UNIVERSITI PUTRA MALAYSIA

SYSTEMATIC STUDIES OF ORCHID GENUS *COELOGYNE* IN PENINSULAR MALAYSIA

YOH KOK HON

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By

YOH KOK HON

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April 2019

Chairman: Professor Rusea Go, PhD
Faculty: Science

Coelogyne is a large genus of about 200 species distributed at pantropical area from the Himalayas, Sri Lanka, India, Southern China and throughout South East Asia to the Papua New Guinea. Most of the species are epiphytic which occur on the large tree of primary forest. In Peninsular Malaysia, this poorly studied group of orchids have fairly large number of small, medium to large-sized flowers with pleasant fragrance, but the flowers are usually short-lived. In this study, 59 *Coelogyne* taxa were collected throughout Peninsular Malaysia and 57 of them were identified to species level. The widely accepted classification system previously was exclusively based on floral morphology, and there were no significant molecular studies of *Coelogyne* that have been carried out in Peninsular Malaysia so far. To study the phylogeny of this genus, morphology characters were utilized together with molecular evidences to generate the systematic hypotheses. The morphological analysis part was performed using both the vegetative and floral characters. Clustered analysis was conducted on morphological data of 22 different species while the molecular cladistic analyses were carried out for the 59 taxa. DNA extraction, PCR amplification and sequencing was performed on the 59 taxa using the four nucleotide sequence datasets from two distinct genomes, such as chloroplastid genes (*rbc*L, *mat*K and *trn*L-F) and nuclear ribosomal gene (nrITS). The resulted sequences from each dataset were used to construct independent and combined cladograms using the Neighbour Joining (NJ) and Maximum Likelihood (ML) methods. The overall morphological analysis showed that three sections of Peninsular Malaysian *Coelogyne*, i.e. *Longifoliae*, *Speciosae* and *Fuliginosae* were sister groups which closely related than with the other sections by forming one clade. While another clade consisted of four other sections, namely *Flaccidae*, *Coelogynae*, *Tomentosae* and *Verrucosae*. The molecular systematics analysis had given a robust estimation on the phylogenetic relationships of *Coelogyne*, which implied that the molecular markers *rbc*L, *mat*K and *trn*L-F and nrITS are reliable for the systematics studies of *Coelogyne* species in Peninsular Malaysia. Even though individual marker was insufficient to solve the evolutionary relationship of a whole genus, but the

combination of chloroplastid markers (*rbc*L, *mat*K and *trn*L-F) and nrITS which from different regions greatly improved the systematic study. Combined molecular data has significantly provided better resolution by producing more resolved trees and stronger bootstrap support. Molecular evidences supported the result reported in morphological analysis where sections *Longifoliae*, *Speciosae* and *Fuliginosae* were closely related as well as sections *Tomentosae* and *Verrucosae*. Section *Flaccidae* and *Coelogynae* were placed further from other sections in the phylogenetic tree. Moreover, SCAR markers were also developed from RAPD fragment (Primer: OPU 08 and OPU 12) to discriminate and authenticate three valuable and rare *Coelogyne* species which are endemic to Peninsular Malaysia, namely *Coelogyne kaliana*, *C. stenochila* and *C. tiomanensis*. These three *Coelogyne* spp. are highly similar in their vegetatively morphology and difficult to distinguish without the reproductive structure. Three SCAR markers were successfully developed in this study. SCAR marker pair, CKL_f / CKL_r was specific to *C. kaliana* where it produced a unique single band of 271 bp but not in *C. stenochila* and *C. tiomanensis*. Whereas SCAR marker pair CST_f / CST_r amplified a single band of 854 bp in *C. stenochila* and two bands of different sizes (372 bp and 858 bp) for *C. tiomanensis* but no amplification in *C. kaliana*. The third SCAR marker pair, CTI_f / CTI_r produced a single band (about 500 bp) for both *C. stenochila* and *C. tiomanensis* but showed no amplification in *C. kaliana*. Although not all SCAR markers are species specific, but combination of the three SCAR markers can efficiently discriminate among these three *Coelogyne* species. The accurate identification provides better understanding of the species and allow proper management plan to be established in the effort of conservation.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Doktor Falsafah

