Sains Malaysiana 51(3)(2022): 643-656
http://doi.org/10.17576/jsm-2022-5103-02

Phylogenetic Relationships of the Orchid Genus *Coelogyne* in Peninsular Malaysia Inferred from Morphological Characteristics and Internal Transcribed Spacer (ITS) Sequence Data

(Hubungan Filogenetik Genus Orkid *Coelogyne* di Semenanjung Malaysia Disimpulkan daripada Ciri Morfologi dan Data Jujukan *Internal Transcribed Spaces* (ITS))

YOH, KOK-HON[1], CHRISTINA SEOK-YIEN YONG[1], JANNA ONG ABDULLAH[2] & RUSEA GO[1,*]

[1]*Department of Biology, Faculty of Science, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor Darul Ehsan, Malaysia*
[2]*Department of Cell and Molecular Biology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor Darul Ehsan, Malaysia*

Received: 3 February 2021/Accepted: 23 July 2021

ABSTRACT

The phylogenetic relationships among the Peninsular Malaysian orchid genus *Coelogyne* were studied by morphological characteristics and sequence data of 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* samples were collected 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 by itself. Another clade consisted of four other sections, namely Flaccidae, Coelogynae, Tomentosae, and Verrucosae. Molecular phylogenies obtained by using the Neighbour-Joining method showed 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: Molecular systematics; neighbour-joining; Orchidaceae; species delimitation

ABSTRAK

Hubungan filogenetik antara orkid genus *Coelogyne* Semenanjung Malaysia telah dikaji berdasarkan ciri morfologi dan data jujukan *internal transcribed region* (ITS) daripada DNA ribosom nukleus (nrDNA). *Coelogyne* adalah genus besar dengan kira-kira 200 spesies yang tersebar di kawasan pantropika dari Himalaya, Sri Lanka, India, China Selatan dan seluruh Asia Tenggara hingga ke Papua New Guinea. Sistem pengelasan yang diterima luas sebelum ini adalah berdasarkan kepada morfologi bunga. Sehingga kini, terdapat hanya sedikit kajian sistematik molekul pada genus *Coelogyne* yang terdapat di Semenanjung Malaysia. Dalam kajian ini, 59 sampel *Coelogyne* dikumpulkan dari seluruh Semenanjung Malaysia dan 57 daripadanya telah dikenal pasti ke peringkat spesies. Untuk mengkaji filogeni genus ini, ciri-ciri morfologi dan bukti molekul digunakan untuk menghasilkan hipotesis sistematik. Analisis kelompok dilakukan dengan menggunakan ciri-ciri vegetatif dan bunga. Hasil kajian menunjukkan bahawa *Coelogyne* Semenanjung Malaysia terdiri daripada tiga seksyen, iaitu Longifoliae, Speciosae dan Fuliginosae yang merupakan kumpulan saudara yang lebih berkait rapat dengan membentuk satu klad tersendiri. Klad lain terdiri daripada empat seksyen lagi, iaitu Flaccidae, Coelogynae, Tomentosae dan Verrucosae. Filogeni molekul yang diperoleh melalui kaedah

Jiran Menyambung mendedahkan hubungan rapat antara seksyen Tomentosae dan Verrucosae, namun penggunaan kaedah kebolehjadian maksimum menunjukkan bahawa tiga seksyen, iaitu Longifoliae, Speciosae dan Fuliginosae adalah kumpulan saudara kerana mereka membentuk satu klad berasingan.

Kata kunci: Jiran menyambung; Orchidaceae; persempadanan spesies; sistematik molekul

Introduction

Coelogyne Lindl. 1821, a genus from the orchid family comprises over 200 species, and is 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 in primary forests. In Peninsular Malaysia, this poorly studied group of orchids has a fairly large number 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 & 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, and three new records are added namely *Coelogyne* rigida C.S.P. Parish & Rchb. f., *Coelogyne* superba R. Rice and *Coelogyne velutina* de Vogel (Rice 2019).

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 proposed a new orchid genus *Thuniopsis* to this subtribe. Nonetheless, very few studies have been conducted on the genus *Coelogyne* and the other genera in subtribe Coelogyninae in Peninsular Malaysia.

