Phylogenetic Relationships 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 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 clad 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-HCl, 50 mM KCl and 0.1 Triton® X-100, Promega, USA), 2.5 mM MgCl2 (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 ddH2O 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 *Coelogyne* species.
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 Coelogyne 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 closely related to form a single clade with bootstrap percentage (BP) 100%. The six 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. stenochnila, which are also species of section Longifoliae, were clustered together with the three species (C. tiamanensis, C. septemcostata, and C. xyrekes) of section Speciosae, forming a monophyletic group. Three other sections (Fuliginosae, Coelogynae, and Flaccidae) and two unidentified species (C. sp1 and C. sp2) each formed individual separate groups in this analysis.

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 closely related to three species (C. stenochnila, 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. tiamanensis, 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. stenochnila, which are also species of section Longifoliae, were clustered together with the three species (C. tiamanensis, C. septemcostata, and C. xyrekes) of section Speciosae, forming a monophyletic group. Three other sections (Fuliginosae, Coelogynae, and Flaccidae) and 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.

| 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. 2012        |
| 14. | *C. pandurata* | Selangor, Malaysia | Verrucosae | UMC 1394 | MK356179                  | Planted in Universiti Malaya | 18 Jul. 2013       |
| No. | Species          | Location            | Collection | GenBank | Author                  | Collection Date |
|-----|------------------|---------------------|------------|---------|-------------------------|-----------------|
| 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 |
|   | Species          | Location            | Collection | Accession Number | Institution                  | Date       |
|---|------------------|---------------------|------------|------------------|------------------------------|------------|
| 38 | C. prasina       | Cameron Highlands, Malaysia | Longifolia | YKH 003          | MK356181 Yoh Kok Hon & Rusea Go (UPM) | 10 Jan. 2012 |
| 39 | C. prasina       | Gunung Tahan, Malaysia | Longifolia | YKH 032          | MK356182 Yoh Kok Hon & Rusea Go (UPM) | 3 Sept. 2013 |
| 40 | C. prasina       | Endau-Rompin, Malaysia | Longifolia | RG 2807          | MK356183 Yoh Kok Hon & Rusea Go (UPM) | 1 Jul. 2012  |
| 41 | C. prasina       | Fraser's Hill, Malaysia | Longifolia | FAN.FH115        | MK356184 Farah Alia & Rusea Go (UPM) | 1 Jul. 2011  |
| 42 | C. prasina       | Pulau Banding, Malaysia | Longifolia | RG 2884          | MK356185 Rusea Go (UPM) | 5 Oct. 2012 |
| 43 | C. prasina       | Gunung Jerai, Malaysia | Longifolia | RG 4390          | MK356186 Yoh Kok Hon & Rusea Go (UPM) | 15 Feb. 2013 |
| 44 | C. radicosa      | Genting Highlands, Malaysia | Longifolia | YKH 013          | MK356166 Yoh Kok Hon & Rusea Go (UPM) | 14 Feb. 2012 |
| 45 | C. radicosa      | Cameron Highlands, Malaysia | Longifolia | YKH 002          | MK356167 Yoh Kok Hon & Rusea Go (UPM) | 10 Jan. 2012 |
| 46 | C. radicosa      | Fraser's Hill, Malaysia | Longifolia | FAN.FH193        | MK356168 Farah Alia & Rusea Go (UPM) | 1 Jul. 2011  |
| 47 | C. radicosa      | Gunung Tahan, Malaysia | Longifolia | RG 4488          | MK356169 Yoh Kok Hon & Rusea Go (UPM) | 3 Sept. 2013 |
| 48 | C. stenochila    | Gunung Tahan, Malaysia | Longifolia | 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 (L) |
|-----------------------------|---------------------|------------------|
| Buffer (5x)                 | 1×                  | 10.00            |
| MgCl₂ (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/μL)     | 0.5 U               | 0.50             |
| DNA template                | 10 to 500 ng        | 1.00             |
| ddH₂O                       | up to final volume 50 L | 29.50          |
| **Total**                   |                     | **50.00 L**      |

[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.
The phylogeny of seven sections of the genus *Coelogyne* in Peninsular Malaysia was studied based on the morphological 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 Longifolae, 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 Coelogyininae, we propose that combined molecular data set of plastid genes such as rbcL, matK and trnL-F with ITS is to be employed in order to provide a better resolution.

