Phylogenetic of the orchid genus *Coelogyne* in Peninsular Malaysia inferred from Morphological Characteristics and Internal Transcribed Spacer (ITS) Sequence Data

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Abstract

The phylogenetic relationships among the Peninsular Malaysian orchid genus *Coelogyne* were studied by morphological characteristics and by sequencing the internal transcribed region (ITS) from the nuclear ribosomal DNA (nrDNA). *Coelogyne* is a large genus of about 200 species distributed in pantropical areas from the Himalayas, Sri Lanka, India, Southern China and throughout South East Asia to Papua New Guinea. The widely accepted previous classification system was exclusively based on floral morphology. There were very few molecular systematic studies of *Coelogyne* done in Peninsular Malaysia thus far. In this study, 59 *Coelogyne* taxa were collected from throughout Peninsular Malaysia and 57 of them were identified to the species level. To study the phylogeny of this genus, morphological characters were utilized together with molecular evidences to generate the systematic hypotheses. Cluster analysis was performed using both the vegetative and floral characters. The results showed that three sections of Peninsular Malaysian *Coelogyne*, namely *Longifoliae*, *Speciosae* and *Fuliginosae* were sister groups which were more closely related by forming one clade than they were with the other sections. Another clade consisted of four other sections, namely *Flaccidae*, *Coelogynae*, *Tomentosae* and *Verrucosae*. Molecular phylogenies obtained by using the Neighbour Joining method revealed the close relationship between the sections *Tomentosae* and *Verrucosae*, whereas usage of the Maximum Likelihood method demonstrated that three sections namely *Longifoliae*, *Speciosae* and *Fuliginosae* were sister groups since they formed a single clade.

Keywords

Coelogyne, phylogeny, morphology, ITS sequence data, Malaysia, taxonomy

Introduction

Coelogyne Lindl. 1821, a genus from the Orchid family comprises of over 200 species, distributed across India, Nepal, China, Southeast Asia to the Fiji islands, with the main centres being in Borneo, Sumatra and the Himalaya mountain range. Most of the species are epiphytic which occur on large trees of primary forests. In Peninsular Malaysia, this poorly studied group

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of orchids have fairly large numbers of small, medium to large-sized flowers with pleasant fragrance, but the flowers are usually short-lived. There are 28 species of *Coelogyne* in Peninsular Malaysia (Seidenfaden and Wood, 1992; Turner, 1995). However, the World Checklist of Selected Plant Families (WCSP, 2020) recognized only 26 species as five from Turner's list are now synonyms, but three new records are added namely *Coelogyne rigida* C.S.P.Parish & Rchb.f., *Coelogyne superba* R.Rice and *Coelogyne velutina* de Vogel.

As some *Coelogyne* species are very similar vegetatively they are very difficult to distinguish morphologically without the flowers. This makes their identification and classification difficult and challenging. *Coelogyne* is among the 21 genera placed under the subtribe Coelogyninae (tribe Arethuseae, subfamily Epidendroideae) and the main difference of this genus is the absence of a saccate lip base, which is found in all other genera of the subtribe (Butzin, 1992). Currently, *Coelogyne* is defined as polyphyletic whereas the subtribe Coelogyninae as monophyletic (Gravendeel *et al*., 2001). The latest phylogenetic study of this subtribe was conducted by Li *et al*. (2015) who discovered and proposed a new orchid genus named *Thuniopsis* to this subtribe. Nonetheless, very few studies had been conducted on the genus *Coelogyne* and other genera in subtribe Coelogyninae in Peninsular Malaysia.

During the pre-molecular era, the fundamentals for species delimitation of this family were based on morphological and anatomical characters, especially of the floral parts such as column organization, anther structure (pollinaria) and pollinium formation. The floral structures are likely to display high degrees of parallelism or convergence as these parts are particularly prone to selective pressure from pollinators (Dodson, 1962; Atwood, 1986). Nowadays, molecular evidences have contributed greatly to the understanding of the phylogenetic relationships of orchids. Molecular systematics employ nucleotide and protein sequence comparisons for estimating phylogenetic relationships. DNA sequences which serve as the basis of molecular systematics make use of the study of different gene markers. The common molecular markers used in plant systematics come from two main sources, which are plastid DNA and nuclear ribosomal DNA (nrDNA).

The nrDNA is a gene that encodes for ribosomal RNA. The nrDNA gene of eukaryotes contains an operon or a tandem repeat of a unit segment comprising of 5'- ETS1, 18S, ITS1, 5.8S, ITS2, 26S, ETS2 -3' tracts. The internal transcribed spacer (ITS) region is known as the spacer located among the large-subunit ribosomal RNA and small-subunit ribosomal RNA genes in the chromosome or is the corresponding transcribed region in the polycistronic rRNA precursor transcript. In eukaryotic cells, there are two ITS regions. ITS1 is situated between the 18S and 5.8S rRNA genes, whereas ITS2 is situated between the 5.8S and 26S rRNA genes (in plants) or the 28S rRNA genes (in animals) (Baldwin, 1992).

The nrDNA is highly suited for a broad range of phylogenetic analyses (Hamby and Zimmer, 1992) due to the varied components of nrDNA which differ in their degrees of conservation. Three nuclear ribosomal cistrons (18S, 5.8S and 26S) are relatively conservative throughout all organisms, both in their nucleotide sequences and in their lengths. However, the ITS regions

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are evolved more rapidly and are much more diverged in their nucleotide sequences. The ITS regions can be easily amplified by polymerase chain reaction (PCR) and is thus one of the most widely used markers in molecular assays.

There had been very limited studies about the sectional relationships of Peninsular Malaysia's *Coelogyne* species thus far. The widely adopted classification system previously done by Seidenfaden and Wood in 1992 was exclusively based on floral morphology. Hence, to confirm and resolve the uncertainties of the taxonomical status of *Coelogyne* species, both morphological and molecular systematics studies of this genus are required. Therefore, the objective of this study was to examine the ability of the nrITS region to resolve the sectional delimitation and evolutionary relationships among the *Coelogyne* species in Peninsular Malaysia.

Materials and Methods

Taxon sampling

A total of 59 *Coelogyne* plant materials were sampled from the field, nursery, botanical gardens, orchid collectors and any source which could provide material for this study. A small piece of fresh young leave sample (3 cm × 3 cm) from every plant was kept in silica gel during sampling, transported to the laboratory and later used for DNA extraction. Table 1 shows the list of samples used in this study.

Morphological Study

A total of 69 morphological characters based on vegetative and reproductive structures were scored. The selected characters for the morphological analysis in this study are listed in Appendix 1. For cluster analysis, morphological data were analysed using the MVSP (Multi Variate Statistical Package) software version 3.1 (Kovach, 2007).