During the pre-molecular era, the fundamental for species delimitation of this family was 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 a high degree of parallelism or convergence as these parts are particularly prone to selective pressure from pollinators (Atwood 1986; Dodson 1962). Nowadays, molecular evidence has 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 (in plants) or 28S rRNA genes (in animals) (Baldwin et al. 1995). The nrDNA is highly suited for a broad range of phylogenetic analyses (Hamby & 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 evolve more rapidly and are much more diverged in the 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 have been very limited sectional relationship studies on the Peninsular Malaysia's *Coelogyne* species thus far. The widely adopted classification system previously done by Seidenfaden and Wood (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 systematic studies for this genus are required. Therefore, the objective of this study was to study the phylogenetic 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 leaf 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. In order to determine the species interrelationships, cluster analysis using the UPGMA method was performed. 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 & Doyle 1987). The nuclear ribosomal ITS region was amplified by using primers 17SE and 26SE designed for *Sorghum* (Sun et al. 1994) of approximately 800-1000 bp in length including 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 (10 mM Tris-HCI, 50 mM KCI and 0.1 Triton® X-100, Promega, USA), 2.5 mM $MgCl_2$ (Promega, USA), 0.05 mM dNTPs mix (Promega, USA), 0.5 µM forward primer, 0.5 µM reverse primer (First Base Laboratory, Serdang, Malaysia), 0.5 U *Taq* DNA polymerase (Promega, USA), 50 ng template DNA and ddH_2O in a total volume 50 µL. Table 2 shows the reaction mixture for the PCR amplification. Lastly, 1.5 µL mineral oil was added to the mixture to prevent 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, a final extension cycle was added at 72 °C for 7 min. The amplified products were soaked at 4 °C before being subjected to agarose gel electrophoresis. The DNA sequencing was 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 DNA region were aligned manually using the MEGA 7 software (Kumar et al. 2016). All characters were weighted equally. *Dendrobium crumenatum* from the same subfamily Epidendroideae but of a different tribe (Dendrobieae) was used as the outgroup. To infer the evolutionary relationships, Neighbour-Joining (NJ) analyses were conducted using the matrix of pairwise evolutionary distances between the aligned sequences. 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 evidence

In this study, 59 samples belonging 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, size of leaf and shape of pseudobulb were noted. For the reproductive structures, colour of petal and sepal, colour of lip and characteristics of 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 scored (Appendix 2) for the cluster analysis. Cluster analysis was performed to classify the *Coelogyne* species based on overall similarity (phenetic system). A total of 69 different morphological state characters both quantitative and qualitative were defined in binary mode (0 or 1). For those species that had two or more plant samples, the mean measurement was taken for species delimitation. The phenogram of morphological characters is shown in Figure 1. The phenogram consisted of two major clusters, one cluster 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 70%. This result was fairly congruent with the classification of Seidenfaden and Wood (1992) in that these four sections were closely related to one another. In this study, *C. viscosa* and *C. trinervis* of the section Flaccidae were closely related to *C. foersterrmannii* and *C. cumingii* of the section Coelogynae with high similarity coefficient 83.6%. These four species 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 68.8%. This result also corresponds well with the classification of Seidenfaden and Wood (1992) 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 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 EVIDENCE

Total DNA was extracted from 59 samples of 22 *Coelogyne* species. All the ITS sequences obtained from this study were submitted to the NCBI GenBank database. The accession numbers are shown in Table 1. The ITS sequences were analysed for 59 samples of the 22 *Coelogyne* species and the outgroup *Dendrobium crumenatum* (accession number: KC701378) obtained from NCBI served 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 ITS data, the NJ analysis (Figure 2) showed that four sections (Tomentosae, Verrucosae, Longifoliae, and Speciosae) of *Coelogyne* formed a single clade with bootstrap percentage (BP) 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 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 groups 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 82%. The ML analysis yielded better resolved phylogenetic tree compared to the NJ analysis. The ML tree showed that species of the sections Fuliginasae, Speciosae, Longifoliae, Coelogynae, and Tomentasae showed monophyletic status with a strong bootstrap value 100%. The ML analysis also split the species under section Verrucosae into similar groupings as the NJ analysis. The two unidentified species (*C*. sp1 and *C*. sp2), *C. trinervis* and *C. viscosa* were placed in separate clades from six other sections.

