) and one nuclear (nrITS) marker sampled from 70 species (varieties) of *Cymbidium*. Four species from the tribe Malaxideae Lindley (Lindley, 1826) and one species from the tribe Vandeae Lindley (Lindley, 1821) were used as outgroups. The selection of outgroups was based on the classification of Chase et al. (2015). In this study, 510 sequences (468 of which were newly sequenced) were obtained, the voucher information and GenBank accession numbers were listed in Table 1, and the specimen was deposited in the herbarium of the National Orchid Conservation Center of China (NOCC).

2.2. Collection of DNA sequences

Total DNA was extracted from fresh material using a modified CTAB procedure of Doyle and Doyle (1987). DNA extraction, PCR amplification, and sequencing were performed according to Chen et al. (2017). The primers used for PCR analysis were listed in Table S1.

2.3. Sequence analysis and alignment

Both forward and reverse sequences referring to the corresponding chromatograms were edited and assembled into contig sequences using SeqMan v.7.1 (DNAStar, USA) with the default "Classic Assembler" parameters (Match Size = 12; Minimum Match Percentage = 80). DNA sequences were aligned with MEGA 5.05 under the Muscle model and manually adjusted to account for obvious or missing inserts (Tamura et al., 2011; Zhang et al., 2013).

2.4. Identification of incongruence

The congruence among the nuclear data (nrITS) and the combined chloroplast DNA data set (*rbcL*, *trnS*, *trnG*, *matK*, *trnL*, *psbA* and *atpI*) was tested using the incongruence length difference (ILD) test (Farris et al., 1995), implemented as the Partition Homogeneity test in PAUP* v.4.0b10, and followed procedures described by Li et al. (2015). Incongruence was also visually inspected for in trees that exhibited contrasting topologies when obtained from different data sets. The thresholds of hard incongruence followed those adopted by Pelser et al. (2010): bootstrap values $\geq$ 80 and/or PP $\geq$ 0.95, as well as ILD $P < 0.01$.

2.5. Phylogenetic analyses

Phylogenetic analyses were performed using Bayesian inference (BI) and maximum-likelihood (ML) methods. The evolutionary models for the ML and BI analyses were determined by jModelTest using the Akaike Information Criterion (AIC). ML analysis was performed using the CIPRES Science Gateway web server (RAxML-HPC2 on XSEDE 8.2.10) (Miller et al., 2010) with 1000 bootstrap replicates and settings that are described in Edgar. (2004). BI analysis was performed using the CIPRES Science Gateway web server (MrBayes 3.2.6 on XSEDE) (Stamatakis et al., 2008). The following settings were used: sampling frequency = 1000; tem = 0.1; burn-in = 2000; and number of Markov chain Monte Carlo generations = 10,000,000 (Li et al., 2015).

Table 1
Taxa studied, voucher information and GenBank accessions. A dash (–) indicates missing data, an asterisk (*) denotes sequences obtained in this study, and the remaining sequences are from GenBank.

