ABILITY OF *trnH-psbA* AND *trnL-trnF* INTERGENIC SPACERS TO DISCRIMINATE BETWEEN CLOSELY RELATED *Paphiopedilum* SPECIES

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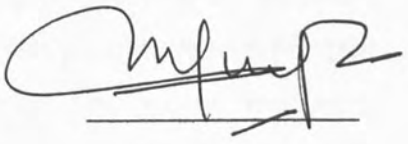
1. SUPERVISOR **(DR. VIJAY KUMAR)**	
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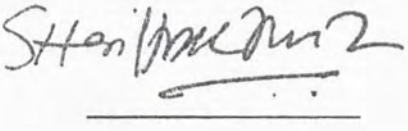
3. DEAN **(SUPT/ KS ASSOCIATE PROFESSOR** **DR. SHARIFF A. K OMANG)**	

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ABSTRACT

Two intergenic spacers, *trnH-psbA* and *trnL-trnF* of the chloroplast DNA (cpDNA) were used to determine whether they can be used to distinguish between three closely related *Paphiopedilum* species namely *P. rothschildianum*, *P. lowii* and *P. dayanum*. Genomic DNA was extracted from the leaves of the three *Paphiopedilum* species without the use of phenol chloroform. Next, the two loci were amplified through PCR using two primers pairs (psbAF – trnHR and trnLF – trnFR). The amplified PCR products were ligated into the pCR 2.1 – TOPO vector, transformed using TOP 10 chemically competent cells. Then, plasmid minipreparation was performed and the plasmids obtained were sequenced. Data analysis was performed using the resulting sequences. A total of 29 indels mutations points with 95 indels were observed, accounting for *trnH-psbA* intergenic spacer length of *P. rothschildianum*, *P. lowii* and *P. dayanum* with a range of 924 to 1000 bp. In the *trnL-trnF* intergenic spacer, 11 indels mutation points with 48 indels have been discovered for the three *Paphiopedilum* species with a range of between 462 to 490 bp. The genetic similarity and differences between the *Paphiopedilum* species was examined by calculating the pairwise nucleotide differences that occurred in each intergenic region. The result showed that *P. rothschildianum* and *P. lowii* were more closely related compare to *P. dayanum*. Single nucleotide polymorphisms (SNP) were then determined and *trnH-psbA* intergenic spacer was found to provide 96 informative sites which indicate the variations that occur in this spacer region was higher compared to *trnL-trnF* spacer region which provide 48 informative sites. The phylogenies of the three species were also examined by constructing phylogenetic trees and confirmed by performing bootstrapping analysis. The results obtained indicate that *trnL-trnF* intergenic spacer is more reliable compared to the *trnH-psbA* intergenic region for distinguishing between the three *Paphiopedilum* species. However, more research should to be conducted on *trnH-psbA* intergenic spacer as it exhibit sufficient nucleotide variations which proved that it is also a potentially good marker for phylogenetics studies and DNA barcoding of other plants.