DNA extraction, PCR amplification and sequencing

Total DNA was extracted from the leaves according to the conventional cetyl trimethyl ammonium bromide (CTAB) method (Doyle and Doyle, 1987). The nuclear ribosomal ITS region was amplified by using primers 17SE and 26SE designed for Sorghum (Sun, Skinnder, Liang and Hulbert, 1994) of approximately 800-1000 bp in length and included the ITS1, ITS2 and 5.8s ribosomal gene. The primers were synthesized by First Base Laboratory, Serdang, Malaysia. The PCR reaction mixture contained 1 × reaction buffer (10mM Tris-HCI, 50mM KCI and 0.1 Triton® X-100, Promega, USA), 2.5 mM of MgCl2 (Promega, USA), 0.05 mM dNTPs mix (Promega, USA), 0.5 µM of forward primer, 0.5 µM of reverse primer (First Base Laboratory, Serdang, Malaysia), 0.5 units of Taq DNA polymerase (Promega, USA), 50 ng of template DNA and ddH2O in a total volume of 50 µl. Table 2 shows the reaction mixture for the PCR amplification. Lastly, about 1.5 µl of mineral oil was added to the mixture to prevent

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evaporation during amplification. The PCR was carried out in a thermal cycler (Eppendorf Master Cycler Gradient, Hamburg, Germany).

The PCR amplification profile for the ITS region consisted of an initial denaturation cycle at 95 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 2 min and extension at 72 °C for 2 min. After 35 cycles, there was a final extension cycle at 72 °C for 7 min. The amplified products were soaked at 4°C before being subjected to agarose gel electrophoresis. The sequencing reactions were done by First Base Laboratory Sdn. Bhd. (Serdang, Malaysia).

Phylogenetic analyses

Both the forward and reverse sequences were first assembled to produce a contig sequence by using the BioEdit software version 7.0.2 (Hall, 1999). All contig sequences of each gene region were aligned manually using the MEGA 7 software (Kumar *et al*., 2016). All characters were weighted equally. *Dendrobium crumenatum* which comes from the same subfamily Epidendroideae but is of a different tribe was used as the outgroup. To infer the evolutionary relationships, Neighbour Joining (NJ) analyses were conducted using a heuristic strategy of branch-swapping by tree bisection-reconnection (TBR) step wise addition with 1000 random-addition replicates. The levels of support were estimated with 1000 bootstrap replicates (BP) by using the TBR algorithm of branch swapping for 10 random-addition replicates per bootstrap replicate. In the Maximum Likelihood (ML) analysis, the T92+G substitution model served as the optimal model in the analysis. The ML method was performed using a heuristic search strategy, with TBR branch-swapping and 10 random sequence additions. The levels of support were estimated with 1000 bootstrap replicates (BP), using the TBR algorithm of branch swapping for 10 random-addition replicates per bootstrap replicate.

Results and Discussion

Morphological Data

In this study, 59 samples which belonged to 22 species of the seven sections described by Seidenfaden and Wood (1992) were collected from various localities in Peninsular Malaysia. Morphological characters from vegetative structures such as size of plant, leave and shape of pseudobulb were noted. For the reproductive structures, size, colour of petal, sepal, lip and keels were observed and studied for the morphological analysis of *Coelogyne* species. A total of 69 different binary morphological characters were defined (Appendix 1) and were scored (Appendix 2) in the cluster analysis.

Cluster analysis was performed to classify the species of the genus *Coelogyne* based on overall similarity (Phenetic system). A total of 69 different morphological state characters both quantitative and qualitative were defined in binary mode (either 0 or 1). For those species that had two or more samples per plant, the mean measurement was taken for species delimitation.

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In order to determine the species interrelationships, cluster analysis using the UPGMA method was performed. The phenogram of morphological characters is shown in Figure 1. The phenogram comprised of two major clusters, one of which contained four sections while the other cluster contained three sections of the genus *Coelogyne*. Overall, the Peninsular Malaysia *Coelogyne* species studied in this research shared 61.9 % similarity.

The first main cluster consisted of 13 species of the sections *Verrucosae*, *Flaccidae*, *Coelogynae* and *Tomentosae* with Similarity Coefficient of 70 %. This result was fairly congruent with the Seidenfaden and Wood (1992) classification in that these four sections were closely related to one another. In this study, two species (*C. viscosa* and *C. trinervis*) of the section *Flaccidae* were closely related to two species (*C. foersterrmannii* and *C. cumingii*) of the section *Coelogynae* with high Similarity Coefficient of 83.6 %. Then the four species mentioned shared 75.1 % of similarity with six other species from the section *Tomentosae*. Another three *Coelogyne* species (*C. mayeriana*, *C. pandurata* and *C. asperata*) of the section *Verrucosae* were placed further away forming another sub-cluster. Members of the section *Verrucosae* usually have large flower sizes (lengths of sepals and petals are 40 mm or longer), one or two bract-like sheaths below floral bracts, rachis, pedicel and ovary without hairs.

The second cluster consisted of seven species of the sections *Speciosae*, *Longifoliae* and *Fuliginosae* with Similarity Coefficient of 68.8 %. This result also corresponded well with the Seidenfaden and Wood (1992) classification in that these three sections were closely related to one another. *Coelogyne fimbriata* of section *Fuliginosae* was placed close to three species (*C. stenochila*, *C. prasina* and *C. radicosa*) of section *Longifoliae* with Similarity Coefficient of 74.3 %. This may be due to the fact that they all have small flower size (length of sepals and petals less than 30 mm) and 2-leaved pseudobulb. Two unidentified species, *C.* sp1 and *C.* sp2 were grouped next to the species of section *Longifoliae* in the cluster analysis based on their vegetative characters with 75.7 % Similarity Coefficient. Next to them was section *Speciosae* forming a subcluster which consisted of three species (*C. xyrekes*, *C. tiomanensis* and *C. septemcostata*) with 85.7 % similarity. Species in section *Speciosae* were with synanthous inflorescence and 1-leaved pseudobulb.

Molecular Data

Total DNA was extracted from 59 samples of 22 species of *Coelogyne*. All the nrITS sequences obtained from this study were submitted to the NCBI GenBank database. The accession numbers are shown in Table 1.

The nrITS sequences were analysed for 59 samples of the 22 *Coelogyne* species and one sequence of *Dendrobium crumenatum* (accession number: KC701378) obtained from NCBI served as the outgroup in the phylogenetic analyses. The evolutionary history was inferred based on the Neighbour Joining (NJ) and Maximum Likelihood (ML) methods. Branches corresponding to partitions reproduced in less than 50 % of the trees were collapsed.