In this investigation, we studied seven different sections of *Coelogyne*. Overall, the phylogenies from

the NJ analysis showed the close relationship between sections Tomentosae and Verrucosae by forming clade B with a moderate BP support 57%. This result is congruent with the earlier classification by Seidenfaden and Wood (1992). However, the phylogenies obtained using the ML analysis demonstrated that three sections i.e. Longifoliae, Speciosae and Fuliginosae were sister groups which were closely related by forming a single clade E with high BP support 97%. The phylogeny from the ML analysis is also fairly congruent with the previous classification of Seidenfaden and Wood (1992). These three sections were introduced together with 10 other sections giving a total of 13 new sections as proposed by Pfitzer and Kraenzlin (1907). This scheme was later maintained by most 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 both 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 DNA marker. The ITS region alone may not be powerful enough to differentiate the circumscription of the seven sections for *Coelogyne*. We believe that more studies are necessary to reconfirm the delineation of *Coelogyne* by employing a combined molecular data set of several genes.

TABLE 1. List of species studied and the GenBank 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

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
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
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

TABLE 2. Reaction mixture for PCR

Chemical stock concentration	Final concentration	Final volume (μL)
Buffer (5×)	1×	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 (5 U/uL)	0.5 U	0.50
DNA template	10 to 500 ng	1.00
ddH_2O	up to final volume 50 μL	29.50
	Total	50.00 μL

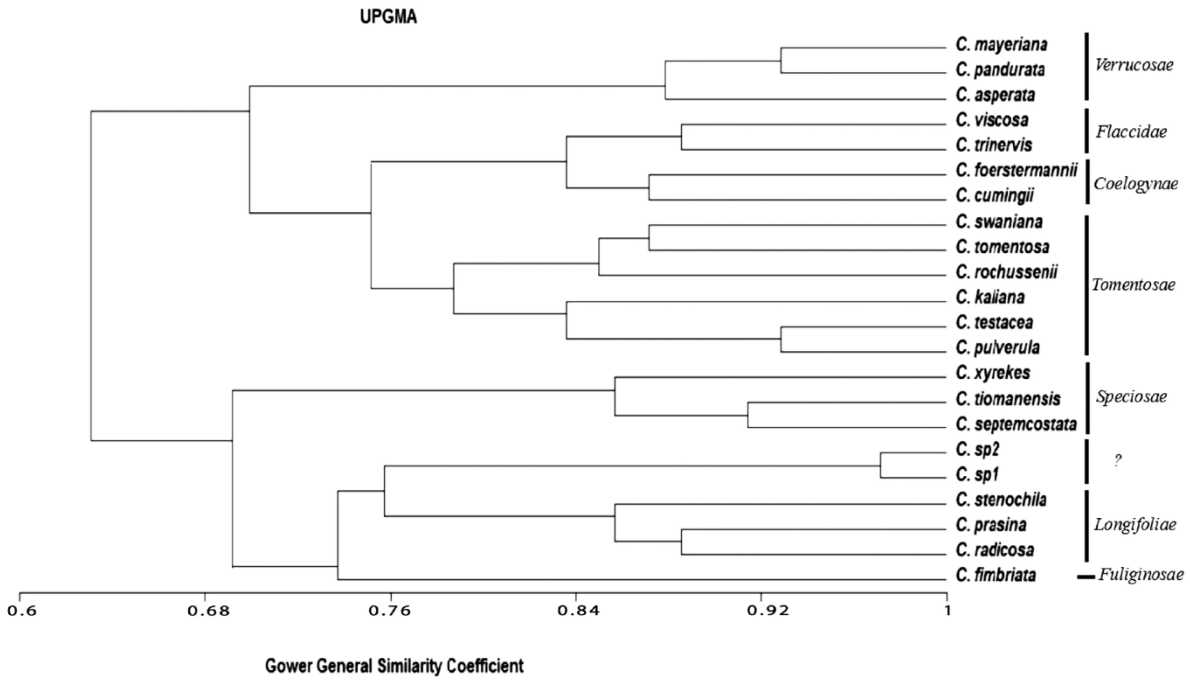

FIGURE 1. UPGMA clustering of *Coelogyne* species based on 69 morphological characters