Species	Voucher	nrITS	*matK*	*rbcL*	*trnL*	*atpI*	*trnS*	*psbA*	*trnG*
Cymbidium aestivum	Z.J.Liu 200254	MK439805*	MK439758*	MK439781*	MK439712*	MK439655*	MK439667*	MK439737*	MK439688*
Cymbidium aloifolium	Z.J.Liu 6591	MF861139*	MF861054*	MF861098*	MF860930*	MF860838*	MF860955*	–	MF860889*
Cymbidium atropurpureum	Z.J.Liu 6592	MF861153*	MF861069*	MF861111*	MF860945*	MF860837*	MF860986*	MF861196*	MF860902*
Cymbidium banaense	Z.J.Liu 5331	MF861160*	MF861076*	MF861118*	MF860952*	–	MF860992*	MF861201*	MF860909*
Cymbidium baoshanense	Z.J.Liu 2581	MK439807*	MK439760*	MK439783*	MK439714*	MK439662*	MK439669*	MK439739*	MK439690*
Cymbidium bicolor		AF284696	KX298601	–	–	–	–	FJ527762	–
Cymbidium candliculatum	Z.J.Liu 6326	MF861161*	MF861078*	–	–	MF860850*	MF860994*	MF861168*	MF860911*
Cymbidium changningense	Z.J.Liu 6430	MF861126*	MF861042*	MF861085*	MF860917*	MF860865*	MF860961*	MF861173*	MF860876*
Cymbidium chloranthum		AF470499	HM137047	–	–	–	–	FJ527761	–
Cymbidium cochleare	Z.J.Liu 2807	MF861130*	MF861045*	MF861089*	MF860921*	MF860846*	MF860964*	MF861176*	MF860880*
Cymbidium cyperifolium	Z.J.Liu 3205	MK439808*	MK439761*	MK439784*	MK439715*	MK439654*	MK439670*	MK439740*	MK439691*
Cymbidium daweishanense	Z.J.Liu 8663	MH59389 *	MH593898*	MH574772*	–	–	–	–	–
Cymbidium dayanum	Z.J.Liu 6437	MF861122*	MF861038*	MF861081*	MF860913*	MF860831*	MF860957*	MF861169*	MF860872*
Cymbidium defoliatum	Z.J.Liu 2554	MF861135*	MF861050*	MF861094*	MF860926*	MF860859*	MF860969*	MF861181*	MF860885*
Cymbidium devonianum	Z.J.Liu 2693	MF861136*	MF861051*	MF861095*	MF860927*	MF860860*	MF860970*	MF861182*	MF860886*
Cymbidium eburneum	Z.J.Liu 2625	MF861124*	MF861040*	MF861083*	MF860915*	MF860833*	MF860959*	MF861171*	MF860874*
Cymbidium eburneum var. *longzhouense*	Z.J.Liu 3032	MF861144*	MF861059*	MF861103*	MF860935*	MF860864*	MF860977*	MF861162*	MF860894*
Cymbidium elegans	Z.J.Liu 6399	MF861147*	MF861062*	MF861106*	MF860938*	MF860870*	MF860980*	MF861190*	MF860897*
Cymbidium ensifolium	Z.J.Liu 6599	MF861138*	MF861053*	MF861097*	MF860929*	MF860849*	MF860972*	MF861184*	MF860888*
Cymbidium erythraeum	Z.J.Liu 2900	MK439809*	MK439762*	MK439785*	MK439716*	MK439651*	MK439671*	MK439741*	MK439692*
Cymbidium erythraeum var. *flavum*	Z.J.Liu 10140	MK439810*	MK439763*	MK439786*	MK439717*	MK439660*	MK439672*	MK439742*	MK439693*
Cymbidium erythrostylum		AF470524	AF470483	–	–	–	–	–	–
Cymbidium faberi	Z.J.Liu 7071	MF861148*	MF861063*	MF861107*	MF860939*	MF860854*	MF860981*	MF861191*	MF860898*
Cymbidium finlaysonianum		AF470514	HM137048	–	–	–	–	FJ527763	–
Cymbidium floribundum	Z.J.Liu 3256	MK439811*	MK439764*	MK439787*	MK439718*	MK439652*	MK439673*	MK439743	MK439694*
Cymbidium gaoligongense	Z.J.Liu 6432	MF861142*	MF861057*	MF861101*	MF860933*	MF860840*	MF860975*	MF861187*	MF860892*
Cymbidium goeringii	Z.J.Liu 2522	MK439812	MK439765	MK439788	MK439719	MK439646	MK439674	MK439744	MK439695
Cymbidium haematodes	Z.J.Liu 10160	MK439813	MK439766	MK439789	MK439720	MK439658	MK439675	MK439745	MK439696
Cymbidium hookerianum	Z.J.Liu 6425	MF861143*	MF861058*	MF861102*	MF860934*	MF860863*	MF860976*	–	MF860893*
Cymbidium insigne	Z.J.Liu 3251	MF861140*	MF861055*	MF861099*	MF860931*	MF860847*	MF860973*	MF861185*	MF860890*
Cymbidium iridioides	Z.J.Liu 6429	MF861141*	MF861056*	MF861100*	MF860932*	MF860845*	MF860974*	MF861186*	MF860891*
Cymbidium kanran	Z.J.Liu 2808	MK439814*	MK439767*	MK439790*	MK439721*	MK439659*	MK439676*	MK439746*	MK439697*
Cymbidium lancifolium	Z.J.Liu 7013	MF861137*	MF861052*	MF861096*	MF860928*	MF860839*	MF860971*	MF861183*	MF860887*
Cymbidium lowianum var. *iansonii*	Z.J.Liu 3029	MF861146*	MF861061*	MF861105*	MF860937*	MF860869*	MF860979*	MF861189*	MF860896*
Cymbidium macrorhizon	Z.J.Liu 200231	MK439815*	MK439768*	MK439791*	MK439722*	MK439666*	MK439677*	–	MK439698*
Cymbidium maguanense	Z.J.Liu 3257	MF861125*	MF861041*	MF861084*	MF860916*	MF860834*	MF860960*	MF861172*	MF860875*
Cymbidium mannii	Z.J.Liu 6590	MF861121*	MF861037*	MF861080*	MF860912*	MF860830*	MF860956*	–	MF860871*
Cymbidium mastersii	Z.J.Liu 2924	MK439816*	MK439769*	MK439792*	MK439723*	MK439653*	MK439678*	MK439747*	MK439699
Cymbidium micranthum	Z.J.Liu 2705	MF861149*	MF861065*	–	MF860941*	MF860842*	MF860982*	MF861193*	–
Cymbidium multiradicatum	Z.J.Liu 2614	MK439817*	–	MK439793*	MK439724*	MK439644*	MK439679*	MK439748*	MK439700*
Cymbidium nanulum	Z.J.Liu 2562	MF861152*	MF861068*	–	MF860944*	MF860862*	MF860985*	MF861195*	–
Cymbidium omeiense	Z.J.Liu 3101	–	MF861064*	MF861108*	MF860940*	MF860855*	–	MF861192*	MF860899*
Cymbidium paucifolium	Z.J.Liu 2112	MF861151*	MF861067*	MF861110*	MF860943*	MF860844*	MF860984*	MF861166*	MF860901*
Cymbidium puerense	Z.J.Liu 10626	MG980600*	MG980601*	MG980602*	MG980604*	MG980599*	MG980603*	–	–
Cymbidium pumilum		AF284699	–	–	–	–	–	–	–
Cymbidium qiubeiense	Z.J.Liu 2555	MF861158*	MF861074*	MF861116*	MF860950*	MF860853*	MF860990*	–	MF860907*
Cymbidium rectum		AF470494	AF470463	–	–	–	–	FJ527767	–
Cymbidium rhizomatosum	Z.J.Liu 2559	MF861150*	MF861066*	MF861109*	MF860942*	MF860843*	MF860983*	MF861194*	MF860900*
Cymbidium schroederi	Z.J.Liu 2837	MF861155*	MF861071*	MF861113*	MF860947*	MF860867*	MF860988*	MF861198*	MF860904*
Cymbidium serratum	Z.J.Liu 2575	MF861134*	MF861049*	MF861093*	MF860925*	MF860856*	MF860968*	MF861180*	MF860884*
Cymbidium sichuanicum	Z.J.Liu 3027	MF861154*	MF861070*	MF861112*	MF860946*	MF860857*	MF860987*	MF861197*	MF860903*
Cymbidium sinense	Z.J.Liu 2503	MF861159*	MF861075*	MF861117*	MF860951*	MF860861*	MF860991*	MF861163*	MF860908*
*Cymbidium sp.*7066	Z.J.Liu 7066	MF861132*	MF861047*	MF861091*	MF860923*	MF860852*	MF860966*	MF861178*	MF860882*
*Cymbidium sp.*5256	Z.J.Liu 5256	MK439820*	MK439772*	MK439796*	MK439727*	MK439645*	–	MK439750*	MK439703*
*Cymbidium sp.*10161	Z.J.Liu 10161	MK439821*	MK439773*	MK439797*	MK439728*	MK439661*	–	MK439751*	MK439704*
*Cymbidium sp.*5774	Z.J.Liu 5774	MK439822*	MK439774*	MK439798*	MK439729*	MK439663*	–	MK439752*	MK439705*
*Cymbidium sp.*10163	Z.J.Liu 10163	MK439823*	–	MK439799*	MK439730*	MK439643*	MK439681*	–	MK439706*
*Cymbidium sp.*5828	Z.J.Liu 5828	MK439824*	MK439775*	MK439800*	MK439731*	MK439664*	MK439682*	–	MK439707*
*Cymbidium sp.*6016	Z.J.Liu 6016	MK439825*	MK439776*	MK439801*	MK439732*	MK439665*	MK439683*	MK439753*	MK439708*
Cymbidium suavissimum	Z.J.Liu 2881	MK439826*	MK439777*	–	MK439733*	MK439649*	MK439684*	MK439754*	–
Cymbidium teretipetiolatum	Z.J.Liu 2949	MK439827*	MK439778*	MK439802*	MK439734*	MK439647*	MK439685*	MK439755*	MK439709*
Cymbidium tigrinum	Z.J.Liu 10115	MK439828*	MK439779*	MK439803*	MK439735*	MK439648*	MK439686*	MK439756*	MK439710*
Cymbidium tortisepalum	Z.J.Liu 6403	MF861133*	MF861048*	MF861092*	MF860924*	MF860858*	MF860967*	MF861179*	MF860883*
Cymbidium tortisepalum var. *longibracteatum*	Z.J.Liu 7008	MF861131*	MF861046*	MF861090*	MF860922*	MF860851*	MF860965*	MF861177*	MF860881*
Cymbidium tracyanum	Z.J.Liu 6426	MF861123*	MF861039*	MF861082*	MF860914*	MF860832*	MF860958*	MF861170*	MF860873*
Cymbidium wenshanense	Z.J.Liu 6431	MF861128*	MF861043*	MF861087*	MF860919*	MF860835*	–	MF861174*	MF860878*
Cymbidium quinquelobum	Z.J.Liu 10113	MK439829*	MK439780*	MK439804*	MK439736*	MK439650*	MK439687*	MK439757*	MK439711*
Cymbidium whiteae		AF470508	AF470474	–	–	–	–	–	–
Cymbidium wilsonii	Z.J.Liu 7025	MF861156*	MF861072*	MF861114*	MF860948*	MF860868*	MF860989*	MF861199*	MF860905*