Based on the nrITS data, the NJ analysis (Figure 2) revealed that four sections (*Tomentosae*, *Verrucosae*, *Longifoliae* and *Speciosae*) of *Coelogyne* formed a single clade with bootstrap

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percentage (BP) of 100%. The six species (*C. tomentosa*, *C. pulverula*, *C. testacea*, *C. rochusenii*, *C. kaliana* and *C. swaniana*) of section *Tomentosae* formed a monophyletic group indicating genetic closeness. Conversely, the three species (*C. mayeriana*, *C. asperata* and *C. pandurata*) of section *Verrucosae* were split into two monophyletic groups. Interestingly, one of the *C. asperata* (collected from Kedah) was grouped in the same clade with *C. pandurata* instead of with other members of *C. asperata*. All *C. prasina* individuals, regardless of sampling localities, formed a single clade with a strong bootstrap value of 92%. Intriguingly, *C. radicosa* and *C. stenochila*, which are also species of section *Longifoliae*, were clustered together with the three species (*C. tiomanensis*, *C. septemcostata* and *C. xyrekes*) of section *Speciosae*, forming a monophyletic group. Three other sections (*Fuliginosae*, *Coelogynae* and *Flaccidae*) and the two unidentified species (*C.* sp1 and *C.* sp2) each formed individual separate group in this analysis.

The ML tree (Figure 3) constructed based on the ITS data placed all the studied species of the genus *Coelogyne* into a monophyletic group with high bootstrap value of 82%. The ML analysis yielded better resolved phylogenetic tree compared to the NJ analysis. The ML tree revealed that species of sections *Fuliginasae*, *Speciosae*, *Longifoliae*, *Coelogynae* and *Tomentasae* showed monophyletic status with a strong bootstrap value of 100%. ML analysis also split the species under section *Verrucosae* into similar groupings as NJ analysis. The two unidentified species (*C.*sp1 and *C.* sp2), *C. trinervis* and *C. viscosa* were placed in separate individual clades from six other sections.

In this investigation we studied seven different sections of *Coelogyne*. Overall, phylogenies using NJ revealed the close relationship between sections of *Tomentosae* and *Verrucosae* by forming clade A1 with a moderate BP support of 57 %. This result was congruent with the the earlier classification done by Seidenfaden and Wood (1992). However, the phylogenies obtained using ML demonstrated that three sections i.e. *Longifoliae*, *Speciosae* and *Fuliginosae* were sister groups which were closely related by forming a single clade E1 with high BP support of 97 %. The ML analyses were also fairly congruent with the previous Seidenfaden and Wood (1992) classification. These three sections were introduced together with 10 other sections giving a total of 13 new sections as published by Pfitzer and Kraenzlin (1907). This scheme was later maintained by almost all authors, except Smith (1933) and Comber (1990) who included *Speciosae* and *Fuliginosae* into *Longifoliae*. However, there are many clear differences among the three sections.

Based on the analyses of both the NJ and ML trees, there were slight differences in the phylogenetic positions of several sections. We believed that the sectional relationship of *Coelogyne* was hardly resolved by using only a single individual marker. As the nrITS region alone may not be powerful enough to differentiate the circumscription of the seven *Coelogyne* sections we propose that more studies are necessary to reconfirm the delineation of *Coelogyne* by employing a combined molecular data set of several genes.

Conclusion

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The phylogeny of seven sections of the genus *Coelogyne* in Peninsular Malaysia were studied based on mophological and nrITS sequence data. The results of the cluster analysis based on morphological data showed that the Peninsular Malaysia *Coelogyne* species were divided into two clades, which were highly congruent with a prior classification done by Seidenfaden and Wood (1992) where species from sections *Longifoliae*, *Speciosae* and *Fuliginosae* formed a single clade, indicating their close relationship. Then species under sections *Flaccidae*, *Coelogynae*, *Tomentosae* and *Verrucosae* were grouped into another clade. The two unidentified species (*C.* sp1 and *C.* sp2) were sister groups to species of section *Longofoliae* based on their vegetative structures only. The cluster analysis results were supported by the Maximum Likelihood analysis of the nrITS sequence data, where sections *Longifoliae*, *Speciosae* and *Fuliginosae* were found to be closely related. As for the species in sections *Coelogyne*, *Tomentosae*, *Verrucosae* and *Flaccidae*, the ML tree showed different groupings to those of the UPGMA clusters. However, a single nrITS marker may not be powerful enough to totally resolve and confirm the sectional delimitation of Peninsular Malaysia's *Coelogyne* species. Hence, for future studies on the systematics of the *Coelogyne* and other species of the subtribe Coelogyninae, we propose that combined molecular data sets of plastid genes such as *rbc*L, *mat*K, *trn*L-F with nrITS be employed in order to provide a better resolution.

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Author Contributions:

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(ii) Kok Hon, **YOH** : Implementing the study, data collection, analysis, drafted the paper, correcting the paper after reviewed.
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(iii) Janna Ong, **ABDULLAH:** Assisting in Molecular works, data analysis, reviewing the paper.

(iv) Christina Seok Yien, **YONG**: Assisting in Molecular works, data analysis, reviewing the paper.

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Table 1: List of species studied and the gene bank accession number.

No	Species	Location	Section	Voucher	Gene bank accession number	Collector name	Date of collection
1.	*C. fimbriata*	Gunung Tahan, Malaysia	*Fuliginosae*	RG 4461	MK356158	Yoh Kok Hon & Rusea Go (UPM)	3 Sept. 2013
2.	*C. fimbriata*	Gunung Jerai, Malaysia	*Fuliginosae*	L006	MK356159	Yoh Kok Hon & Rusea Go (UPM)	15 Feb. 2013
3.	*C. fimbriata*	Kedah, Malaysia	*Fuliginosae*	YKH 022	MK356160	Yoh Kok Hon & Rusea Go (UPM)	15 Feb. 2013
4.	*C. fimbriata*	Terengganu, Malaysia	*Fuliginosae*	FRI 71463	MK356161	Ong Poh Teck (FRIM)	26 Apr. 2011
5.	*C. asperata*	Kedah, Malaysia	*Verrucosae*	YKH 025	MK356162	Yoh Kok Hon & Rusea Go (UPM)	15 Feb. 2013
6.	*C. asperata*	Perak, Malaysia	*Verrucosae*	L012	MK356163	Yoh Kok Hon & Rusea Go (UPM)	28 Sept. 2012
7.	*C. asperata*	Terengganu, Malaysia	*Verrucosae*	L013	MK356164	Yoh Kok Hon & Rusea Go (UPM)	9 Aug. 2012
8.	*C. asperata*	Selangor, Malaysia	*Verrucosae*	L014	MK356165	Yoh Kok Hon & Rusea Go (UPM)	19 Jan. 2012
9.	*C. mayeriana*	Cameron Highlands, Malaysia	*Verrucosae*	YKH 011	MK356195	Yoh Kok Hon & Rusea Go (UPM)	10 Jan. 2012
10.	*C. mayeriana*	Selangor, Malaysia	*Verrucosae*	UMC 1415	MK356196	Planted in Universiti Malaya	18 Jul. 2013
11.	*C. pandurata*	Genting Highlands, Malaysia	*Verrucosae*	L009	MK356176	Yoh Kok Hon & Rusea Go (UPM)	14 Feb. 2012
12.	*C. pandurata*	Cameron Highlands, Malaysia	*Verrucosae*	YKH 010	MK356177	Yoh Kok Hon & Rusea Go (UPM)	10 Jan. 2012
13.	*C. pandurata*	Terengganu, Malaysia	*Verrucosae*	L011	MK356178	Yoh Kok Hon & Rusea Go (UPM)	9 Aug.t 2012
14.	*C. pandurata*	Selangor, Malaysia	*Verrucosae*	UMC 1394	MK356179	Planted in Universiti Malaya	18 Jul. 2013

