GENETIC ANALYSIS OF *SPATHOGLOTTIS* SPECIES IN PENINSULAR MALAYSIA

FLORENCE C GINIBUN @ CAMILLUS

FACULTY OF SCIENCE
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ABSTRACT

A study was conducted to characterize the five native Malaysian *Spathoglottis* species of *Spathoglottis aurea*, *Spathoglottis affinis*, *Spathoglottis gracilis*, *Spathoglottis microchilina* and *Spathoglottis plicata* by morphological characteristics and molecular techniques. Three molecular markers were used namely; Inter Retrotransposon Amplified Polymorphism (IRAP), DNA barcoding and Amplified Fragment Length Polymorphism (AFLP).

Two distinct morphological traits of flower colour and lip shape were selected from 47 qualitative characteristic to distinguish the species. The morphological characterisation was supported by molecular technologies using nuclear and chloroplast DNA markers. The nuclear marker consists of IRAP and AFLP technique. Both techniques amplified high polymorphism banding pattern where they form 341 polymorphic bands and later produce 891 polymorphic bands. The markers were also supported by the chloroplast DNA marker with the same clustering pattern based on the species. First cluster comprised of *S. plicata* while the second cluster comprised of S. *aurea*, *S. gracilis*, *S. affinis* and *S. microchilina*. The dendrogram generated by three different molecular markers showed consensus in the clustering pattern. The clustering pattern for the five native *Spathoglottis* species was in accordance with their flower colour where *S. plicata* is purple in colour and the other four species of *Spathoglottis* are yellow in colour.

Spathoglottis plicata was a chosen species for a population study to evaluate the genetic structure based on geographical location for conservation and management purposes. A total of 11 AFLP markers of *EcoRI*+3 bases/*MseI*+3 base primer combinations give rise to 279 polymorphis bands. Two distinct clades generated from UPGMA dendrogram further investigate in Bayesian analysis using STRUCTURE

software and estimated the population structure at optimal value *K*=4. Result show a present of four genetic structures in *S. plicata* population. Pahang and Terengganu population revealed a higher genetic variation (60.25%) indicating a possible hybridization between the Northern (Kedah), Southern (Negeri Sembilan and Johor) and Central (Selangor) region population. It is clear that geographical distance is the first factor to contribute distant population structures.

The finding from the morphological and molecular data, show the conservation measures to cryopreserved *S. plicata* germplasm are important. However, due to limitation of time, optimization and lack of domestication measures implemented to grow the *S. plicata* ex situ resulted in low survival rate of the protocorm even though the encapsulation-vitrification technique has been established. Therefore, this study will need to be revisited in future once domestication process for this species has been implemented.

This study resulted in characterisation of *spathoglottis* species using morphological and molecular marker for ease identification introducing the concept of varietal protectionand conservation strategy and legislation for commercial of this valued species.

ABSTRAK

Satu kajian telah dijalankan untuk memperkelaskan lima spesies *Spathoglottis* Malaysia iaitu *Spathoglottis aurea*, *Spathoglottis affinis*, *Spathoglottis gracilis*, *Spathoglottis microchilina* dan *Spathoglottis plicata* menggunakan ciri-ciri morfologi dan teknik molekul. Tiga penanda molekul yang digunakan dalam kajian ini adalah Inter Retrotransposon Amplified Polymorphism (IRAP), DNA barcoding and Amplified Fragment Length Polymorphism (AFLP).