ABSTRAK

Dua *intergenic spacer*, *trnH-psbA* dan *trnL-trnF* dari DNA kloroplas dikaji untuk menentukan sama ada dua *intergenic spacer* ini sesuai digunakan untuk membezakan antara tiga spesies *Paphiopedilum* yang saling berkait rapat iaitu *P. rothschildianum*, *P. lowii* dan *P. dayanum*. DNA genomik diekstrak daripada daun tiga spesies *Paphiopedilum* tanpa menggunakan fenol kloroform. Kemudian, dua *spacer* tersebut diamplifikasikan menggunakan dua pasangan primer (psbAF – trnHR dan trnLF – trnFR). Produk PCR yang terhasil diklon dengan vektor pCR 2.1 – TOPO and ditransformasikan dalam TOP 10 *competent cell*. Selepas itu, plasmid diekstrak dan penjujukan DNA dilakukan. Analisis data dilakukan dengan menggunakan jujukan DNA yang diperolehi. Bagi *trnH-psbA intergenic spacer*, sejumlah 29 kawasan tumpuan mutasi dengan 95 *indels* telah diperhatikan dalam kawasan *spacer* yang bersaiz dalam lingkungan 924 hingga 1000 pasangan bes bagi ketiga-tiga spesies *Paphiopedilum*. Manakala bagi *trnL-trnF intergenic spacer* pula, 11 kawasan tumpuan mutasi dengan 48 *indels* telah dikenalpasti daripada tiga spesies yang bersaiz dalam lingkungan 462 hingga 490 pasangan bes. Persamaan dan perbezaan genetik diantara spesies-spesies ditentukan dengan mengira perbezaan nukleotid secara berpasangan antara tiga spesies tersebut bagi kedua-dua *intergenic spacer*. Keputusan yang diperolehi menunjukkan bahawa *P. lowii* dan *P. rothschildianum* mempunyai persamaan yang paling ketara berbanding *P. dayanum*. *Single nucleotide polymorphisms (SNP)* kemudiannya ditentukan dan didapati *trnH-psbA intergenic spacer* mempunyai bilangan kawasan informatif yang lebih banyak yakni 96 berbanding *trnL-trnF intergenic spacer* yang mempunyai 48 kawasan informatif. Bilangan kawasan informatif yang lebih banyak dalam *trnH-psbA intergenic spacer* menunjukkan *spacer* ini mempunyai lebih variasi berbanding *trnL-trnF intergenic spacer*. Hubungan filogenetik diantara tiga spesies *Paphiopedilum* dikaji dengan menggunakan pokok filogenetik dan kemudian dengan analisis *bootstrap*. Hasil kajian menunjukkan bahawa *trnL-trnF intergenic spacer* adalah lebih sesuai digunakan untuk membezakan antara tiga spesies *Paphiopedilum*. Namun, lebih banyak kajian keatas *trnH-psbA spacer* patut dijalankan kerana, *spacer* ini juga menujukkkan variasi nukleotid yang tinggi dan berpotensi untuk dijadikan penanda molekular untuk kajian filogenetik and barkod DNA bagi tumbuhan lain.

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LIST OF ABBREVIATIONS

WCE	Sabah Wildlife Conservation Enactment
CBOL	The Consortium for the Barcode of Life
CITES	Convention on International Trade in Endangered Species
RM	Malaysian Ringgit
COI	*Cytochrome c oxidase subunit I* gene
DNA	Deoxyribonucleic acid
gDNA	Genomic deoxyribonucleic acid
mtDNA	Mitochondrial deoxyribonucleic acid
cpDNA	Chloroplast deoxyribonucleic acid
rRNA	Ribosomal ribonucleic acid
tRNA	Transfer ribonucleic acid
RNase	Ribonuclease
DNase	Deoxyribonuclease
BLAST	Basic Local Alignment Search Tool
SNP	Single nucleotide polymorphisms
SSR	Simple Sequence Repeat/ Microsatellite
RFLP	Restriction Fragment Length Polymorphism
RAPD	Random Amplified Polymorphism DNA
AFLP	Amplified Fragment Length Polymorphism
PCR	Polymerase Chain Reaction
NaCl	Natrium chloride
NaOH	Sodium hydroxide
EDTA	Ethylenediamine tetraacetic acid
SDS	Sodium dodesil sulphate
KCl	Potassium acetate
TBE	Tris-Borate-EDTA
LB	Luria-Bertani
BSA	Bovine Serum Albumin
E. coli	*Escherichia coli*
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
UV	ultra-violet

A	Adenine
T	Thymine
C	Cytosine
G	Guanine
g	gram
rpm	round per minute
min	minute (s)
s	second (s)
kb	kilo base pair
bp	base pair
M	molar
mM	milimolar
mmol	milimol
pmol	picomol
ml	mililiter
µl	microliter
L	liter
V	volt
U	unit
%	percent
°C	degree Celsius

CHAPTER 1

INTRODUCTION

1.1 Preface

Malaysia is a home to about 3000 species of exotic, attractive and unique orchid species and many of these are native to Malaysia. Orchids usually grow well in the jungles, forests, mountains and islands. On mountainous areas, the orchids usually grow at the altitude of 1000 to 6000 meters above sea level. For instance, the largest species of all orchids, the *Grammatophyllum* can be found in the mountainous areas of the Main Range of Peninsular Malaysia (Fadelah *et al.*, 2001). In Sabah and Sarawak, unique orchid species such as the *Paphiopedilum* species orchids can be found.

The genus *Paphiopedilum* belongs to the family of Orchidaceae and subfamily of *Cyripedioideae*. In fact, *Paphiopedilum* orchids are closely related to *Cypripedium*, *Phragmipedium* and *Selenipedium*. They are all referred to as *Cypripedium* or 'Cyps' prior to being separated to their respective genera by Rolfe in 1896. The distribution of *Paphiopedilum* orchids extend from India to Burma, Southeast Asia, South China,

Malaysia, Java, Borneo, Philippines and Papua New Guinea to the Solomon Islands (Schoser, 1981). There are about 50 species of *Paphiopedilum* that are native to tropical Asia and five of which are found growing in Malaysia.