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15.	*C. cumingii*	Gunung Jerai, Malaysia	*Coelogynae*	RG 4389	MK356170	Yoh Kok Hon & Rusea Go (UPM)	15 Feb. 2013
16.	*C. cumingii*	Kelantan, Malaysia	*Coelogynae*	YKH 012	MK356171	Yoh Kok Hon & Rusea Go (UPM)	14 Feb. 2012
17.	*C. cumingii*	Kedah, Malaysia	*Coelogynae*	YKH 026	MK356172	Yoh Kok Hon & Rusea Go (UPM)	15 Feb. 2013
18.	*C. foerstermannii*	Gunung Arong, Malaysia	*Coelogynae*	RG 3993	MK356204	Yoh Kok Hon & Rusea Go (UPM)	8 Apr. 2013
19.	*C. foerstermannii*	Gunung Jerai, Malaysia	*Coelogynae*	L005	MK356205	Yoh Kok Hon & Rusea Go (UPM)	15 Feb. 2013
20.	*C. foerstermannii*	Setiu, Malaysia	*Coelogynae*	YKH 028	MK356206	Yoh Kok Hon & Rusea Go (UPM)	9 Aug. 2012
21.	*C. rochussenii*	Taiping's Hill, Malaysia	*Tomentosae*	L007	MK356173	Rusea Go (UPM)	28 Sept. 2012
22.	*C. rochussenii*	Gunung Jerai, Malaysia	*Tomentosae*	L008	MK356174	Yoh Kok Hon & Rusea Go (UPM)	15 Feb. 2013
23.	*C. rochussenii*	Fraser's Hill, Malaysia	*Tomentosae*	UMC 673	MK356175	Planted in Universiti Malaya	18 Jul. 2013
24.	*C. pulverula*	Cameron Highlands, Malaysia	*Tomentosae*	YKH 009	MK356155	Yoh Kok Hon & Rusea Go (UPM)	10 Jan. 2012
25.	*C. pulverula*	Fraser's Hill, Malaysia	*Tomentosae*	L002	MK356156	Farah Alia & Rusea Go (UPM)	1 July 2011
26.	*C. pulverula*	Genting Highlands, Malaysia	*Tomentosae*	L015	MK356157	Yoh Kok Hon & Rusea Go (UPM)	14 Feb. 2012
27.	*C. testacea*	Kedah, Malaysia	*Tomentosae*	YKH 023	MK356202	Yoh Kok Hon & Rusea Go (UPM)	15 Feb. 2013
28.	*C. testacea*	Terengganu, Malaysia	*Tomentosae*	KGB 20081942	MK356203	Ong Poh Teck (FRIM)	1 Sept. 2010
29.	*C. swaniana*	Perak, Malaysia	*Tomentosae*	L001	MK356207	Yoh Kok Hon & Rusea Go (UPM)	28 Sept. 2012
30.	*C. swaniana*	Gunung Jerai, Malaysia	*Tomentosae*	L002	MK356208	Yoh Kok Hon & Rusea Go (UPM)	15 Feb. 2013

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31.	*C. tomentosa*	Genting Highlands, Malaysia	*Tomentosae*	YKH 018	MK356187	Yoh Kok Hon & Rusea Go (UPM)	14 Feb. 2012
32.	*C. tomentosa*	Cameron Highlands, Malaysia	*Tomentosae*	L010	MK356188	Yoh Kok Hon & Rusea Go (UPM)	10 Jan. 2012
33.	*C. tomentosa*	Fraser's Hill, Malaysia	*Tomentosae*	FAN.FH293	MK356189	Farah Alia & Rusea Go (UPM)	1 Jul. 2011
34.	*C. tomentosa*	Endau-Rompin, Malaysia	*Tomentosae*	RG 2809	MK356190	Yoh Kok Hon & Rusea Go (UPM)	1 Jul. 2012
35.	*C. kaliana*	Genting Highlands, Malaysia	*Tomentosae*	YKH 020	MK356193	Yoh Kok Hon & Rusea Go (UPM)	14 Feb. 2012
36.	*C. kaliana*	Cameron Highlands, Malaysia	*Tomentosae*	YKH 004	MK356194	Yoh Kok Hon & Rusea Go (UPM)	10 Jan. 2012
37.	*C. prasina*	Genting Highlands, Malaysia	*Longifoliae*	YKH 014, YKH 015, YKH 016	MK356180	Yoh Kok Hon & Rusea Go (UPM)	14 Feb. 2012
38.	*C. prasina*	Cameron Highlands, Malaysia	*Longifoliae*	YKH 003	MK356181	Yoh Kok Hon & Rusea Go (UPM)	10 Jan. 2012
39.	*C. prasina*	Gunung Tahan, Malaysia	*Longifoliae*	YKH 032	MK356182	Yoh Kok Hon & Rusea Go (UPM)	3 Sept. 2013
40.	*C. prasina*	Endau-Rompin, Malaysia	*Longifoliae*	RG 2807	MK356183	Yoh Kok Hon & Rusea Go (UPM)	1 Jul. 2012
41.	*C. prasina*	Fraser's Hill, Malaysia	*Longifoliae*	FAN.FH115	MK356184	Farah Alia & Rusea Go (UPM)	1 Jul. 2011
42.	*C. prasina*	Pulau Banding, Malaysia	*Longifoliae*	RG 2884	MK356185	Rusea Go (UPM)	5 Oct. 2012
43.	*C. prasina*	Gunung Jerai, Malaysia	*Longifoliae*	RG 4390	MK356186	Yoh Kok Hon & Rusea Go (UPM)	15 Feb. 2013
44.	*C. radicosa*	Genting Highlands, Malaysia	*Longifoliae*	YKH 013	MK356166	Yoh Kok Hon & Rusea Go (UPM)	14 Feb. 2012
45.	*C. radicosa*	Cameron Highlands, Malaysia	*Longifoliae*	YKH 002	MK356167	Yoh Kok Hon & Rusea Go (UPM)	10 Jan. 2012