Dua ciri morfologi iaitu warna bunga dan bentuk bibir telah dipilih daripada 47 ciri-ciri kualitatif bagi membezakan spesies ini. Pencirian morfologi ini disokong dengan teknologi molekul menggunakan penanda DNA nuklear dan kloroplas. Penanda nuklear terdiri daripada teknik IRAP dan AFLP. Kedua-dua teknik ini menghasilkan corak pita polimorfik yang tinggi dimana teknik IRAP menghasilkan sebanyak 341 pita polimorfik dan AFLP menghasilkan sebanyak 891 pita polimorfik. Petanda ini juga disokong oleh penanda DNA kloroplas yang menunjukkan corak kelompok yang sama berdasarkan kepada spesies. Kelompok pertama terdiri daripada *S. picata* manakala kelompok kedua terdiri daripada *S. aurea*, *S. gracilis*, *S. affinis* dan *S. microchilina*. "Dendrogram" yang dihasilkan oleh ketiga-tiga penanda molekul menunjukkan persamaan dalam corak kelompok itu. Corak kelompok lima spesies *Spathoglottis* adalah mengikut warna bunga dimana *S. plicata* berwarna ungu dan empat spesies *Spathoglottis* lain berwarna kuning.

Spathoglottis plicata adalah spesis yang dipilih untuk kajian populasi melalui penilaian struktur genetik berdasarkan lokasi geografi bagi tujuan pemuliharaan dan pengurusan. Sebanyak 11 penanda AFLP gabungan primer *EcoRI*+3 bases/*MseI*+3 bases menghasilkan sebanyak 279 pita polimorfik. "Dendrogram" UPGMA telah menghasilkan dua kelompok yang jelas berbeza, seterusnya kelompok ini dikaji menggunakan analisis Bayesian melalui perisian STRUKTUR dimana struktur populasi

dianggarkan pada nilai optimum *K*=4. Keputusan kajian menunjukkan terdapat empat struktur genetik dalam populasi *S. plicata*. Populasi di Pahang dan Terengganu menghasilkan variasi genetik yang tinggi (60.25%), menunjukkan kemungkinan penghibridan berlaku di antara kawasan populasi Utara (Kedah), Selatan (Negeri Sembilan dan Johor) dan Tengah (Selangor). Ini dengan jelas menunjukkan bahawa jarak geografi merupakan faktor utama dalam menyumbang kepada perbezaan struktur populasi ini.

Hasil kajian daripada data morfologi dan molekul, menunjukkan langkah-langkah pemuliharaan janaplasma *S. plicata* secara krioawaten adalah penting. Walau bagaimanapun, disebabkan oleh batasan masa, kekurangan langkah-langkah pengoptimuman dan penyesuaian penanaman untuk pertumbuhaan *S. plicata* secara *ex situ* menyebabkan kadar kemandirian protocorm yang rendah walaupun teknik pengekacaan pengkapsulan telah diwujudkan. Oleh itu, kajian ini perlu dikaji semula pada masa akan datang selepas proses penyesuaian adaptasi penanaman untuk spesies ini dilaksanakan.

Kajian ke atas pencirian spesies *Spathoglottis* menggunakan penanda morfologi dan molekul adalah untuk mempermudahkan strategi pemuliharaan dan memperkenalkan konsep perlindungan varieti serta perundangan bagi perdagangan spesies ini.

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AFLP	:	amplified fragment length polymorphism
AMOVA	:	analysis of molecular variance
ANOVA	:	analysis of variance
BARE-1	:	barley retro element
bp	:	base pair
cpDNA	:	chloroplast DNA
cm	:	Centimeter
$CaCl_2.2H_2O$	:	Calcium chloride
$Ca(NO_3)_2$	:	calcium nitrate
$CoCl_2.6H_2O$	:	cobaltus chloride
$CuSO_4.5H_2O$	:	cupric sulphate
df	:	degree of freedom
dH_2O	:	distilled water
DMSO	:	dimethyl sulphoxide
DMRT	:	duncan's multiple range test
dNTPs	:	deoxyribonucleoside triphosphates
DNA	:	deoxyribonucleic acid
EcoRI	:	restriction enzyme isolated from Escherichia coli strain R
EDTA	:	ethyldiaminotetraacetic acid
$FeSO_4.7H_2O$	:	ferrous sulphate
g	:	Gram
g/l	:	gram/liter
H_3BO_3	:	boric acid
HCI	:	hydrochloric acid