The name of *Paphiopedilum* is derived from a Greek word *paphos*, which means the 'island with a temple dedicated to Venus' and also *pedilon* which means 'sandal' (Teo, 1985). The common name of the ladyslipper orchid is given due to the appearance of the lip of the *Paphiopedilum* flower which resembles a lady's slipper. Due to the unique appearance of the flowers, *Paphiopedilum* species are always in high demand and popular among orchid lovers.

In Sabah, *Paphiopedilum* is zealously protected by the Mount Kinabalu National Park since the number of *Paphiopedilum* plants that are growing in the wild has been decreasing drastically and is facing extinction. The *Paphiopedilum* orchids in Sabah are facing enormous threats because the Kinabalu National Park borders are constantly being encroached by mining, lumbering, agricultural projects and also the practice of shifting agriculture by illegal squatters (Cribb, 1998). There are nine species of *Paphiopedilum* orchids that are listed under the Sabah Wildlife Conservation Enactment (WCE). The penalty for owning one of the species listed can be a fine of a maximum of RM 50 000, five years jail or both (Kaur and John, 2006).

Other than threats caused by development activities as mentioned, many orchid sellers and some handicrafts owners are not aware of the value of the endangered *Paphiopedilum* orchids. These orchids are planted or collected from the wild to be sold to tourists and orchid lovers at very low price rather than the actual

price which they are worth. On the other hand, most of the orchid lovers are aware of the penalty for owning the *Paphiopedilum* species orchid. However, the protected status of these orchids has only stir up more interest in them for possessing the plant. It has also been reported that these precious orchids are smuggled out of Sabah and to be sold in other countries (Kaur and John, 2006). The *Paphiopedilum* orchids are in very high demand especially in the temperate countries and they can fetch up to very high price (Cribb, 1998).

The continuous smuggling activities of *Paphiopedilum* have proved that the protection by the Kinabalu National Park and the penalty implemented are insufficient to protect the "Paph" orchids from being removed from its natural habitat. Efforts to prevent smuggling of the protected *Paphiopedilum* species are often not effective as the smugglers of these precious orchids can easily get away from the law as they can deny for possessing or selling the endangered *Paphiopedilum* plants. This is due to the reason as some of these endangered *Paphiopedilum* species has similar appearance like the ones which are non-protected species. In most cases, people are unable to distinguish between the endangered *Paphiopedilum* species and the non-protected ones.

Even at present, most of the orchid experts and experienced researchers are still differentiating *Paphiopedilum* orchids by the physical characteristics on the plants as there are no established or convincing methods to distinguish between similar looking species. Due to this reason, the use of DNA barcoding, in particular the intergenic region *trnH-psbA*, could be used to determine whether it is able to

discriminate between morphologically similar or closely allied *Paphiopedilum* species.

Using the concept of DNA barcoding, a uniform region of a genome in animals or plants is used to distinguish between different animals or plants species. Generally, the *cytochrome c oxidase subunit I* (*COI*) gene is used to tag animals whereas the *trnH-psbA* intergenic spacer is has been used in tagging angiosperms. DNA barcoding enable different animals and plants species to be clearly distinguished. Thus, at the same time it can also help in solving problems regarding closely related animals or plants species.

Applying the DNA barcoding concept in this research, the *trnH-psbA* intergenic spacer can be used to determine whether it would serve as a good marker to discriminate between few closely allied *Paphiopedilum* species. The *Paphiopedilum* orchid species that will be used are *Paphiopedilum rothschildianum, P. dayanum,* and *P. lowii* which are currently available at Sabah Park Orchid Conservatory. Based on the concept of DNA barcoding, the nucleotide sequence in the *trnH-psbA* intergenic region in all the three species of *Paphiopedilum* will be determined. Each species of *Paphiopedilum* species is expected to produce a set of unique nucleotides sequence of their *trnH-psbA* intergenic region.

The specific sets of nucleotide sequences of the *trnH-psbA* intergenic spacer will serve as barcodes and tag each of the species, thus help in solving ambiguities between closely related *Paphiopedilum* species. Moreover, should any problem arise in the future whether a person is possessing or selling endangered *Paphiopedilum*

orchids, the uncertainty can be solve by checking the barcodes of the *Paphiopedilum* plants. Other than tagging closely related *Paphiopedilum*, knowing the sequences of the *trnH-psbA* intergenic spacer will also show the relationship between the species. Generally, a species is said to be closely allied to another species if there is high percentage of nucleotide sequences similarity between the two species.