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46.	*C. radicosa*	Fraser's Hill, Malaysia	*Longifoliae*	FAN.FH193	MK356168	Farah Alia & Rusea Go (UPM)	1 Jul. 2011
47.	*C. radicosa*	Gunung Tahan, Malaysia	*Longifoliae*	RG 4488	MK356169	Yoh Kok Hon & Rusea Go (UPM)	3 Sept. 2013
48.	*C. stenochila*	Gunung Tahan, Malaysia	*Longifoliae*	YKH 031	MK356153	Yoh Kok Hon & Rusea Go (UPM)	3 Sept. 2013
49.	*C. septemcostata*	Endau-Rompin, Malaysia	*Speciosae*	RG 2787, RG2801	MK356191	Yoh Kok Hon & Rusea Go (UPM)	1 Jul. 2012
50.	*C. septemcostata*	Terengganu, Malaysia	*Speciosae*	FRI 71373	MK356192	Ong Poh Teck (FRIM)	1 Sept. 2010
51.	*C. xyrekes*	Genting Highlands, Malaysia	*Speciosae*	YKH 029	MK356197	Yoh Kok Hon & Rusea Go (UPM)	14 Feb. 2012
52.	*C. xyrekes*	Cameron Highlands, Malaysia	*Speciosae*	YKH 006, YKH 007	MK356198	Yoh Kok Hon & Rusea Go (UPM)	10 Jan. 2012
53.	*C. tiomanensis*	Gunung Kajang, Malaysia	*Speciosae*	FRI 75329	MK356154	Ong Poh Teck (FRIM)	8 Aug. 2013
54.	*C. trinervis*	Kelantan, Malaysia	*Flaccidae*	L004	MK356199	Rusea Go (UPM)	30 Oct. 2013
55.	*C. trinervis*	Kedah, Malaysia	*Flaccidae*	YKH 024	MK356200	Yoh Kok Hon & Rusea Go (UPM)	15 Feb. 2013
56.	*C. trinervis*	Terengganu, Malaysia	*Flaccidae*	L003	MK356201	Yoh Kok Hon & Rusea Go (UPM)	9 Aug. 2012
57.	*C. viscosa*	Cameron Highlands, Malaysia	*Flaccidae*	YKH 001	MK356152	Yoh Kok Hon & Rusea Go (UPM)	10 Jan. 2012
58.	*C.* sp 1	Setiu, Malaysia	?	RG 2827	-	Yoh Kok Hon & Rusea Go (UPM)	9 Aug. 2012
59.	*C.* sp 2	Setiu, Malaysia	?	RG 2828	-	Yoh Kok Hon & Rusea Go (UPM)	9 Aug. 2012

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Table 2: Reaction mixture for PCR

Chemical Stock Concentration	**Final Concentration**	**Final Volume (µl)**
Buffer (5X)	1 X	10.00
$MgCl_2$ (25 mM)	2.5 mM	5.00
dNTPs mix (10 mM)	0.05 mM	2.00
Forward Primer (100 µM)	0.5 µM	1.00
Reverse Primer (100 µM)	0.5 µM	1.00
DNA Polymerase (5U/ul)	0.5 unit	0.50
DNA template	10 to 500 ng	1.00
ddH_2O	up to final volume of 50 µl	29.50
	Total	50.00 µl

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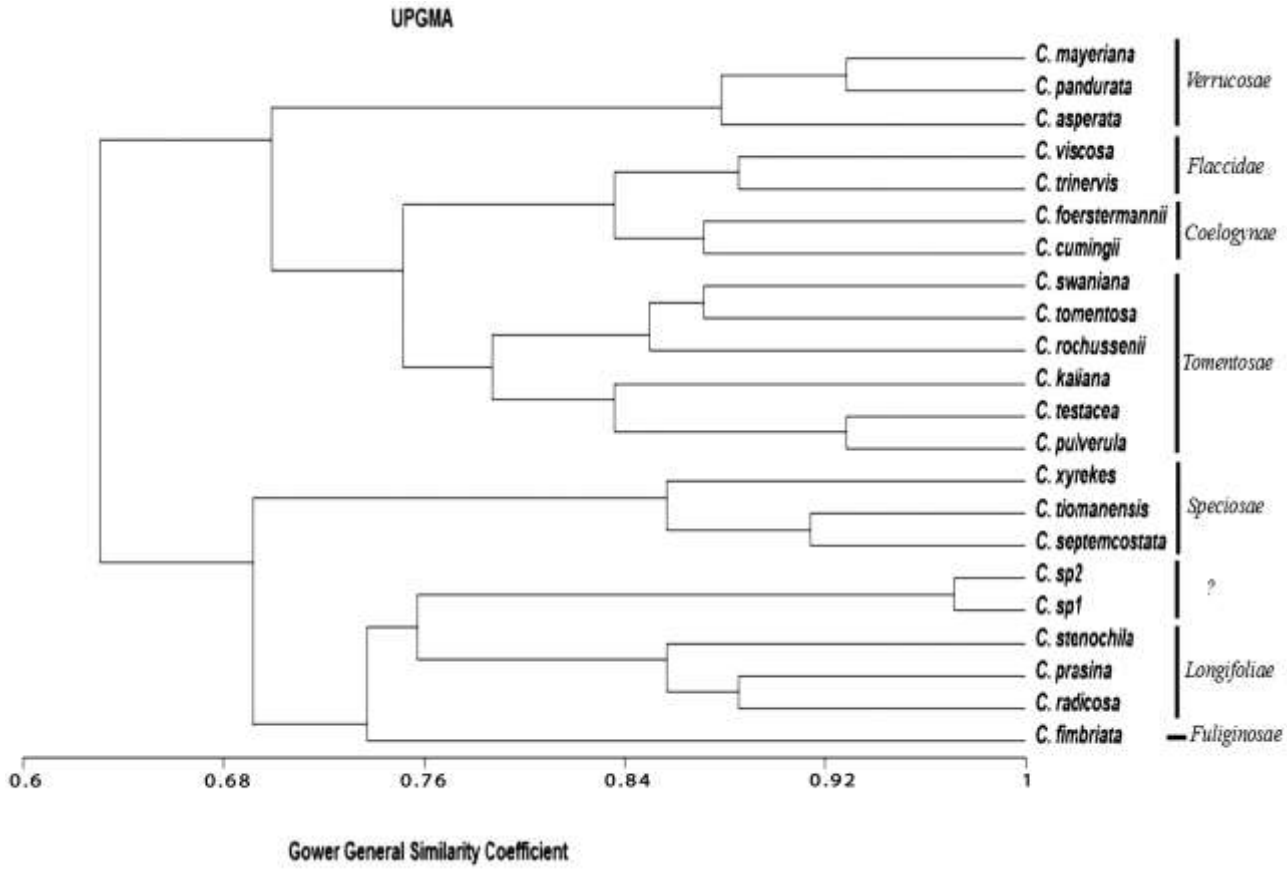

Figure 1: UPGMA clustering of *Coelogyne* species using 69 different morphological characters state.