IRAP	:	Inter-retrotransposon amplified polymorphism
kbp	:	kilo base pair
KCI	:	potassium chloride
KH_2PO_4	:	potassium dihydrogen orthophosphate
KI	:	potassium iodide
KNO_3	:	potassium nitrate
L	:	Liter
LN	:	liquid nitrogen
LTR	:	long terminal repeats
m	:	Meter
M	:	Molar
MC	:	moisture content
MCMC	:	Markov Chain Monte Carlo
mg	:	Milligram
mg/ml	:	milligram/millilitre
ml	:	Milliliter
mM	:	Millimolar
$MgSO_4.7H_2O$	:	magnesium sulphate
$MgCl_2$	:	magnesium chloride
$MnSO_4.4H_2O$	:	manganese sulphate
MS	:	Murashige and Skoog media
MseI	:	restriction enzyme isolated from Escherichia coli strain R
NaCl	:	sodium chloride
$Na_2EDTA._2H2O$	:	sodium EDTA
$Na_2MoO_4.2H_2O$	:	sodium molybdate
NaOH	:	sodium hydroxide

ng	:	Nanogramme
nm	:	Nanometer
NH_4NO_3	:	ammonium nitrate
PCR	:	polymerase chain reaction
PCoA	:	principal coordinate analysis
PVS2	:	plant vitrification solution 2
p.s.i.	:	pound per square inch
RCBD	:	randomized complete block design
REMAP	:	retrotransposon microsatellite amplified polymorphism
RTNs	:	Retrotransposon
RT	:	reverse transcriptase
SAS	:	statistical analysis system
s	:	Second(s)
T_a	:	annealing temperature
TBE	:	tris borate EDTA
TE	:	tris-EDTA
TEMED	:	Tetramethylethylenediamine
U	:	Unit
UPGMA	:	unweighted pair group method with arithmetic mean
UV	:	ultra violet
wt	:	Weight
w/v	:	weight by volume
$ZnSO_4$	:	zinc sulphate

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CHAPTER 1: GENERAL INTRODUCTION

Orchidaceae is the largest family of flowering plants widely distributed around the world with 19,500 species in 804 genera (Dressler, 1993). About 854 species covering 141 genera are indigenous to Peninsular Malaysia (Seidenfaden & Wood, 1992). Most of the orchid species are listed as endangered species (Ried & Miller, 1989) including *Spathoglottis* species. Orchids as cut flowers have a high economic potential due to the domestic growth and export market. *Dendrobium*, *Aranda*, *Oncidium* and *Mokara* are the most popular cultivated orchid type. The total production of orchid cut flowers in 2000 was 24,262,974 cutting/ pot/ plant where 19,200 were of *Spathoglottis* species. But there is no report on the production of *Spathoglottis* orchid after 2000. However, the production of orchid either as cuttings or as potted increased from 154,595,882 in 2009 to 182,274,565 in 2013 and in 2014 the production was estimated at about 191,390,691 (MOA, 2014).

The *Spathoglottis* genus is one of the most popular terrestrial orchids consisting of about 40 species widely distributed in regions from Northern India, Southern Japan and China down through the Malay Archipelago, the islands of the Pacific; New Guinea, Samoa, New Caledonia and Northern Australia (Holttum, 1953; Seidenfaden & Wood, 1992). Papua New Guinea has the largest recorded numbers with 21 *Spathoglottis* species. Malaysia was reported to have six native *Spathoglottis* species; *S. plicata, S. aurea, S. microchilina, S. affinis, S. gracilis and S. hardingiana* that grow in the wild throughout the country (Seidenfaden & Wood, 1992; Beltrame, 2006). However, only five species are available with one or two species in each location. For example, *S. affinis* is found in Gunung Jerai, Kedah; *S. aurea* in Cameron Highlands, Pahang; *S. microchilina* in Genting Highlands, Pahang; *S. gracilis* in Endau Rompin, Johor whereas S. *plicata* is available in all locations mentioned and also in other areas.