Table 1 (*continued*)

Species	Voucher	nrITS	*matK*	*rbcL*	*trnL*	*atpI*	*trnS*	*psbA*	*trnG*
Cymbidium dianlan	Z.J.Liu 6039	MK319538*	MK319536*	MK319537*	–	–	–	–	–
Eulophia graminea		AF284727	FJ565159	KF358040	–	–	–	FJ564680	–
Galeandra devoniana		EU877142	KF660268	AF074171	–	–	EU877105	–	–
Paraholcoglossum amesianum		KX29864	JN106350	–	JN106343	JX202637	JX202760	JX202707	–
Phalaenopsis lamelligera		AY912233	EU179845	AY389387	AY265765	–	–	–	–
Tsiorchis kimballiana		HQ404400	JN106345	HQ404490	HQ452931	JX202640	JX202763	HQ404450	–

2.6. Network analyses

To visualize conflicts among gene trees, SPLITSTREE4 v.4 (Huson and Bryant, 2006) was used to generate a consensus tree (seven cpDNAs and one nuclear gene).

3. Results

3.1. Sequences and alignment

In the present study, 468 new *Cymbidium* sequences were obtained from 15 sections (except section *Borneensia*) of three subgenera, the division of sections within *Cymbidium* was adopted according to Liu et al. (2006). However, we were unable to amplify the chloroplast regions of a few accessions; we treated these as missing data. Aligned sequence length were as follows: 700 bp for the nrITS region (111 bp parsimony-uninformative and 189 bp parsimony-informative in the data set), 1293 bp for *rbcL*, 1626 bp for *matK*, 768 bp for *trnG*, 1027 bp for *trnS*, 1314 bp for *trnL*, 955 bp for *psbA*, and 838 bp for *atpI*. A total of 869 bp of the combined plastid regions (7524 bp) were parsimony-uninformative; 1004 bp were parsimony-informative. The numbers of variable and parsimony informative sites were listed in Table 2. Details pertinent to the best-fit model of molecular evolution could be found in Table 3.

3.2. Phylogenetic analysis of the combined cpDNA data set

Our phylogenetic analyses of cpDNA focused on the combined data set. Seventy-one taxa were included in this cpDNA matrix, five of which were outgroups. Four major groupings within *Cymbidium* were recovered with moderate support (Fig. 1). Clade A consisted of one species of subgenus *Cymbidium*, three species of subgenus *Jensoa* and 23 species of subgenus *Cyperorchis*. Clade B was composed of one species of subgenus *Cymbidium* and one species of subgenus *Jensoa*, *Cymbidium dayanum* Rchb. f. and *Cymbidium omeiense* Y.S. Wu et S.C. Chen. Clade C was composed of nine species, seven species of subgenus *Cymbidium,* and two species of subgenus *Cyperorchis* (PP = 0.91). Clade D contained three species of subgenus *Cymbidium*, one species of subgenus *Cyperorchis*, and 24 species of subgenus *Jensoa* (PP = 0.91).