1.2 Research Objectives

The objectives of this research are:

1.2.1 To obtain the DNA nucleotide sequences of the *trnH-psbA* intergenic regions of *Paphiopedilum dayanum, P. lowii* and *P. rothschildianum*, respectively.

1.2.2 To perform multiple sequence alignment of the *trnH-psbA* intergenic spacer and to determine the number of single nucleotide polymorphism in *P. dayanum, P.* lowii and *P. rothschildianum*.

1.2.3 To examine the genetic relationship of the three *Paphiopedilum* species by constructing a phylogenetic tree based on the genetic distances obtained from pairwise nucleotide differences.

CHAPTER 2

LITERATURE REVIEW

2.1 *Paphiopedilum* Orchids

The *Paphiopedilum* orchids are sympodial plants with narrow and elongated leaves. The leaves are green color or mottled and the surface of the leaves always appear waxy. New buds and plantlets often arise from the rhizome of the plant. Other than that, the ladyslipper orchids are also pseudobulbless and stemless. Even if they have stems, the stems are too short and it stops growing after some time. Generally, the *Paphiopedilum* species are able to flower throughout the year. Flowers of *Paphiopedilum* can be single or multiple and the flowers are usually in wide variety of form and color and can last for a very long period.

The *Paphiopedilum* orchids often grow well in the mountain forest where the grounds were covered with fallen leaves and twigs, limestone cliffs and on the granite cliffs. These habitats provide essential requirement for the *Paphiopedilum* roots to grow. *Paphiopedilum* plants should not get completely dry or too wet either as they are unable to store water. Most of the *Paphiopedilum* are often found growing best in

the monsoon areas which have dry and wet season instead of the tropical rain forests which receive plenty of rain throughout the year.

Temperature plays a very important role in determining the blossom of *Paphiopedilum* flowers. Generally, *Paphiopedilum* species grow best between the temperature range of 15 °C to 30 °C. However, to be more specific, the origin of each *Paphiopedilum* species determines the optimum temperature for the particular species to grow well. Often, the ones that originated from the lowland need higher temperatures like the tropical temperatures and as for the highland species, they need lower temperatures.

This orchid is a protected genus under Malaysia legislation and also listed on Appendix I of the Convention on International Trade in Endangered Species (CITES). Therefore, the trade of *Paphiopedilum* is strictly banned and ownership of these orchids is regulated with permits (Kaur and John, 2006). The endangered and protected *Paphiopedilum* species such as *Paphiopedilum rothschildianum* are found growing well on Mount Kinabalu, Sabah. Other than *P. rothschildianum*, other *Paphiopedilum* species orchids that were found grow on Mount Kinabalu since 1980s include *P. dayanum*, *P. lowii*, *P. volonteanum*, *P. petri*, *P. stonei*, *P. virens*, *P. hookerae*, *P. philippinense* and many other species.

2.1.1 *Paphiopedilum rothschildianum*

The *P. rothschildianum* orchid which originated from Borneo was named after Baron Ferdinand de Rothschild (Fowlie and Lamb, 1983). This orchid was introduced by Frederick Sander in 1888 and was crowned the 'King of Orchids' (Bechtel, 1983). *P. rothschildianum* population is one of the rarest in the *Paphiopedilum* genus as it is a species that only can be found in Mount Kinabalu, Sabah and they are protected by the Mount Kinabalu National Park. Unfortunately, this orchid species is facing great threats as their habitat is seriously destructed by development activities such as logging, mining, agriculture projects and illegal squatters that carry out shifting agriculture as part of their living (Cribb, 1998). Recently, it is also reported that this precious species was sold to tourists with extremely low price by the local handicraft owners (Kaur and John, 2006).

P. rothschildianum grow well at the slopes of Mount Kinabalu at about 1500 feet to 3000 feet above sea level and perform best in cool and shady conditions. In nature, it is seen in the steep ravines in the streams or on the ultrabasic rocks (Fowlie and Lamb, 1983). Seedlings of *P. rothschildianum* often sprout on lythophytic mosses and when mature, they are rooted lithophytically onto the cliff trees.

The leaves of mature *P. rothschildianum* can grow up to two feet long by 2½ inches in breadth. These hard and long-petal leaves are droopier looking compared to the leaves of *P. lowii*. The leaves catch leaf litters and debris which adds to the compost for the adult plants. Normally, the fallen leaves of other trees, for instance,

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