Spathoglotis hardingiana is a very rare species and does not exist now as reported by a taxonomist, however, it may be found in Langkawi Island.

Spathoglottis is well known as a ground orchid. It is hardy and beautiful as potted and garden orchid or as landscaping plants. *Spathoglottis* species can be differentiated by their morphological characteristics based on their flower colour and the shape of the lip. It produces small to large attractive flowers with varying colours ranging from white to mauve and in shades ranging from yellow to gold.

Spathoglottis orchids are constantly exposed to dangers such as erosion, landslides, construction and other environmental factors. This orchid was listed in the CITES under Appendix II (http://www.cites.org/eng/app/appendices.php.) as endangered species. Taxonomists and breeders have collected the species for herbarium and hybridization since the late 18th and early 19th century; yet the species continue to be endangered as they are still being collected directly from their natural habitat and not propagated *in vitro*. Therefore, conservation strategies for this wild species remain a priority.

Many of the *Spathoglottis* species have been widely cultivated for some time and have been successfully used for hybridization (Holttum, 1964; Teoh, 1980; Beltrame, 2006) and are worthy to be preserved as germplasm for breeding. Beltrame (2006) reported that there are 84 registered Grex names for *Spathoglottis* and initiated a hybridization programme in 1997. To date, the registration has increased to 107 at the Royal Horticultural Society, the international orchid register.

In general, *Spathoglottis* has the potential to be very valuable and in demand commercially as potted or garden plant. Therefore, knowledge and information on the species especially the Malaysian species are really important. The plant genetic resources of *Spathoglottis* should be conserved for future use. Holttum (1953); Seidenfaden and Wood (1992) have described and have written the key for the Malaysian *Spathoglottis* with illustrations. It is now increasingly important to estimate

the variation that still exists within this genus. This can be done with respect to their morphological characteristics based on Bioversity International's (IPGRI) descriptor and UPOV development of test guidelines. Morphological characteristics are also an important method used to distinguish a species or variety in granting plant breeders' right.

Morphological markers are still important as a technique applied to measure a genetic diversity in the plants. However, plant morphology, physical characteristics and plant growth characteristics could be influenced by environmental conditions. Morphological characteristics alone are not sufficient and sometimes difficult to distinguish the variability between and within species. Due to this limitation, it has become a problem for taxonomists and breeders to differentiate between two or more species or varieties. However, the problem could be solved and supported through molecular technologies.

Molecular studies on *Spathoglottis* can be applied for DNA fingerprinting, species identification, genetic relationships and genetic diversity. An Inter-retrotransposon amplified polymorphism (IRAP) is a method that has been developed and used widely to study genetic diversity and relationships of plants (Kalender et al., 2011; Kalender et al., 2010; Kalender et al., 1999). A number of PCR based techniques can be applied for this purpose. A second approach uses chloroplast DNA (cpDNA) markers as DNA barcode; it is a method that can be rapidly and accurately used to identify and describe a species just using DNA sequences from a small fragment (Lahaye et al., 2008). This can also be used as a forensic tool for identification of plants when the morphology of the sample is absent. A third method is the Amplified Fragment Length Polymorphism (AFLP); developed in 1995 which combines the use of restriction enzymes and oligonucleotides for PCR without needing to know the sequence (Vos et al., 1995). It has been used widely in the assessment of genetic diversity and genetic relationships in

a wide range of orchid species (Kim et al., 2013). It is also a choice tool to look at variation within and between populations (Qian et al., 2013).

Wild species of *Spathoglottis* have been utilized in hybridization but their valuable germplasm could become rare and extinct because of natural habitat loss. Therefore, there is an urgent need to conserve the still existing *Spathoglottis* germplasm in both *in situ* and *ex situ* conservation. *In vitro* culture technique is an option available for *ex situ* germplasm conservation and cryopreservation is a viable method for long term storage by conserving the germplasm at low temperature of liquid nitrogen (-196^{o}C).