KAJIAN SISTEMATIK ANGGERIK GENUS *COELOGYNE* DI SEMENANJUNG MALAYSIA

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Coelogyne merupakan satu genus yang besar dimana terdapat kira-kira 200 spesies di kawasan pantropika dari Himalaya, Sri Lanka, India, China Selatan dan seluruh Asia Tenggara ke Papua New Guinea. Kebanyakan spesies adalah epifit dijumpai di atas pokok besar dalam hutan primer. Di Semenanjung Malaysia, kumpulan orkid yang kurang dikaji ini menghasilkan bunga yang bersaiz kecil, sederhana hingga besar dengan aroma yang menyenangkan, walau bagaimanapun biasanya tidak kekal lama. Dalam kajian ini, 59 taksa *Coelogyne* dikumpul dari seluruh Semenanjung Malaysia dan 57 daripada itu telah dikenalpasti ke peringkat spesies. Sistem klasifikasi dahulu yang diterima secara meluas adalah berdasarkan morfologi bunga sahaja dan tidak terdapat kajian molekul yang signifikan dilakukan ke atas *Coelogyne* Semenanjung Malaysia setakat ini. Untuk mengkaji filogeni genus ini, ciri-ciri morfologi digabungkan dengan bukti molekul untuk tujuan pengelasan sistematik. Kajian morfologi dilakukan dengan menggunakan kedua-dua struktur vegetatif dan bunga. Analisis Berkelompok dijalankan ke atas data morfologi 22 spesies yang berbeza manakala analisis kladistik molekul telah dilakukan ke atas 59 taksa. Pengekstrakan DNA, amplifikasi PCR dan penjujukan dilakukan ke atas 59 taksa dengan menggunakan empat set data jujukan nukleotida daripada dua genom yang berbeza iaitu gen kloroplas (*rbc*L, *mat*K dan *trn*L-F) dan gen ribosomal nuklear (nrITS). Jujukan yang dihasilkan dari setiap dataset digunakan untuk membina kladogram individu dan gabungan dengam kaedah Neighbour Joining (NJ) dan Maximum Likelihood (ML). Secara keseluruhan, analisis morfologi menunjukkan bahawa tiga seksyen *Coelogyne* Semenanjung Malaysia, iaitu *Longifoliae*, *Speciosae* dan *Fuliginosae* adalah kumpulan saudara yang berkait rapat berbanding seksyen lain dengan membentuk satu klad. Sedangkan klad lain terdiri daripada empat seksyen yang lain, iaitu *Flaccidae*, *Coelogynae*, *Tomentosae* dan *Verrucosae*. Analisis sistematik molekul telah memberi anggaran yang kukuh dalam hubungan filogenetik *Coelogyne*, mencadangkan bahawa penanda molekul *rbc*L, *mat*K dan *trn*L-F dan nrITS boleh digunakan bagi kajian sistematik spesies *Coelogyne* di Semenanjung Malaysia. Walaupun individu gen tidak mencukupi untuk menyelesaikan hubungan evolusi genus keseluruhan, tetapi gabungan penanda kloroplastid (*rbc*L, *mat*K dan *trn*L-F) dan nrITS

yang dari genom berlainan telah menambah baik keputusan kajian sistematik. Gabungan data molekul telah memberi resolusi yang lebih baik dengan menghasilkan lebih banyak cabang penyelesaian dan sokongan bootstrap yang lebih kuat. Bukti-bukti molekul menyokong keputusan yang dilaporkan dalam analisis morfologi di mana seksyen *Longifoliae*, *Speciosae* dan *Fuliginosae* adalah berkaitan rapat begitu juga seksyen *Tomentosae* dan *Verrucosae*. Seksyen *Flaccidae* dan *Coelogynae* pula terletak lebih jauh dari sekyen lain dalam pokok filogenetik. Selain itu, penanda SCAR juga dibangunkan daripada fragmen RAPD (Primer: OPU 08 dan OPU 12) untuk membezakan dan mengesahkan tiga spesies *Coelogyne* yang jarang ditemui dan endemik kepada Semenanjung Malaysia, iaitu *Coelogyne kaliana*, *C. stenochila* dan *C. tiomanensis*. Ketiga-tiga spesies *Coelogyne* ini sangat serupa dari segi morfologi vegetatif dan sukar dibezakan tanpa struktur pembiakan. Tiga penanda SCAR berjaya dihasilkan dalam kajian ini. Pasangan penanda SCAR, CKL_f / CKL_r khusus untuk ***C. kaliana*** di mana ia menghasilkan satu jalur tunggal yang unik sebesar 271 bp tetapi tidak dalam *C. stenochila* dan *C. tiomanensis*. Manakala pasangan penanda SCAR, CST_f / CST_r menjanakan satu jalur 854 bp dalam *C. stenochila* dan dua jalur yang berlainan saiz (372 bp dan 858 bp) untuk *C. tiomanensis* tetapi tiada penjanaan dalam *C. kaliana*. Pasangan penanda SCAR yang ketiga, CTI_f / CTI_r menghasilkan satu jalur (kira-kira 500 bp) untuk kedua-dua *C. stenochila* dan *C. tiomanensis* tetapi tiada amplifikasi dalam *C. kaliana*. Walaupun bukan semua penanda SCAR adalah spesifik spesies, tetapi kombinasi ketiga-tiga penanda SCAR dapat membezakan antara tiga spesies *Coelogyne* ini dengan mudah. Pengenalpastian yang tepat memberikan pemahaman yang lebih baik tentang species-spesies tersebut dan membolehkan pelan pengurusan yang sesuai dapat dirancang untuk pemuliharaan.