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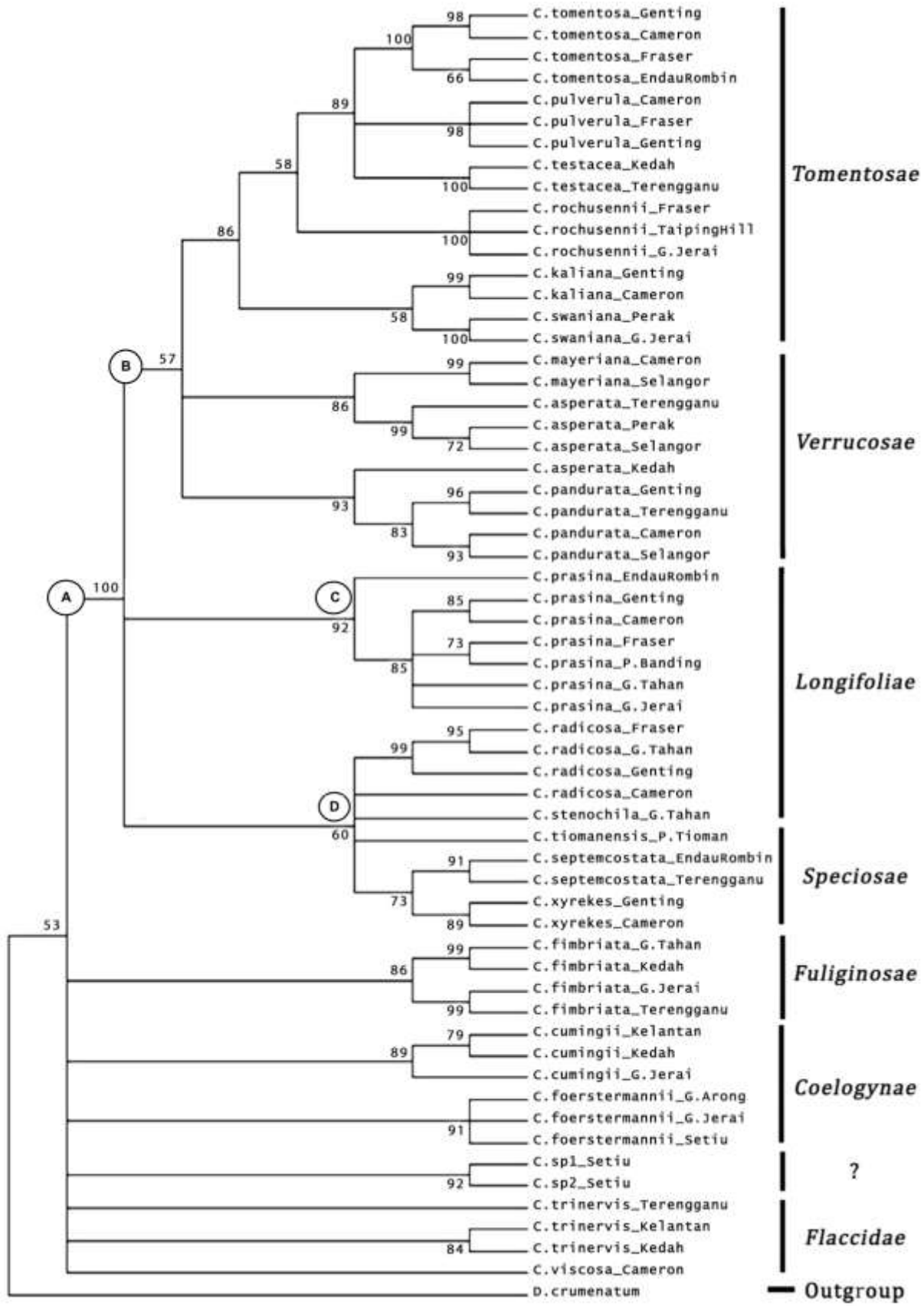

Figure 2: The Neighbour Joining (NJ) tree for nrITS region. Bootstrap percentage ≥ 50 are indicated at the nodes.

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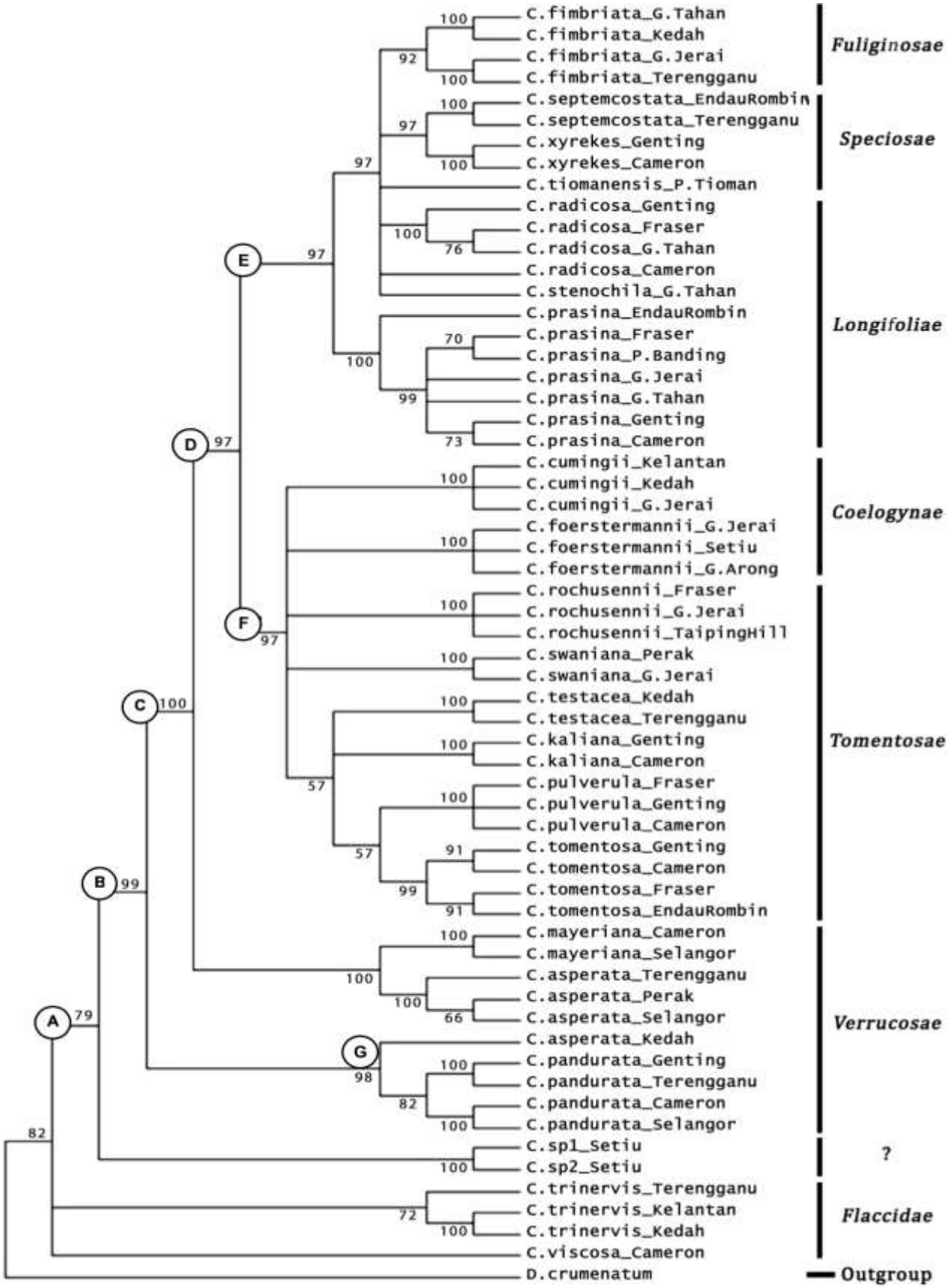

Figure 3: The Maximum Likelihood (ML) tree for nrITS region. Numbers at nodes represent percent recovery in bootstrap analysis (1000 replicates).