This study focussed on the *Spathoglottis* species and *S. plicata* population located in different geographical region in Peninsular Malaysia. Their morphological characterisation and genetic variation will be assessing using IRAP, DNA Barcoding and AFLP molecular technique. This study will assess whether or not the morphological and genetic characterisation could differentiate the species and assess the genetic structure in *S.plicata* populations. This study also aims to evaluate the possibility of vitrification technique for cryopreservation of the *S.plicata* for germplasm conservation in future.

1.1 Research questions

1.1.1 Can the morphological characteristic be used to differentiate the *Spathoglottis* species?

1.1.2 Can the morphological characteristics of the *Spathoglottis* species be differentiated to different taxonomically and biological species based on the nuclear and chloroplast DNA markers.

1.1.3 *Spathoglottis plicata* is the most common, abundant and commercial important species. Is there any posible genetic structure based on geographical located to allow conservation management to be implemented.

1.1.4 Can establish vitrification technique be measures for cryopreservation of the *S. plicata* species.

1.2 Objectives

1.2.1 To observe and characterize the morphology of five Malaysian *Spathoglottis* species.

1.2.2 To study and identify *Spathoglottis* species using a suitable DNA marker technique (IRAP, DNA Barcoding and AFLP).

1.2.3 To evaluate the genetic diversity within and among *Spathoglottis plicata* populations in different locations of Peninsular Malaysia using AFLP marker.

1.2.4 To evaluate the potential of vitrification technique for cryopreservation of *Spathoglottis plicata*.

1.3 Hypothesis

1.3.1 Morphology and molecular technique could be a potential tool in characterising the *Spathoglottis* species and the *Spathoglottis plicata* population.

1.3.2 Vitrification by encapsulating the sample could be a potential technique to be applied in cryopreservation.

CHAPTER 2: LITERATURE REVIEW

2.1 The botany and taxonomic classification of *Spathoglottis*

Spathoglottis belongs to the family Orchidaceae and is reported to be in the terrestrial orchid group. In 1825, Carl Blume proposed it as a genus and gave the generic name of *Spathoglottis*. It is derived from two Greek words, 'spathe' a spoon shape or a broad blade and 'glossa/glotta' meaning a tongue. Thus, it refers to the characteristic of the broad spatula or spoon-like lip of the flower and the broad midlobe of the lip or labellum of the flower (Teo, 1985; Aziz et al., 2001).

Spathoglottis plants are found growing on the ground in well-drained soil. It is small to large, erect and perennial terrestrial herb. The *Spathoglottis* in the wild is associated with disturbed sites such as landslips, exposed open field, streams or edges of rocks. Generally, the *Spathoglottis* flower colours range from purple to yellow and to white. The morphological characteristic of *Spathoglottis* comprised of ovoid pseudo bulbs each bearing a few plicate leaves. The inflorescence grows from the basal leaf axils. It has long peduncles and the rachis bears a succession of several flowers placed close together. Normally, the flowers open wide and the sepals and petals are similar. The lip is trilobed which indicates that the sidelobes are narrow, oblong and curved upwards. Its midlobe has two small calluses at the base of the claw and two small laterally spreading teeth. The column is long and thin, slender, curved and without foot but there are eight pollinia (Holttum, 1953; Seidenfaden & wood, 1992; Comber, 2001).

