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DNA BARCODING OF ENDANGERED PENINSULAR MALAYSIAN SLIPPER ORCHIDS, THE GENUS PAPHIOPEDILUM

MICHEAL CHARLES RAJARAM

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DNA BARCODING OF ENDANGERED PENINSULAR MALAYSIAN SLIPPER ORCHIDS, THE GENUS *PAPHIOPEDILUM*

By

MICHEAL CHARLES RAJARAM

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DNA BARCODING OF ENDANGERED PENINSULAR MALAYSIAN SLIPPER ORCHIDS, THE GENUS *PAPHIOPEDILUM*

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Chair : Rusea Go, PhD
Faculty : Science

Paphiopedilum is a genus of the orchid family that is highly prized for its beautiful and long lasting flowers. Due to its attractiveness, many plants have been collected from the wild to meet the demands of the market and thus reduced the populations till the point of endangerment. Thus, all *Paphiopedilum* are protected by CITES Appendix I. Even though they produce beautiful and distinctive flowers, the vegetative form of this genus, however, lacks sufficient distinguishing features that enable species discrimination, creating a problem for accurate species identification for biodiversity inventory and in regulating the control of its trade. DNA barcoding uses standardized regions of DNA that can supplement the current taxonomic identification data. Five species have been identified to occur in Peninsular Malaysia and IUCN Redlist categorized all species as Endangered. Some of the threats recognized to affect *Paphiopedilum* survival in the wild are poaching, deforestation and logging in Malaysia. The samples were collected in localities throughout Peninsular Malaysia. To produce species-specific barcodes, DNA extraction was performed using the CTAB method. Selected barcode regions were amplified using PCR and the protocols were optimized specifically for all barcodes chosen. Amplified products were sequenced using Sanger bi-directional sequencing method. *rbcL* and *matK* has the highest sequence quality and followed by *trnH-psbA* and *ITS*. Sequences obtained were edited, aligned and its effectiveness was measured by using several approaches: presence of monophyletic groups in Neighbour-Joining trees, barcoding gap (global and local) and BLASTn sequence similarity. *matK* showed the highest species resolution with 100% correct match in NCBI BLASTn database followed by *ITS* (52.9%), *trnH-psbA* (26.3%) and lastly *rbcL* (23.5%). All barcodes were correct at the genus level. Phylogenetic trees were constructed using the Neighbour-joining method with Kimura-2-parameter metric and supported by 1000 bootstrap replicates in MEGA 6.0. Trees constructed using *matK* and *ITS*

barcodes grouped similar species into a clade and congruent with currently accepted taxonomy while those constructed with *rbcL* and *trnH-psbA* were unable to resolve the differences between the species. Differences between barcode efficiency are attributed to differing molecular rate of evolution of the individual loci. Global barcoding gap exists only in *matK* sequences but could be the result of sampling bias and may not be a reliable indicator for species delimitation. Local barcoding gap exists for all species studied using *matK* sequences except between *P. barbatum* and *P. callosum* var. *sublaeve*. The shared similarities and uneven sampling between both species may contribute to the lack of local barcoding gap. Based on the findings of the study, *matK* is the most suitable barcode for identifying *Paphiopedilum* species in Peninsular Malaysia. DNA barcoding presents an effective and simple method for species level identification for *Paphiopedilum* species.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