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Appendix 1: Morphological Characters State

1. Rhizome: 0 = absent / 1 = present
2. Rhizome, growth form: 0 = monopodial / 1 = sympodial
3. Pseudobulbs: 0 = close (less than 3 cm apart) / 1 = distant (more than 3 cm apart)
4. Pseudobulb: 0 = smooth / 1= ribbed
5. Pseudobulb, laterally flattened: 0 = no / 1 = yes
6. Pseudobulbs, number of leaves: 0 = one-leaved / 1 = two-leaved
7. Pseudobulbs shape, ovoid (conical): 0 = no / 1 = yes
8. Pseudobulbs shape, elliptical: 0 = no / 1 = yes
9. Pseudobulbs shape, spherical: 0 = no / 1 = yes
10. Pseudobulbs shape, fusiform (spindle shape): 0 = no / 1 = yes
11. Leaf sheath: 0 = absent / 1 = present
12. Leaf blade/ lamina: 0 = smooth / 1 = pleated
13. Leaf length: 0 = small to intermediate (less than 30 cm) / 1 = large (more than 30 cm)
14. Leaf width (at middle): 0 = narrow (less than 3 cm) / 1 = broad (more than 3 cm)
15. Leaf shape, elliptical: 0 = no / 1 = yes
16. Leaf shape, lanceolate: 0 = no / 1 = yes
17. Leaf shape, linear: 0 = no / 1 = yes
18. Leaf shape, ovate: 0 = no / 1 = yes
19. Leaf bases, acute: 0 = no / 1 = yes
20. Leaf bases, cuneate: 0 = no / 1 = yes
21. Leaf bases, obtuse: 0 = no / 1 = yes
22. Leaf apex, acute: 0 = no / 1 = yes
23. Leaf apex, obtuse: 0 = no / 1 = yes
24. Leafmargin: 0 = entire / 1 =crisped
25. Inflorescence, pendulous: 0 = no / 1 = yes
26. Inflorescence insertion, synanthous: 0 = no / 1 = yes
27. Inflorescence insertion, hysteranthous: 0 = no / 1 = yes
28. Inflorescence insertion, heteranthous: 0 = no / 1 = yes
29. Inflorescence insertion, proteranthous: 0 = no / 1 = yes
30. Scape: 0 = without persistent bracts / 1 = with persistent bracts
31. Scape, shape in cross section: 0 = not flattened / 1 = flattened
32. Flower: 0 = single / 1 = multi-flowered
33. Flower: 0 = open in succession / 1 = all opening at the same time (simultaneously)
34. Flower, bract: 0 = caducous (deciduous) / 1 = persistent
35. Flower size, small (diameter less than 35 mm): 0 = no / 1 = yes
36. Flower size, medium (diameter 35-50 mm): 0 = no / 1 = yes
37. Flower size, large (diameter more than 50 mm): 0 = no / 1 = yes
38. Flower, fragrant: 0 = no / 1 = yes
39. Petal andsepal colour, white: 0 = no / 1 = yes
40. Petal and sepal colour, yellow: 0 = no / 1 = yes
41. Petal and sepal colour, green: 0 = no / 1 = yes
42. Petal and sepal colour, salmon pink: 0 = no / 1 = yes
43. Petal, length: 0 = up to 25 mm / 1 = more than 25 mm

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44. Petal, width (at middle): 0 = up to 5 mm / 1 = more than 5 mm
45. Petal shape, elliptical: 0 = no / 1 = yes
46. Petal shape, lanceolate: 0 = no / 1 = yes
47. Petal shape, ovate-oblong: 0 = no / 1 = yes
48. Petal shape, linear: 0 = no / 1 = yes
49. Sepal length: 0 = up to 25 mm / 1 = more than 25 mm
50. Sepal width (at middle): 0 = up to 5 mm / 1 = more than 5 mm
51. Sepal shape, elliptical: 0 = no / 1 = yes
52. Sepal shape, lanceolate: 0 = no / 1 = yes
53. Sepal shape, ovate-oblong: 0 = no / 1 = yes
54. Sepal shape, falcate: 0 = no / 1 = yes
55.Lip, length: 0 = short (up to 35 mm) / 1 = long (more than 35 mm)
56. Lip, margin, hairy: 0 = no / 1 = yes
57. Lip, colouration, green: 0 = no / 1 = yes
58. Lip, colouration, salmon pink: 0 = no / 1 = yes
59. Lip, colouration, white: 0 = no / 1 = yes
60. Lip, colouration, yellow: 0 = no / 1 = yes
61. Lip, colouration, brown: 0 = no / 1 = yes
62. Lip, colouration, black: 0 = no / 1 = yes
63. Lip, number of keels: 0 = one to three / 1 = more than three
64. Keels, all emerged from base: 0 = no / 1 = yes
65. Keels, smooth: 0 = no / 1 = yes
66. Keels, wavy: 0 = no / 1 = yes
67. Keels, papillose: 0 = no / 1 = yes
68. Keels, toothed: 0 = no / 1 = yes
69. Keels, warty: 0 = no / 1 = yes

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Appendix 2: Scoring of morphological characters state