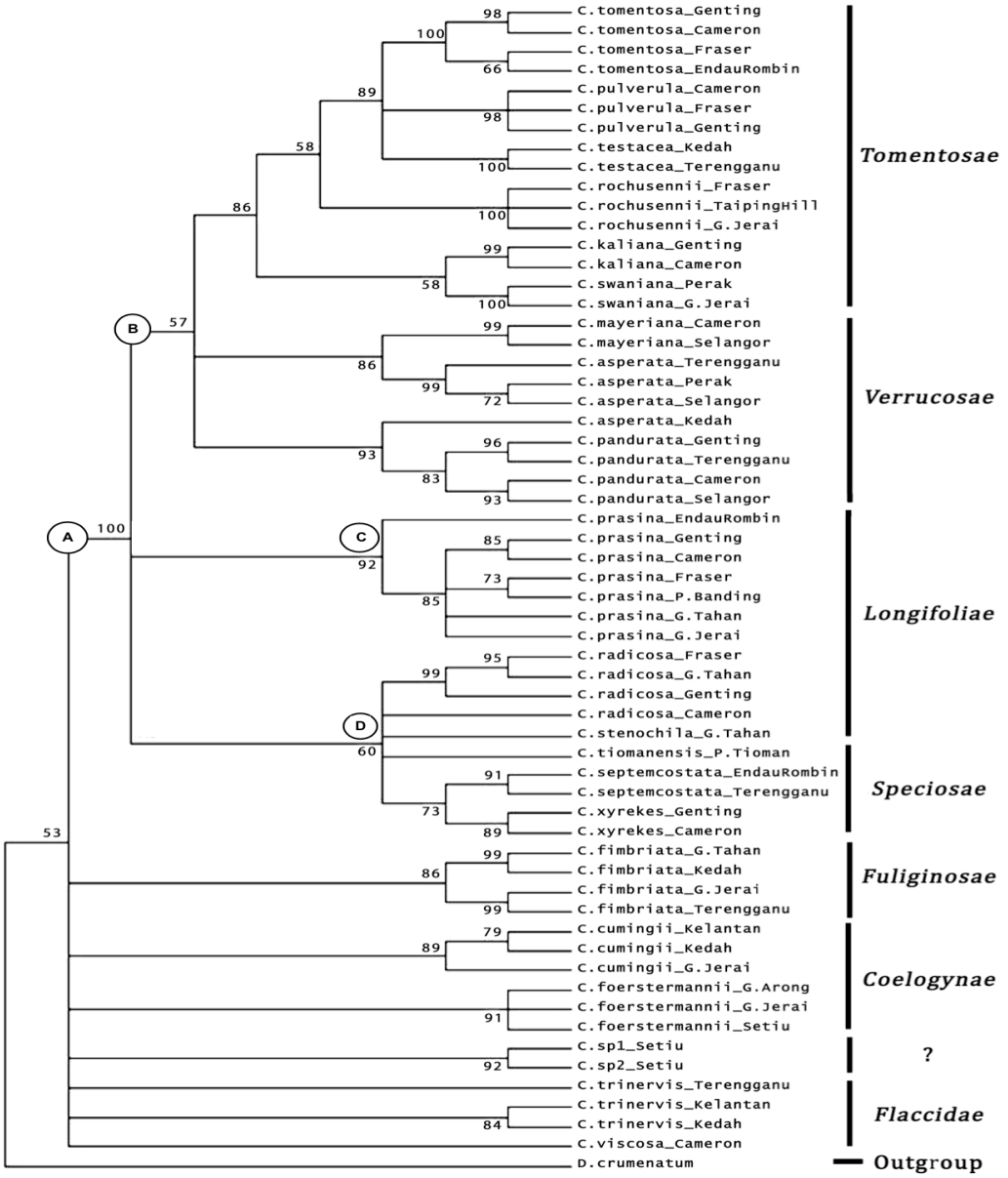

FIGURE 2. The NJ tree based on ITS sequence data. Bootstrap percentages ≥50 are indicated at the nodes