Table 2
Statistics from the analyses.

Information	nrITS	Combined Plastid
No. of taxa	75	71
Aligned length (bp)	710	7805
No. parsimony-uninformative	111	869
No. parsimony-informative	189	1004
Tree length	563	4302
Consistency index	0.72	0.54
Retention index	0.55	0.65

3.3. Phylogenetic analysis of the nrITS data set

Seventy-five samples, including five species identified as outgroups, were sampled for the nrITS analysis, and three differentiation clades (clades A-C) were recovered (Fig. 2).

(1) Clade A included nine species from two sections of subgenus *Cymbidium* clustered in a single lineage (PP = 0.9, BS = 68): *C. rectum* Ridley., *C. mannii* Richb. f., *C. paucifolium* Z.J. Liu et S.C. Chen, *C. bicolor* Lindl., *C. canaliculatum* R.Br., and *C. aloifolium* (L.) Sw. *C. finlaysonianum* Lindl., *C. atropurpureum* (Lindl.) Rolfe, and *C. puerense* Z.J. Liu et S.R. Lan.
(2) Clade B consisted of 27 species from two subgenera and was poorly resolved with low support (PP = 0.55, BS = 52). It was subdivided into eight subclades (subclades 1–8). Subclade 1, which was sister to the other seven subclades, and included two subgenera: *Cymbidium* and *Cyperorchis*, with weak support. Subclade 2 contained eight species from five sections: *Iridorchis*, *Eburnea*, *Annamaea*, *Cyperorchis*, and *Parishiella* with weak support. The subclade 3 consisted of two species of section *Cyperorchis*: *Cymbidium wenshanense* and *C. quinquelobum*, with strong support (PP = 1, BS = 100). Subclade 4 only included one species of section *Himantophyllum* from subgenus *Cymbidium*, *C. dayanum*, with strong support (PP = 0.91). The subclade 5 (PP = 0.91, BS = 66) was composed of four species from section *Iridorchis.* Subclade 6 included two species from section *Iridorchis.* Subclade 7 was composed of two species of section *Cyperorchis* and one species of section *Iridorchis* and was sister to subclade 8, which consisted of four species of section *Eburnea*; however, the relationships had low support.
(3) Clade C (PP = 0.52, BS = 68) was composed of 27 species and seven un-identified species from two subgenera, subgenus *Cymbidium* and subgenus *Jensoa.* Six divergent subclades (subclades 9–14) were recovered in this clade. Subclade 9 included six species of subgenus *Cymbidium*, with strong support (PP = 1, BS = 92). The subclade 10 was composed of two species of section *Geocymbidium* and one species of section *Pachyrhizon.* Subclade 11 only included one species of section *Pachyrhizon.* Subclade 12 was composed of nine species from three sections (sections *Axillaria*, *Jensoa*, and *Nanula*) with strong support (PP = 1, BS = 97), but the interrelationships were poorly resolved with low support. Subclade 13 consisted of *Cymbidium cyperifolium* Wall. et Lindl., *C. defoliatum* Y.S. Wu et S.C. Chen, and *C. faberi* Rolfe, and one unidentified species was sister to the remaining members of this clade. Subclade 14 consisted of 11 species from two subgenera (*Jensoa* and *Cymbidium*), but the interrelationships were poorly resolved with low support.

3.4. Incongruence tests

Our molecular analyses using nrITS and a combined cpDNA data sets indicated many topological conflicts, some of which

Table 3
Best-fit model and parameter for the analysis data sets.

Region	AIC select model	Base frequencies				Substitution model (rate matrix)						I	G
		A	C	G	T	A-C	A-G	A-T	C-G	C-T	G-T		
nrITS	TIM3+G	0.1878	0.2905	0.3607	0.1609	0.6159	2.7987	1.0000	0.6159	6.5213	1.0000	0.0000	0.4760
cpDNA	TVM + I + G	0.3227	0.1599	0.1699	0.3476	1.1776	1.2415	0.6934	0.2902	1.2414	1.0000	0.5340	0.3770