Species	Characteristics																						
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
C. fimbriata	1	1	1	0	0	1	1	0	0	0	0	1	0	1	1	0	0	0	1	0	0	1	0
C. septemcostata	1	1	0	1	0	0	1	0	0	0	0	1	0	1	1	0	0	0	1	0	0	0	1
C. xyrekes	1	1	0	1	0	0	1	1	0	0	0	1	0	1	0	1	0	0	1	0	0	0	1
C. tiomanensis	1	1	0	1	0	0	1	0	0	0	0	1	0	1	0	1	0	0	1	0	0	0	1
C. radicosa	1	1	0	1	0	1	0	0	0	1	0	1	0	1	1	0	0	0	1	0	0	1	0
C. prasina	1	1	1	1	0	1	1	0	0	0	0	1	0	1	1	0	0	0	1	0	0	1	0
C. stenochila	1	1	0	1	0	1	1	0	0	0	0	1	0	0	1	0	0	0	1	0	0	1	0
C. pulverula	1	1	0	1	0	1	1	0	0	0	0	1	1	1	1	1	0	0	1	0	0	1	0
C. testacea	1	1	0	1	0	1	1	0	0	0	0	1	0	1	0	1	0	0	1	0	0	1	0
C. rochussenii	1	1	0	1	0	1	1	0	0	0	0	1	0	1	0	1	0	1	1	0	0	1	0
C. tomentosa	1	1	0	1	0	1	1	0	0	0	0	1	1	1	1	0	0	1	1	0	0	1	0
C. kaliana	1	1	0	1	0	1	1	0	1	0	0	1	0	1	1	1	0	0	1	0	0	1	0
C. swaniana	1	1	0	1	0	1	1	0	0	0	0	1	0	1	1	0	0	1	1	0	0	1	0
C. asperata	1	1	0	1	1	1	1	0	0	0	0	1	1	1	0	1	0	0	1	0	0	1	0
C. pandurata	1	1	0	1	1	1	1	0	1	0	0	1	1	1	1	1	0	0	0	1	0	1	0
C. mayeriana	1	1	1	1	1	1	1	0	1	0	0	1	1	1	0	1	0	0	1	0	0	1	0
C. cumingii	1	1	0	1	0	1	1	0	0	0	1	1	0	1	0	1	0	0	0	1	0	1	0
C. foerstermannii	1	1	0	1	0	1	1	0	0	0	1	1	0	1	0	1	0	0	0	1	0	1	0
C. trinervis	1	1	0	1	0	1	1	0	0	0	0	1	1	1	0	1	0	0	0	1	0	1	0
C. viscosa	1	1	0	1	0	1	1	0	0	0	0	1	1	0	0	1	1	0	0	1	0	1	0
C. sp1	1	1	0	1	0	1	1	0	0	0	0	1	1	1	1	1	0	0	0	1	0	1	0

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C. sp2	1	1	0	1	0	1	1	0	0	0	0	1	0	1	0	1	0	0	0	1	0	1	0

Species	**Characteristics**																						
	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46
C. fimbriata	0	0	0	1	0	0	1	0	1	0	0	1	0	0	0	0	1	0	0	0	0	0	0
C. septemcostata	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	1	1	0	1	0	0	0
C. xyrekes	0	0	1	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	1	1	0	0	0
C. tiomanensis	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	1	0	0	0
C. radicosa	1	0	0	1	0	0	0	1	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0
C. prasina	1	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0
C. stenochila	1	0	1	0	0	0	0	1	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0
C. pulverula	0	1	1	0	0	1	1	0	1	1	1	0	1	0	0	0	1	0	0	1	1	0	0
C. testacea	0	1	1	0	0	1	1	0	1	1	1	0	1	0	0	0	1	0	0	1	1	0	0
C. rochussenii	1	1	0	0	1	0	1	0	1	1	1	0	1	0	1	0	1	0	0	0	0	0	1
C. tomentosa	1	1	0	0	1	0	1	0	1	1	1	0	1	0	1	0	1	0	0	0	1	0	1
C. kaliana	0	1	0	0	1	0	1	0	1	1	1	0	1	0	0	1	0	0	0	1	1	1	0
C. swaniana	0	1	0	0	1	0	1	0	1	1	1	0	1	0	0	1	0	0	0	0	1	0	1
C. asperata	0	1	0	0	0	1	1	0	1	1	1	0	0	1	1	1	0	0	0	1	1	0	1
C. pandurata	0	1	0	0	0	1	1	0	1	1	1	0	0	1	1	0	0	1	0	1	1	0	1
C. mayeriana	0	1	0	0	0	1	1	0	1	1	1	0	0	1	1	0	0	1	0	1	1	0	1
C. cumingii	0	1	1	0	0	0	0	0	1	1	1	1	0	0	1	1	0	0	0	1	0	0	0
C. foerstermannii	0	1	0	0	1	0	1	0	1	1	1	1	1	0	1	1	0	0	0	1	1	1	1
C. trinervis	0	1	0	0	1	0	1	0	1	1	1	1	0	0	1	1	0	0	0	0	0	0	0
C. viscosa	0	1	0	0	1	0	1	0	1	1	1	1	0	0	1	1	0	0	0	1	0	0	0

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C. sp1	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C. sp2	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Species	**Characteristics**																						
	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69
C. fimbriata	0	1	0	1	0	1	0	0	0	1	0	0	0	1	1	0	1	0	0	0	0	1	0
C. septemcostata	0	1	1	1	0	1	0	0	1	0	0	0	0	1	1	0	1	1	0	0	0	0	0
C. xyrekes	0	1	1	1	0	1	0	0	1	0	0	1	0	0	1	0	0	1	0	1	1	0	0
C. tiomanensis	0	1	1	1	0	1	0	0	1	0	0	0	0	1	1	0	0	1	0	1	0	0	0
C. radicosa	0	1	0	1	0	1	0	0	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0
C. prasina	0	1	0	1	0	1	0	0	0	0	1	1	0	0	0	0	0	1	1	0	0	0	0
C. stenochila	0	1	0	1	0	1	0	0	0	0	0	0	1	0	0	0	0	1	1	0	0	1	0
C. pulverula	0	1	1	1	0	1	0	0	1	0	0	0	1	1	1	0	1	0	0	0	0	0	1
C. testacea	0	1	1	1	0	1	0	0	1	0	0	0	1	0	1	0	1	0	0	0	0	1	0
C. rochussenii	0	0	0	0	0	1	0	1	0	0	0	0	1	1	1	0	1	0	0	0	0	1	0
C. tomentosa	0	0	0	1	0	0	1	0	1	0	0	0	1	1	1	0	1	0	0	0	1	1	0
C. kaliana	0	0	1	1	1	1	0	0	1	0	0	0	1	1	1	0	1	0	0	0	0	1	0
C. swaniana	0	0	0	1	0	1	0	0	1	0	0	0	1	0	1	0	1	0	0	0	0	1	0
C. asperata	0	0	1	1	0	1	0	0	1	0	0	0	1	0	1	0	0	1	0	0	0	0	1
C. pandurata	0	0	1	1	0	1	0	0	1	0	1	0	0	0	0	1	0	1	0	0	0	0	1
C. mayeriana	0	0	1	1	0	1	0	0	1	0	1	0	1	0	0	1	0	1	0	0	0	0	1

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C. cumingii	0	1	1	1	0	1	0	0	0	0	0	0	1	1	0	0	1	0	0	0	0	1	0
C. foerstermannii	0	0	1	1	0	1	0	0	1	0	0	0	1	1	0	0	1	0	0	0	0	1	0
C. trinervis	0	1	0	1	0	1	0	0	0	0	0	0	1	1	1	0	1	0	0	0	0	0	1
C. viscosa	0	1	1	1	0	1	0	0	0	0	0	0	1	1	1	0	0	1	0	0	0	1	0
C. sp1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C. sp2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-