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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 bases, acute: 0 = no / 1 = yes
19. Leaf bases, cuneate: 0 = no / 1 = yes
20. Leaf bases, obtuse: 0 = no / 1 = yes
21. Leaf apex, acute: 0 = no / 1 = yes
22. Leaf apex, obtuse: 0 = no / 1 = yes
23. Leaf margin: 0 = entire / 1 = crisped
24. Inflorescence, pendulous: 0 = no / 1 = yes
25. Inflorescence insertion, synanthous: 0 = no / 1 = yes
26. Inflorescence insertion, hysteranthous: 0 = no / 1 = yes
27. Inflorescence insertion, heteranthous: 0 = no / 1 = yes
28. Inflorescence insertion, proteranthous: 0 = no / 1 = yes
29. Scape: 0 = without persistent bracts / 1 = with persistent bracts
30. Scape, shape in cross section: 0 = not flattened / 1 = flattened
31. Flower: 0 = single / 1 = multi-flowered
32. Flower: 0 = open in succession / 1 = all opening at the same time (simultaneously)
33. Flower, bract: 0 = caducous (deciduous) / 1 = persistent
34. Flower size, small (diameter less than 35 mm): 0 = no / 1 = yes
35. Flower size, medium (diameter 35-50 mm): 0 = no / 1 = yes
36. Flower size, large (diameter more than 50 mm): 0 = no / 1 = yes
37. Flower, fragrant: 0 = no / 1 = yes
38. Petal and sepal colour, white: 0 = no / 1 = yes
39. Petal and sepal colour, yellow: 0 = no / 1 = yes
40. Petal and sepal colour, green: 0 = no / 1 = yes
41. Petal and sepal colour, salmon pink: 0 = no / 1 = yes
42. Petal, length: 0 = up to 25 mm / 1 = more than 25 mm
43. Petal, width (at middle): 0 = up to 5 mm / 1 = more than 5 mm
44. Petal shape, elliptical: 0 = no / 1 = yes
45. Petal shape, lanceolate: 0 = no / 1 = yes
46. Petal shape, ovate-oblong: 0 = no / 1 = yes
47. Petal shape, linear: 0 = no / 1 = yes
48. Sepal shape, ovate-oblong: 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
| 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 | 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 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 0 |
| C. xyrekes       |           | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 0 |
| C. tiomanensis   |           | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 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 |
| C. prasina       |           | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 0 |
| C. stenochila    |           | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 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 |
| C. testacea      |           | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 1 |
| C. rochussenii   |           | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 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 |
| C. kaliana       |           | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 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 |
| C. asperata      |           | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 1 |
| C. pandurata     |           | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 1 |
| C. mayeriana     |           | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 1 |
| C. cumingii      |           | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| C. foerstermannii|           | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 1 |
| C. trinervis     |           | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 0 |
| C. viscosa       |           | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 1 |
| C. sp1           |           | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 1 |
| C. sp2           |           | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 1 |
| Species       | 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  | 1  | 0  | 0  | 0  | 1  | 0  | 1  | 0  | 0  | 1  | 0  | 0  | 0  | 1  | 0  | 0  | 0  | 0  | 0  | 0  | 0  |
| *C. septemcostata* | 0  | 0  | 1  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 1  | 0  | 0  | 0  | 1  | 1  | 0  | 1  | 0  | 0  | 0  | 0  |
| *C. xyrekes* | 0  | 0  | 1  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 1  | 0  | 0  | 1  | 0  | 0  | 0  | 0  | 0  | 1  | 1  | 0  | 0  |
| *C. tiomanensis* | 0  | 0  | 1  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 1  | 0  | 0  | 0  | 0  | 1  | 0  | 1  | 0  | 0  | 0  | 0  |
| *C. radicosa* | 1  | 0  | 0  | 1  | 0  | 0  | 0  | 1  | 0  | 0  | 0  | 1  | 0  | 0  | 0  | 0  | 0  | 1  | 0  | 0  | 0  | 0  | 0  |
| *C. prasina* | 1  | 0  | 1  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 1  | 0  | 0  | 0  | 0  | 0  | 0  | 1  | 1  | 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  | 1  | 1  | 1  | 0  | 1  | 0  | 1  | 0  | 0  | 1  | 0  | 0  | 0  | 0  | 0  | 0  |
| *C. tomentosa* | 1  | 1  | 0  | 0  | 1  | 0  | 1  | 1  | 1  | 0  | 1  | 0  | 1  | 0  | 1  | 0  | 0  | 0  | 0  | 1  | 0  | 1  |
| *C. kaliana* | 0  | 1  | 0  | 0  | 1  | 0  | 1  | 1  | 1  | 1  | 0  | 1  | 0  | 0  | 1  | 0  | 0  | 0  | 1  | 1  | 1  | 0  |
| *C. swaniana* | 0  | 1  | 0  | 0  | 1  | 0  | 1  | 1  | 1  | 0  | 1  | 0  | 0  | 1  | 0  | 0  | 0  | 0  | 0  | 1  | 0  | 1  |