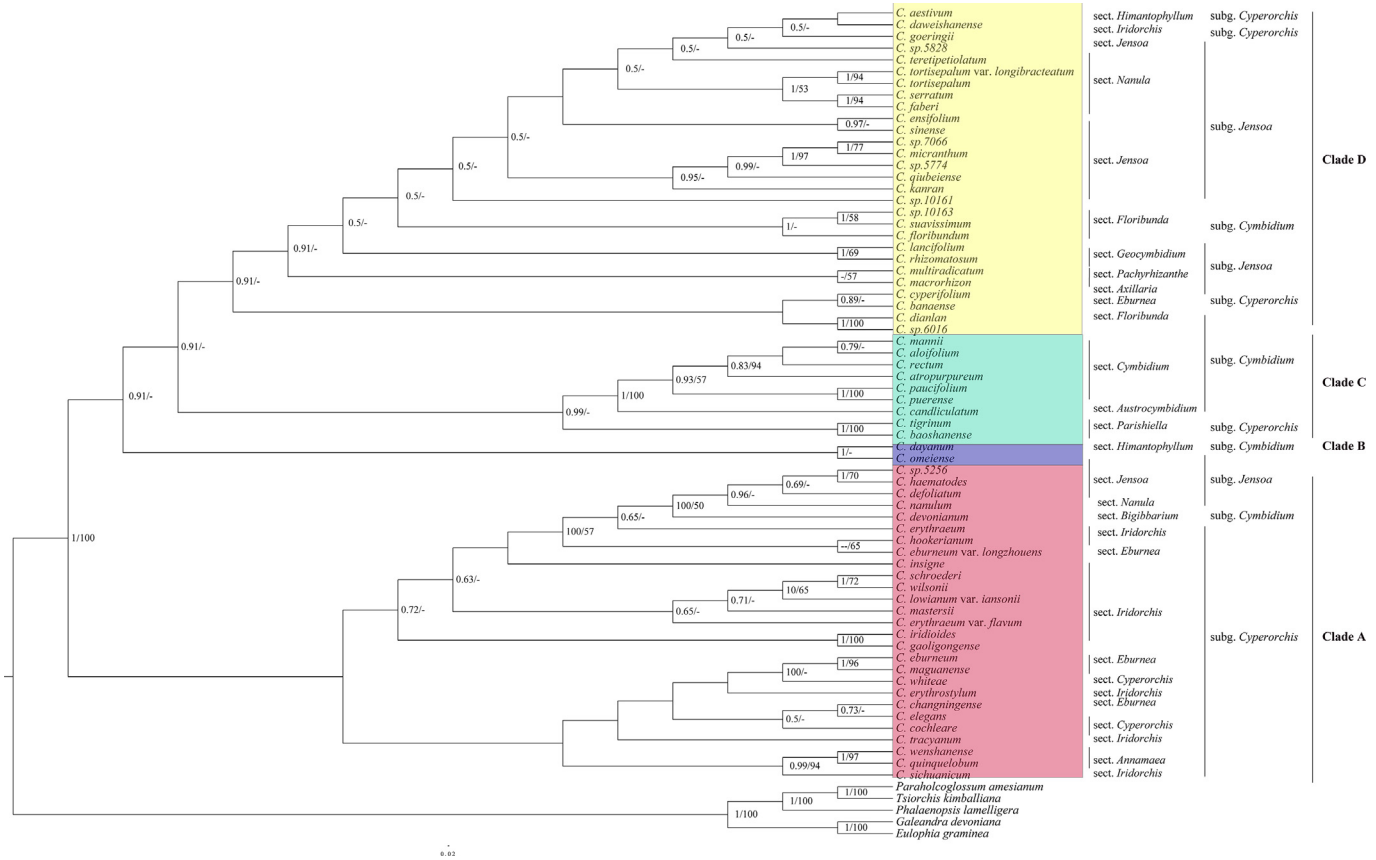

Fig. 1. Phylogenetic relationships of *Cymbidium* based on the plastid DNA (*rbcL*, *trnS*, *trnG*, *matK*, *trnL*, *psbA*, and *atpI*). The numbers near the nodes are bootstrap percentages (PP left, BS right). A dash (−) indicates values less than 50%.

appear to be quite strong judging by support values. The phylogenetic tree based on the nrITS data was divided into three clades, but it could be divided into four clades based on the seven cpDNA sequences. The nuclear gene trees indicated that subgenus *Cymbidium* diverged first, followed by subgenus *Cyperorchises* with two species of subgenus *Cymbidium* and finally the subgenus *Jensoa* with two species of subgenus *Cymbidium* and one species of subgenus *Cyperorchis*. However, the cpDNA gene trees indicated that subgenus *Cyperorchis* diverged first with one species of subgenus *Cymbidium* and three species of subgenus *Jensoa*, followed by the clade *C. dayanum*-*C. omeiense*, followed by the subgenus *Cymbidium* with two species of subgenus *Cyperorchis*, and finally the subgenus *Jensoa* with three species of subgenus *Cymbidium* and one species of subgenus *Cyperorchis*.

Moreover, there were many topological conflicts within sections. For example, the section *Floribunda* was monophyletic in the nrITS gene tree, with strong support (PP = 0.99, BS = 97). This section was subdivided into two clades in the cpDNA tree. The section *Cymbidium* was monophyletic in the cpDNA tree and was sister with *Cymbidium canaliculatum*. However, in the nrITS gene tree, *C. canaliculatum* nested within this section, creating a polyphyletic group. There were also many topological conflicts in species, such as *Cymbidium faberi*, *Cymbidium aestivum*, and *Cymbidium daweishanense*. The ILD test for the nrDNA and combined cpDNA data resulted in $P < 0.01$ and indicated incongruence between the two data sets; therefore, we did not concatenate these two data sets.

3.5. Network analysis

The generated networks revealed inter- and intrasectional reticulations in *Cymbidium* (Fig. 4). The three subgenera (*Cymbidium*, *Cyperorchis*, and *Jensoa*) that exhibited discordant phylogenetic positions in the separate gene trees formed complex networks, suggesting that hybridization events occurred between these three subgenera (Fig. 4a). When some species with highly diverged