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LIST OF ABBREVIATIONS

AFLP	Amplified Fragment Length Polymorphism
BLAST	Basic Local Alignment Search
BP	Bootstrap Replicates
bp	Base pair
CBOL	Consortium for the Barcode of Life
cpDNA	Chloroplast DNA
CTAB	Cetyltrimethyl Ammonium Bromide
ddH_2O	Double-distilled water
DNA	Deoxyribonucleic Nucleic Acid
dNTPs	Deoxynucleotide triphosphates
EDTA	Ethylenediaminetetraacetate
ISSR	Inter-Simple Sequence Repeat
ITS	Internal Transcribed Spacer
KEP	Kepong Herbarium
g	Gram(s)
kb	Kilobases
*mat*K	MaturaseK gene
min	Minute (s)
ml	Millilitre (s)
ML	Maximum Likelihood
mM	Millimolar
mtDNA	Mitochondrial DNA
NJ	Neighbour Joining
NHN-*L*	Netherlands-*Leiden* University branch

nrITS	Nuclear Ribosomal Internal Transcribed Spacer
PCR	Polymerase Chain Reaction
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic Acid
rpm	Revolutions per minute
sec	Second(s)
SCAR	Sequence Characterized Amplified Region
SING	Singapore Herbarium
sp	species
TBE	Tris-Borate EDTA (buffer)
TBR	Tree Bisection Reconnection
TRIS	Tris(hydroxymethyl)amino-methane
µg	Microgram(s) = 10^{-6} gram
µl	Microliter(s) = 10^{-6} liter
µM	Micromolar
UV	Ultra Violet
V	Volts
WCSP	World Checklist of Selected Plant Families
%	Percentage
×	Times
°C	Degree Celsius

CHAPTER 1

INTRODUCTION

The family Orchidaceae, with 736 genera (Chase *et al.*, 2015) and estimated to have 28, 000 species, is one of the two largest families of flowering plants, as well as of the most diverse and widespread family with colourful, fragrant blooms and special floral structures (Dressler and Dodson, 1960; Pridgeon, Cribb, Chase and Rasmussen, 2003). During pre-molecular era, the fundamental for species delimitation of this family were based on morphological and anatomical characters, especially at 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 systematic employ the nucleotide and protein sequence comparisons for estimating phylogenetic relationships. DNA sequence which serve as the basis of molecular systematic make use of the study of different gene markers. The common molecular markers used in plant systematic come from two main sources, which are the plastid DNA and nuclear ribosomal DNA. Protein-coding plastid markers such as *rbc*L and *mat*K were primarily used for classification at the family level. Non-coding plastid markers (*trn*L intron and *trn*L-F intergenic spacer) and nuclear ribosomal ITS gene (ITS1 + 5.8S + ITS2) often studied for classification at lower taxonomic level (tribal and below). There are also phylogenetic analyses based on combination of different markers from plastids (*rbc*L, *mat*K, *trn*L-F, *atp*B) and nuclear (ITS).