DNA BARCODING SPESIS TERANCAM ORKID SELIPAR DI SEMENANJUNG MALAYSIA, GENUS _PAPHIOPEDILUM_

Oleh

MICHEAL CHARLES RAJARAM

Mac 2018

Pengerusi : Rusea Go, PhD
Fakulti : Sains

Paphiopedilum adalah genus dari keluarga orkid yang terdiri daripada spesis-spesis yang menghasilkan bunga yang cantik dan mampu bertahan lama. Namun disebabkan tarikan kecantikannya, orkid ini telah diambil dari habitatnya secara besar-besaran bagi menampung kehendak pasaran flora tetapi pada masa yang sama kegiatan ini telah banyak mengurangkan populasi *Paphiopedilum* di habitat semula jadinya. Oleh itu, semua spesis *Paphiopedilum* dilindungi CITES Appendix I. Meskipun bunga yang dihasilkan oleh setiap spesis berbeza-beza dan adalah ciri pengenalan utama bagi orkid, *Paphiopedilum* jarang sekali berbunga dan keadaan vegetatif selalunya tidak membolehkan pengenalan pada tahap spesis yang menyukarkan inventori biodiversiti dan kawalan pasarannya. DNA barcoding adalah kaedah molekular yang menggunakan jujukan nukleotid dari segmen DNA yang telah ditetapkan bagi menambahbaik data taksonomi yang sedia ada. Lima spesis telah dikenalpasti di Semenanjung Malaysia dan setiap spesis ini dikategorikan sebagai terancam oleh IUCN Redlist. Antara ancaman terhadap *Paphiopedilum* adalah pengambilan secara haram, penebangan hutan dan pembalakan. Sampel-sampel diambil di lokaliti-lokaliti di seluruh Semenanjung Malaysia. Bagi menghasilkan kod bar yang spesifik pada setiap spesis, DNA diekstrak menggunakan kaedah CTAB dan lokus kod bar yang terpilih dalam DNA tersebut digandakan melalui teknik PCR. Penjujukan DNA dijalankan melalui kaedah pejujukan dua hala Sanger. Lokus *rbcL* dan matK menghasilkan kualiti jujukan DNA yang terbaik dan in diikuti oleh *trnH-psbA* dan akhirnya *ITS*. Setiap jujukan nukleotida yang dihasilkan telahpun diedit, dijajarkan dan keberkesannya dinilai melalui beberapa pendekatan iaitu kumpulan monofiletik dalam filogeni Neighbour-Joining, jurang kod bar (global dan lokal) dan padanan dalam laman sesawang NCBI BLASTn. *matK* menunjukkan resolusi tertinggi dengan padanan tepat 100% berdasarkan pangkalan data BLASTn dan diikuti oleh *ITS* (52.9%), *trnH-psbA* (26.3%) dan (23.5%). Walaubagaimana pun, semua kod bar boleh mengenalpasti tepat pada peringkat genus. Filogeni yang dihasilkan menggunakan kaedah

Neighbour-Joining bermetrikkan Kimura-2-parameter dan disokong oleh 1000 replikat bootstrap. Filogeni *matK* dan *ITS* menyamai taksonomi terkini tetapi filogeni yang dihasilkan menggunakan jujukan *rbcL* dan *trnH-psbA* tidak dapat membezakan diantara spesis. Jurang kod bar global wujud dalam jujukan *matK* tetapi ini mungkin disebabkan oleh persampelan yang berat sebelah, oleh itu jurang ini tidak sesuai dijadikan sebagai penanda untuk penspesisan. Jurang kod bar lokal pula wujud untuk tiga daripada 4 spesis menggunakan jujukan *matK*. Tiada jurang lokal antara *P. barbatum* dan *P. callosum* var. *sublaeve*. Persamaan morfologi yang tinggi di antara dua spesis tersebut dan ketidakseimbangan dalam bilangan sampel menyumbang kepada ketiadaan jurang tersebut. Berdasarkan kajian ini, didapati sebagai lokus kod bar terbaik bagi mengenalpasti spesis dalam *Paphiopedilum* di Semenanjung Malaysia. DNA barcoding merupakan kaedah yang mudah dan efektif bagi pengenalpastian pada tahap spesis.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

Rusea Go, PhD
Professor
Faculty of Science
Universiti Putra Malaysia
(Chairman)

Christina Yong Seok Yien, PhD
Senior Lecturer
Faculty of Science
Universiti Putra Malaysia
(Member)

ROBIAH BINTI YUNUS, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

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Signature: _____
Name of Chairman of Supervisory Committee: Prof. Dr. Rusea Go

Signature: _____
Name of Member of Supervisory Committee: Dr. Christina Yong Seok Yien

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LIST OF ABBREVIATIONS

BOLD	Barcoding of Life Database
BLAST	Basic Local Alignment Search Tool
CITES	Convention on International Trade in Endangered Species of Wild Fauna and Flora
CTAB	cetyltrimethyl ammonium bromide
DNA	deoxyribonucleic acid
et al.	abbreviation for the Latin et alia, meaning “and the others”
ITS	Internal transcribed spacer
matK	maturase K
NCBI	National Center for Biotechnology
NJ	Neighbour-Joining
NN	Nearest-Neighbour
PCR	Polymerase chain reaction
rbcL	ribulose biphosphate carboxylase large chain precursor
RNA	ribonucleic acid