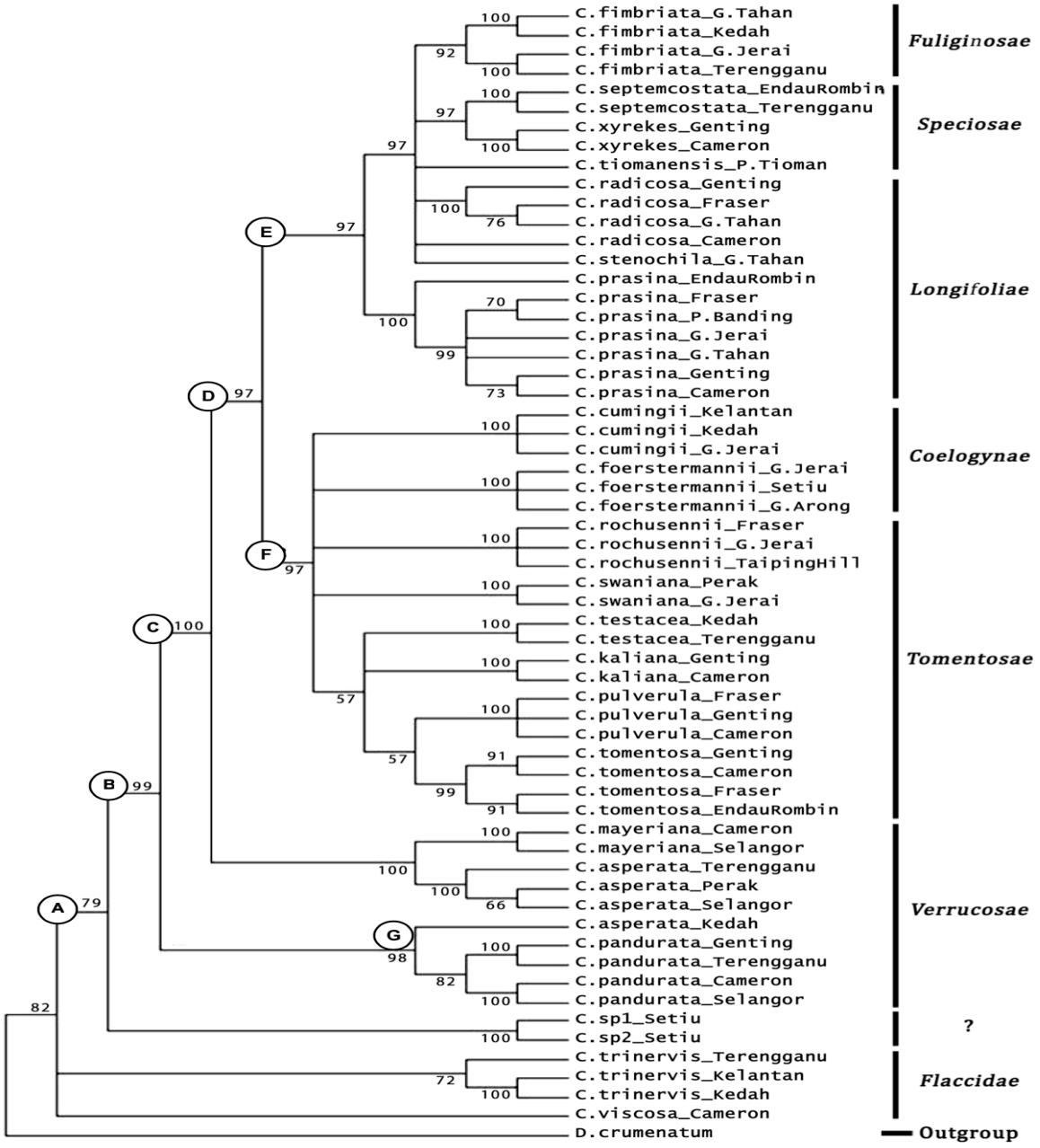

FIGURE 3. The ML tree based on ITS sequence data. Numbers at nodes represent percent recovery in bootstrap analysis (1000 replicates)

CONCLUSION

The phylogeny of seven sections of the genus *Coelogyne* in Peninsular Malaysia was studied based on the mophological characters and ITS 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 the preceding Peninsular Malaysia orchid classification where species from sections Longifoliae, Speciosae, and Fuliginosae formed a single clade, indicating their close relationships. The 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 the species of section Longifoliae based on the vegetative structures only. The cluster analysis results were supported by the Maximum Likelihood analysis of the ITS sequence data, where the sections Longifoliae, Speciosae, and Fuliginosae were found to be closely related. As for the species in sections Coelogynae, Tomentosae, Verrucosae, and Flaccidae, the ML tree showed different groupings to those of the UPGMA clusters. The ITS marker alone 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 *Coelogyne* and other species of the subtribe Coelogyninae, we propose that combined molecular data set of plastid genes such as *rbc*L, *mat*K and *trn*L-F with ITS is to be employed in order to provide a better resolution.

ACKNOWLEDGEMENTS

This study was financially supported by the grant from Universiti Putra Malaysia through Research University Grant Scheme (RUGS) no. 9374500 and 9413603. The authors are also grateful to the Forest Research Institute Malaysia (FRIM) for providing the *Coelogyne tiomanensis* plant samples and Forest Department of Peninsular Malaysia for giving permission to access the study areas where the other samples were collected with permit numbers (a) National Park, Pulau Tioman - JPHL&TN(IP):80-4/2 Jld 17(11) dated 22 October 2013 and (b) National Park, Gunung Tahan - JPHL&TN(IP):80-4/2 Jld 16 dated 3 June 2013.