There are increasing knowledge on the classification of Orchidaceae, through the introduction of molecular methods which were not considered by traditional classification scheme, it may appear that perhaps one day will come out a comprehensive classification system that every taxonomist agrees with (Seidenfaden and Wood, 1992). So far, the latest orchid classification was updated by Chase *et al*. (2015), the classification of all currently recognized 736 genera has been revised. Compared to other earlier findings, Chase *et al*. (2015) made no changes in the subfamilies Apostasioideae and Cypripedioideae, while Vanilloideae was separated into two tribes. As in subfamily Orchidoideae, there were only some small generic changes and most of the changes happened in the subfamily Epidendoideae.

Coelogyne Lindl. 1821, a genus from the Orchid family comprising of over 200 species, distributed across India, Nepal, China, Southeast Asia to the Fiji islands, with the main centres in Borneo, Sumatera and the Himalaya mountain range. There are 28 species of *Coelogyne* in Peninsular Malaysia (Seidenfaden and Wood, 1992; Turner, 1995). However, the World Checklist of Selected Plant Families (WCSP, 2017) recognized only 24 species as 4 species are now synonyms. According to the Checklist of Orchids of

Peninsular Malaysia (Ong, 2017), 26 species were recognized. In Malaysia, there are some very beautiful species which are frequently cultivated. *Coelogyne* species such as *Coelogyne rochussenii*, *C. mayeriana* and *C. pandurata* have high commercial value among wild orchid collectors and enthusiast leading to the illegal and indiscriminate collection of plants from the wild. Many endemic species are severely threatened. There are also some smaller flowered mountain *Coelogyne* spp. that are currently insufficiently known in Malaysia and need further study.

As some *Coelogyne* spp. are very similar vegetatively and very difficult to distinguish morphologically without flower, making the 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), they discovered and proposed a new orchid genus named *Thuniopsis* to this subtribe. Nonetheless, there is still very limited study on the genus *Coelogyne* and other genera in subtribe Coelogyninae in Peninsular Malaysia. Therefore, to confirm and resolve the uncertainty of the taxonomical status of *Coelogyne* spp., molecular systematics study of this genus is required. The molecular approach used in this study has successfully resolved the sectional delimitation and evolutionary relationships among *Coelogyne* spp.

Sequence Characterized Amplified Regions (SCAR) marker developed by Paran and Michelmore (1993) is a robust, current established and reliable method used to identify and differentiate closely related samples or species using PCR amplification by amplifying product at different sizes, or it leads to negative amplification in non-targeted samples and positive amplification in targeted sample. For the past few years, this method has been widely adopted in the authentication of morphologically similar but genetically different organisms. A number of robust and reliable RAPD-based SCAR marker has been successfully developed for *Lettuca* (Paran and Michelmore, 1993), *Pisum sativum* (Srivastava, Mishra, Singh and Mohapatra, 2012) and *Sorghum halepense* (Zhang *et al.*, 2013). In orchids, such markers have been developed for *Phalaenopsis* (Niknejad, Kadir, Kadzimin, Abdullah and Sorkheh, 2009) and *Paphiopedilum* (Sun, Liao, Hung, Chang and Sung, 2011). However, SCAR markers have not been developed for *Coelogyne* spp. in Malaysia.

In this study, novel RAPD-SCAR markers had been developed successfully to authenticate three morphologically similar and closely related Peninsular Malaysia endemic *Coelogyne* spp. RAPD primers were used to amplify the DNA samples of the three endemic species to identify reproducible species-specific bands. The species-specific bands were then cloned, sequenced and SCAR markers were designed based on these sequences. This is the first study on the development of RAPD-based SCAR marker to authenticate the endemic orchid's genus *Coelogyne* in Peninsular Malaysia.

1.1 Objectives

The objectives of this study were:

1. To determine the phylogenetic relationships among *Coelogyne* species in Peninsular Malaysia.

2. To compare morphological classification with molecular classification.

3. To design and test the species-specific SCAR markers as additional tool for rapid identification of *Coelogyne* species in Peninsular Malaysia.

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LIST OF PUBLICATIONS

Rusea, G., Janna, O. A., Wong, W. N., Nor Izzati, S., Abdullah, N. A. P., Mohd-Akmal, M. R., Adilah, A., Christina, Y. S. Y., **Yoh, K. H**. and Fatimah, S. M. I. (2013). Orchid diversity in North Selangor Peat Swamp Forest. In JPSM & MNS (Eds.), Prosiding Ekspedisi Saintifik Biodiversiti Hutan Paya Gambut Selangor Utara. Shah Alam, Selangor: Jabatan Perhutanan Semenanjung Malaysia & Malaysia Nature Society, 136-144.

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