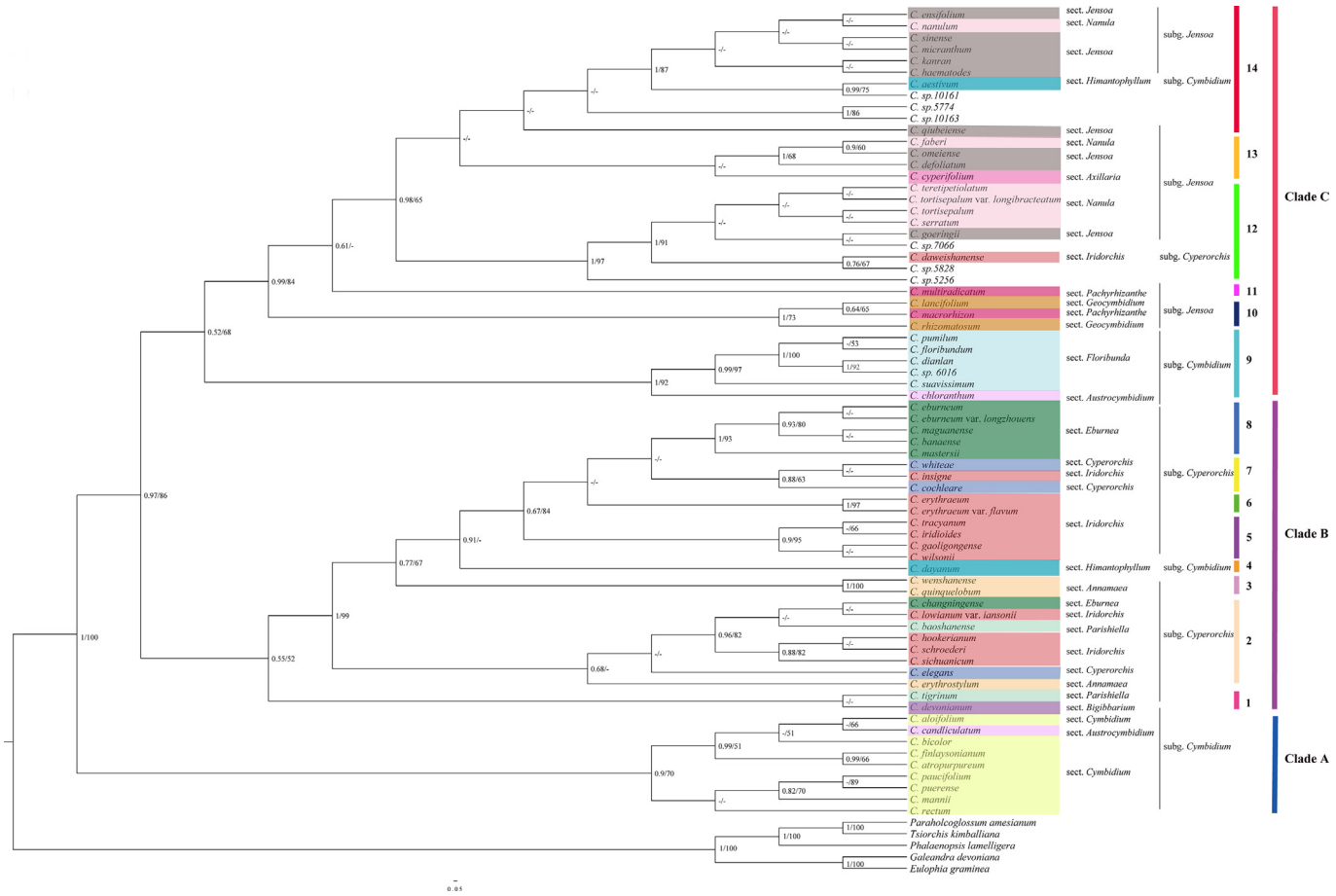

Fig. 2. Phylogenetic relationships of *Cymbidium* based on nrITS. The numbers near the nodes are bootstrap percentages (PP left, BS right). A dash (–) indicates values less than 50%. The classification follows Liu et al. (2006).

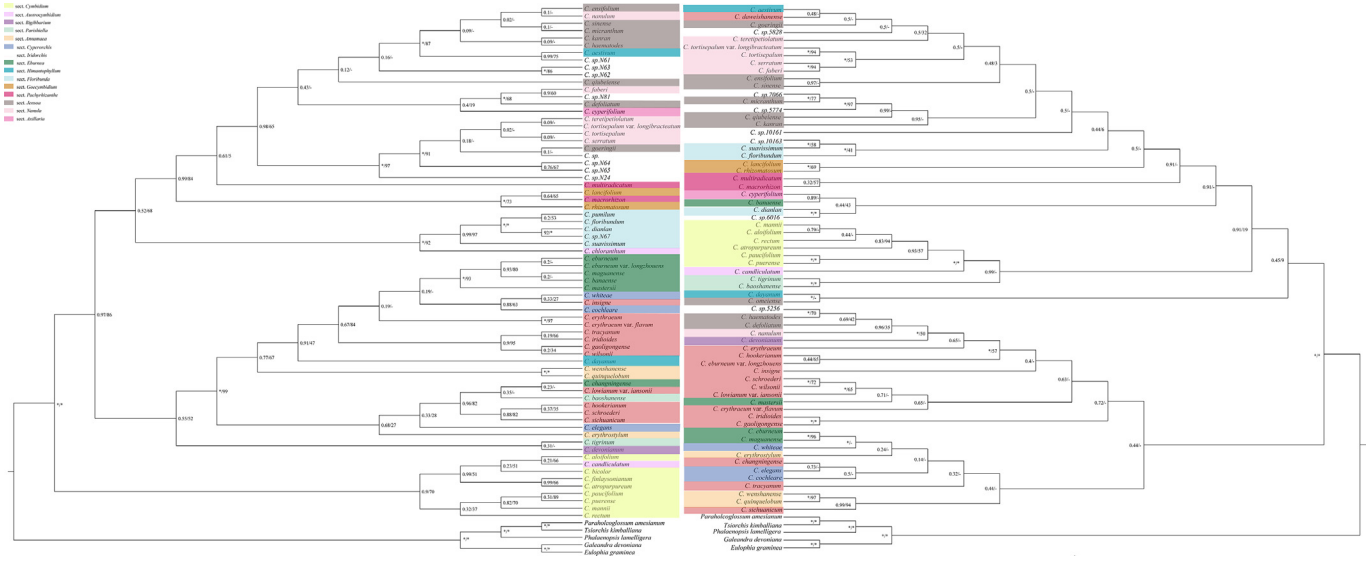

Fig. 3. ITS (left) and combined plastid (right) phylogenies of *Cymbidium*. Bayesian consensus cladograms were generated based on data of the nrITS (left) and the seven plastid loci (*matK*, *rbcL*, *trnL*, *trnS*, *trnG*, *psbA*, and *atpI*) (right). The classification follows Liu et al. (2006).