CHAPTER 1

INTRODUCTION

1.1 General Introduction

The family Orchidaceae is the most diverse of all flowering plants boasting of 25 000 species found in most parts of the world except in deserts and the poles (Swarts & Dixon, 2009). Orchids are a group of flowering plants that is widely distributed. They can be found in a variety of habitats with forms and living habits that are even more diverse. Orchids can be found on all the continents except at the Arctic and Antarctica. They can live in temperate forests but the major orchid diversity is found in the tropical rainforests (Dixon *et al*., 2003; Roberts & Dixon, 2013). Tropical rainforests provide a suitable habitat for species to thrive in. Indeed, of the 25 hotspots listed, tropical rainforests tops the list and dominate in terms of endemism present (Myers *et al.*, 2000). Plenty of sunshine and availability of water allow more species to live in and evolve, thus increasing species richness.

Of all the variety and beauty that orchids are bestowed with, slipper orchids are one of the most intriguing species to have occurred. According to Cribb (1998), there are collectively less than 150 species of slipper orchids that can be found all over the world. Majority of the species occur in areas that have tropical climate and only one genus occurs in the temperate climate. There are 5 genera in this subfamily namely *Selenipedium, Phragmipedium, Mexipedium, Cypripedium* and *Paphiopedilum.* The last mentioned genus is the largest in the subfamily and the only one found in tropical Asia.

Malaysia has been blessed with excellent conditions for orchids to thrive in. With approximately 2000 species of orchids occur within its borders, it is a region of immense biodiversity and also of concern for its preservation (Myers *et al*., 2000; Rusea *et al*., 2009). Up to date, 21 accepted species of *Paphiopedilum* has been described to occur in this country, including some of the rarest and most magnificent looking slipper orchids like *Paphiopedilum rothschildianum* and *Paphiopedilum sanderianum* just to name two (Govaerts *et al*., 2018). Due to its beautiful and unique flowers, slipper orchids have been collected and poached for ornamental purposes but this has caused its number to dwindle in the wild. Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) has placed *Paphiopedilum* in Appendix I which prevents its trade across borders of the country it belongs to. Morphological characters of the plants that are very much similar to each other vegetatively have hindered correct identification of species especially in the absence of flowers (Go *et al.*, 2011).

Taxonomy has been a driving force in species inventory of the world's biodiversity but the progress made after 250 years of work is slow and limited in terms of its heavy reliance on morphology (de Boer *et al*., 2014). Compounded also by issues like the lack of specialized taxonomist in certain plant groups, falling out of fashion as a science due to lack of prospective taxonomists, misidentifications in the literature and the difficulty to publish in high impact journals, traditional taxonomy no longer is able to cope with the demands placed on it (Pyšek *et al*., 2013; Ely *et al*., 2017). As such is the problem, DNA barcoding has been proposed as a simple yet effective means by which identification of the individual species can be done without the need for a specialist to routinely identify them. Each barcode is unique to each species and gives clear-cut identification even between closely related species (Chase & Fay, 2009), therefore relieving the specialist from routine identification duties. Though other molecular methods are available for identification, none have been able to offer the specificity of identification at the cost of using universal markers.

1.2 Problem Statements

The genus *Paphiopedilum* in Peninsular Malaysia has not been studied for molecular identification despite being described since the 19th century except for *P. lowii* and *P. dayanum*. The current taxonomy that depends solely on morphological descriptions is not dependable for accurate identifications. Therefore, a more reliable method for taxonomy is needed.

1.3 Objectives of Study

The objectives of this study are as follows;

1. To identify *Paphiopedilum* species from Peninsular Malaysia, their distribution and the conservation status according to IUCN Redlist of Endangered Plant Categories and Criteria.

2. To develop species-specific DNA barcode for all the collected *Paphiopedilum* and deposit them in CBOL and NCBI.

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BIODATA OF STUDENT

Micheal Charles Rajaram was raised in Senawang, Negeri Sembilan and completed the primary and secondary educational levels in SK Senawang and SMK Senawang respectively before joining SMK Seri Ampangan to continue STPM or Form 6 for pre-university. Since then, he has completed B.Sc in Biology in Universiti Putra Malaysia in 2014 and upon graduation enrolled for M.Sc in Universiti Putra Malaysia.

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