REFERENCES

Atwood, J.T. 1986. The size of the Orchidaceae and the systematic distribution of epiphytic orchids. *Selbyana* 9: 171-186.

Baldwin, B.G., Sanderson, M.J., Porter, J.M., Wojciechowski, M.F., Campbell, C.S. & Donoghue, M.J. 1995. The ITS region of nuclear ribosomal DNA - A valuable source of evidence on angiosperm phylogeny. *Annals of the Missouri Botanical Garden* 82: 247-277.

Butzin, F. 1992. *Coelogyne* Lindl. In *Die Orchideen*, edited by Brieger, F.G., Maatsch, R. & Senghas, K. Berlin: Verlag Paul Parey. 1A: 919-940.

Comber, J.B. 1990. *Orchids of Java.* Royal Botanic Gardens, Kew. p. 407.

Dodson, C. 1962. The importance of pollination in the evolution of the orchids of tropical America. *American Orchid Society Bulletin* 31: 525-554.

Doyle, J.J. & Doyle, J.L. 1987. A rapid procedure for DNA purification from small quantities of fresh leaf tissue. *Phytochemical Bulletin* 19: 11-15.

Gravendeel, B., Chase, M.W., de Vogel, E.F., Roos, M.C., Mes, T.H.M. & Bachmann, K. 2001. Molecular phylogeny of *Coelogyne* (Epidendroideae, Orchidaceae) based on plastid RELPs, *mat*K and nuclear ribosomal ITS sequences: Evidence for polyphyly. *American Journal of Botany* 88: 1915-1927.

Hall, A.T. 1999. BioEdit: A user-friendly biological sequence alignment editor and analysis performer windows 95/98/NT. *Nucleic Acid Symposium* 41: 95-98.

Hamby, R.K. & Zimmer, E.A. 1992. Ribosomal RNA as a phylogenetic tool in plant systematics. In *Molecular Systematics of Plants,* edited by Soltis, P.S., Soltis, D.E. & Doyle, J.J. New York, London: Routledge, Chapman and Hall, Inc. pp. 50-91.

Kovach, W.L. 2007. *MVSP - A MultiVariate Statistical Package for Windows,* ver. 3.1. Kovach Computing Services, Pentraeth, Wales, U.K.

Kumar, S., Stecher, G. & Tamura, K. 2016. MEGA 7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution* 33: 1870-1874.

Li, L., Ye, D.P., Niu, M., Yan, H.F., Wen, T.L. & Li, S.J. 2015. *Thuniopsis*: A new orchid genus and phylogeny of the tribe Arethuseae (Orchidaceae). *PLoS ONE* 10: e0132777.

Pfitzer, E. & Kraenzlin, F. 1907. Clavis generum Coelogyninarum. In *Das Pflanzenreich*, edited by Engler, H.G.A. Berlin: Akademie-Verlag.

Rice, R. 2019. *Photo Intro to Asian Bulbophyllum, Coelogyne & Dendrobium Orchids with Floristic Observations of Subtribe Coelogyninae*. Nature & Travel Books Press. p. 220.

Seidenfaden, G. & Wood, J.J. 1992. *The Orchids of Peninsular Malaysia and Singapore*. Fredensborg: Olsen and Olsen.

Smith, J.J. 1933. *Coelogyne* Lindl. Feddes Repert. Spec. Nov. Regni Veg. Beih. 32: 166.

Sun, Y., Skinnder, D.Z., Liang, G.H. & Hulbert, S.H. 1994. Phylogenetic analysis of *Sorghum* and related taxa using internal transcribed spacers of nuclear ribosomal DNA. *Theoretical and Applied Genetics* 89: 26-32.

Turner, I.M. 1995. A catalogue of the vascular plants of Malaya. *The Gardens Bulletin Singapore* 47(1): 559-620.

WCSP. 2020. *World Checklist of Selected Plant Families*. Facilitated by the Royal Botanical Gardens, Kew. Published on the Internet, http://apps.kew.org/wcsp/, retrieved on 30 October 2020.

*Corresponding author; email: rusea@upm.edu.my

APPENDIX 1. Morphological character states

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
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

APPENDIX 2. Scoring of morphological characters

Species	Character																						
	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
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	Character																						
	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