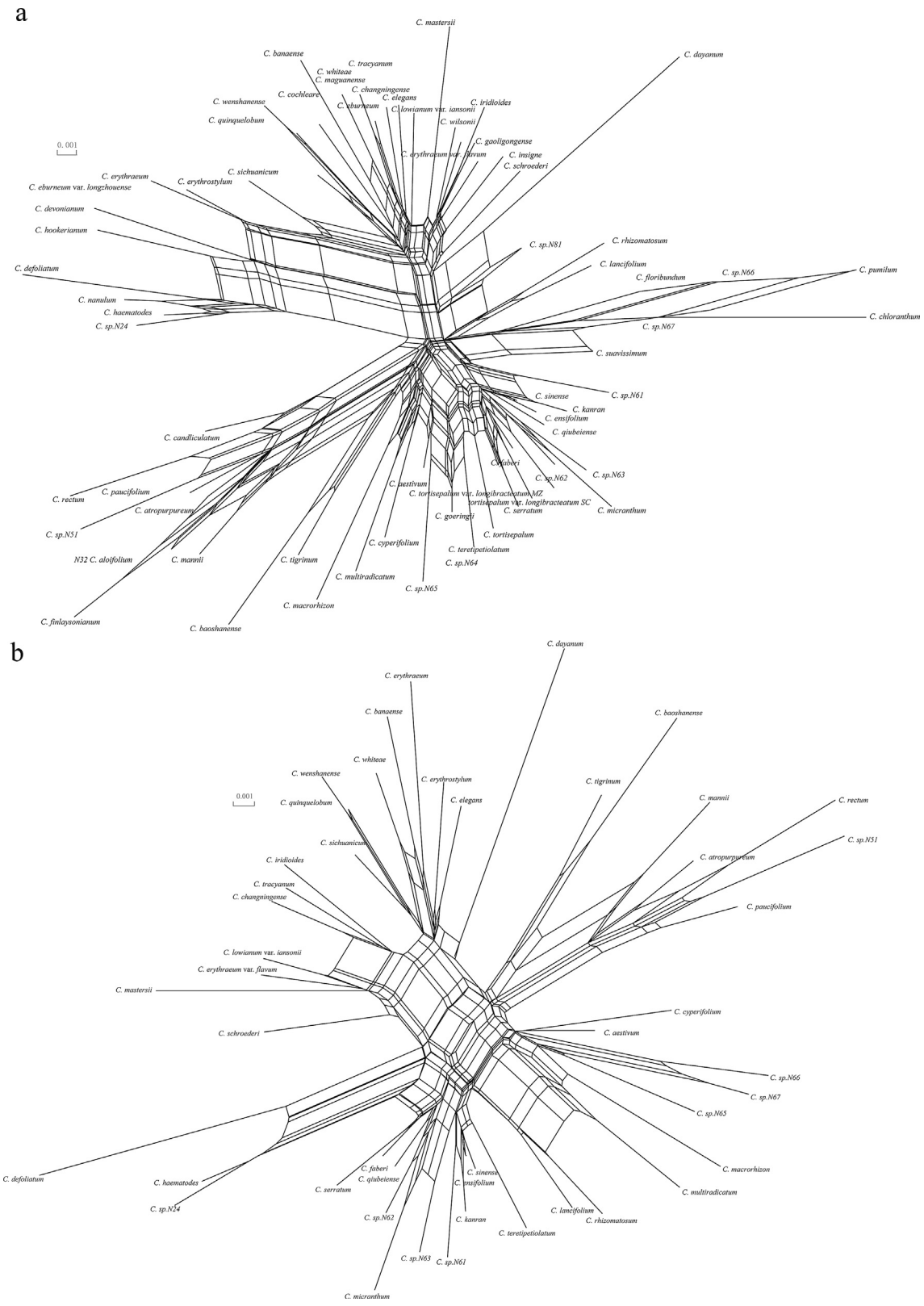

Fig. 4. Filtered super-networks constructed from separate cpDNA and nuclear gene trees. (a) All species were included. (b) Twenty-four species were excluded.

alleles were excluded, the network became much simpler, but reticulation was still observed (Fig. 4b).

4. Discussion

4.1. The phylogeny of the Cymbidium

In the present study, an updated phylogeny of *Cymbidium* was proposed based on comprehensive sampling of 75 species (varieties) (five of which were outgroup) and 468 new DNA sequences. The overall results of the phylogenetic analysis of *Cymbidium* were consistent with previously published results (Berg, 2002; Yukawa and Stern, 2002; Yang et al., 2012). Based on the nuclear DNA (nrITS) data set, *Cymbidium* was found to be composed of three major clades, while it was divided into four clades in the cpDNA tree.

We investigated the relationships among the subgenera of *Cymbidium*. The monophyly of subgenus *Cymbidium* was broken, divided into five clades, four of which were nested in two other subgenera (*Cyperorchis* and *Jensoa*). The monophyly of subgenus *Jensoa* was broken by the nested position of subgenus *Cymbidium* section *Himantophyllum*, *C. aestivum*, and subgenus *Cyperorchis*, *C. daweishanense*. The principal synapomorphic character of the subgenus *Jensoa* is having four pollinia (Liu et al., 2006; Du Puy and Cribb, 2007); however, *C. aestivum* and *C. daweishanense* do not share this character. If it were not for the positions of *Cymbidium devonianum* and *C. dayanum*, subgenus *Cyperorchis* would be monophyletic. Transfer of *C. devonianum* and *C. dayanum* to a member of subgenus *Cyperorchis* resulted in the loss of a prominent synapomorphic character of subgenus *Cyperorchis*, that is, a fused basal part of the lip with the column. Taking the morphological characteristics and phylogenetic relationships into account, subdivisions of *Cymbidium* at the subgeneric level were not useful. We did not find any stable synapomorphic characters that are featured in each subgenus clarified in this study. The delimitation of sections within *Cymbidium* were also problematic, and most sections were found to be polyphyletic. In summary, the currently defined subgenera and sections of *Cymbidium* are not monophyletic. Furthermore, the phylogenetic relationships among *Cymbidium* have not been resolved. Additional markers or second- or third-generation sequencing may be required for further study.

4.2. Reticulate evolution in Cymbidium

The evolutionary relationships at the species level and above could be idealistically represented with bifurcating phylogenetic trees based on the theory of universal common descent. Some evolutionary events, such as horizontal gene transfer, lineage sorting, rapid radiation, hybridization and introgression, may result in conflicts among gene trees, and therefore, phylogenetic networks are more suitable to model the real relationships among species (Geuten et al., 2004; Russell et al., 2010; Yang et al., 2012; Guo et al., 2015).

Phylogenies of *Cymbidium* obtained from nrITS and cpDNA sequences were concordant in certain respects and discordant in others (Fig. 3). Both phylogenies support non-monophyly of each of the three subgenera of *Cymbidium*, as well as sections. The generated networks revealed inter- and intrasectional reticulations in *Cymbidium* (Fig. 4). The three subgenera (*Cymbidium*, *Cyperorchis*, and *Jensoa*) that exhibited discordant phylogenetic positions in the separate gene trees formed complex networks, suggesting that hybridization events occurred between these three subgenera (Fig. 4a). When we deleted the 24 highly divergent alleles, the reticulation relationship among *Cymbidium* was still very complex, indicating that species within *Cymbidium* underwent complex reticulate evolution (Fig. 4b). Actually, due to the sympatric distribution, weak reproductive isolation of the species, and synchronous flowering, the natural interspecific hybridization within orchids occurs commonly (Cribb, 1998; Liu et al., 2009; Guo et al., 2015). Moreover, there have been 15 natural hybrid species reported in the *Cymbidium* (http://www.emonocot.org/). Furthermore, thousands of artificial interspecific hybrids are listed by the Royal Horticultural Society (http://apps.rhs.org.uk/horticulturaldatabase/orchidregister/orchidregister.asp). This phenomenon, which is caused by absence of strong interspecific reproductive barriers and hybrid zones, has also been found in other orchid genera, such as *Orchis* Tourn. ex L. (Bateman et al., 2008), *Ophrys* L. (Cortis et al., 2009), *Epidendrum* Pav. ex Lindl (Pinheiro et al., 2010), and *Paphiopedilum* Pfitzer (Guo et al., 2015). Accordingly, hybridization may play an important role in orchid speciation.

4.3. The possible causes of tree incongruence and the low value

When comparing the nuclear and combined plastid trees (Fig. 3), we immediately observed conflicting branches, but the most branches did not have strong support. The ILD test for the nrDNA and combined cpDNA data resulted in $P < 0.01$, indicating incongruence between the two data sets. In addition, to further determine incongruence of phylogeny tree, we generated the network, which showed that there were complex networks in *Cymbidium*. Tree incongruence and low support values may be explained by hybridization in *Cymbidium* and the lack of informative characters.

4.3.1. Hybridization

Hybridization has long been reported in plant lineages and appreciated to be a key mechanism in plant evolution, as many extant taxa have likely recently originated from hybridizations (Zhang et al., 2012; Guo et al., 2015; Francine et al., 2017). Sequences from different genomes of hybrid species usually reflect different lines of inheritance (e.g., mitochondrion genes from the paternal line, plastid genes from the maternal line, and nuclear genes from both parental lines), which could result in the incongruence between these different data sources (Yu et al., 2013; Zhai et al., 2014; Guo et al., 2015; Kanzi et al., 2020). In *Cymbidium*, we speculated that hybridization may be a cause of tree incongruence. Good examples are described taxa such as *Cymbidium* × *nishiuchianum*, *Cymbidium* × *purpuratum*, *Cymbidium* × *latifolium*, *Cymbidium* × *uniflorum*, *Cymbidium* × *oblancifolium* and *Cymbidium* × *nujiangense*. This phenomenon is also found in *Calanthe* (Zhai et al., 2014), *Epidendrum* (Pinheiro et al., 2010), and *Paphiopedilum* (Guo et al., 2015).

4.3.2. The lack of informative characters

Although we used eight genes to construct the phylogenetic relationships of *Cymbidium*, the informative characters available in these eight genes were limited, and they might represent only a small piece of the evolutionary story in *Cymbidium*. Moreover, the support values for most clades were low. The lack of informative characteristics may be one cause of topological incongruence between the plastid and nuclear phylogenies (Tang et al., 2015).

5. Conclusion

The present work clarified the phylogenetic relationships within *Cymbidium* through molecular evaluations. There were incongruent results in the topology of the combined chloroplast and nrITS trees, and the support values of clades were low. These findings may be the result of natural hybridization and a lack of informative characters. In addition, we detected reticulate evolution in *Cymbidium*. These results add valuable insights into the evolution of *Cymbidium*. Additional studies based on second- or third-generation sequencing are needed, with a focus on geographic and ecological patterns and the tempo and mode of evolution in the genus. This genus is mainly

distributed in subtropical and tropical areas of Asia, and the investigation of diversification patterns of this genus will shed light on biodiversity evolution in this region.

Author contributions

GQZ and JWZ designed the experiments. JH and XYW performed the experiments. GQZ and GZC contributed to the data analysis and molecular system construction. LJC drew the figures. GZC wrote the manuscript. WHR contributed to the collection and morphological identification of some samples. ZJL, SRL, DHP, and MHL provided suggestions on the experimental design and discussion sections. All the authors read and approved the final manuscript.

Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as potential conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pld.2021.08.002.

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