The genus *Spathoglottis* consists of about 40 species and is widely distributed from Northern India across Southeast Asia, Southern China, Japan, Malay Archipelago, Philippines, New Guinea, the islands of Pacific and Australia (Holttum, 1953; Teo, 1985; Seidenfaden & Wood, 1992 and Beltrame, 2006). Papua New Guinea has been reported to have the largest numbers of *Spathoglottis* species with 21 species by Beltrame (2006); Seidenfaden and Wood (1992). Peninsular Malaysia, Borneo and

Philippines have six species; while Thailand, Java, Sumatera and Australia have five, three and two species respectively. Each *Spathoglottis* species is native to its country of origin e.g., India, Myanmar, Thailand, Indonesia, Malaysia, Papua New Guinea, Philippines, Australia, Southwestern Pacific, New Caledonia, Samoa, Solomon Island and Tonga. Howcroft (1992) has listed and described 86 *Spathoglottis* species and varieties which distributed all over South-east Asia to Australia and the Pacific Islands since 1850 to 1979 and has also revised the taxonomy of *Spathoglottis* genus in Papuasia. *Spathoglottis* is known by various common names such as garden orchid, boat orchid, ground orchid and the Philippines ground orchid. In Malaysia, it is known as palm orchid or "orkid pinang" in the Malay language as the leaves of this orchid is very similar to a palm leaf. The taxonomic classification of *Spathoglottis* is as follows:

Kingdom : Plantae

Division : Magnoliophyta

Class : Liliopsida

Order : Asparagales

Family : Orchidaceae

Subfamily :Epidendroideae

Tribe : Arethuseae

Subtribe : Bletiinae

Alliance : Calanthe

Genus : *Spathoglottis*

2.2 The *Spathoglottis* species in Malaysia

2.2.1 Native species

Malaysia particularly Peninsular Malaysia is reported to have six species of *Spathoglottis*. The species are *S. plicata* Blume, *S. aurea* Lindl., *S. affinis* de Vriese, *S. gracilis* Rolfe ex Hook. f., *S. microchilina* Kraenzl. and *S. hardingiana* Par. & Reichb. f.. It is native and widely distributed throughout the country as verified by the herbarium collection list from the botanical gardens in University Malaya, Forestry Research Institute Malaysia, Botanical Garden Singapore, Botanical Garden Edinburgh, Botanical Garden Kew, UK and Leiden University Herbarium since the late 1800's, 1900's to date. Some of *Spathoglottis* herbarium collections are listed in Appendix A.

Species is a group of living organisms consisting of similar individuals capable of exchanging genes or interbreeding. It does also mean a group of plants that are similar and can produce young plants: a group of related plants that is smaller than a genus. To further understand the *Spathoglottis* species found in Peninsular Malaysia, each of the species locality and habitat are described briefly.

Spathoglottis plicata Blume is the most common species and is distributed throughout Malaysia. It is a hardy and extremely variable species found growing wild in open fields, along streams or edges of rocks either in lowlands or elevated areas (Teo, 1985). Seidenfaden and Wood (1992) have reported *Spathoglottis affinis* de Vriese as a mountain plant species and native to the montane areas of North Peninsular Malaysia. It is reported to be only found in Gunung Jerai, Kedah at 600-900 m in open rocky places (p.161). *Spathoglottis aurea* Lindl. is a species that was originally discovered on Gunung Ledang and found between 900 and 1500 m in many parts of Malaysia (p.163). *Spathoglottis gracilis* Rolfe ex Hook.f. is found growing wild in Pulau Tioman, Pahang at 900 m (p.163). *Spathoglottis microchilina* Kraenzl is reported to be found in Gunung Jerai, Kedah, Taiping Hills, Brinchang, Cameron Highlands and Genting Highlands,

characterizing all species diversity. Both Mayden (1997) and de Queiroz (1998) synthesizing the similarities among different species concepts and recognise most of the species concepts are not primary definations but are secondary definitions or species criteria by describing the necessary traits of a species.

Generally, species are separately evolved population level lineages that may require unique traits during evolution (de Queiroz, 2007). The species concepts criteria are used to quantitatively access the degree of lineages that have diverged and used it as an evidence to describe species. Due to the emerging consensus on the definitive properties of species, researches are shifted to more focus on a way to delimit species in nature (de Queiroz, 2007; Weins & Penkrot, 2002; Sanders et al., 2006).

There are numerous methods used in delineating species and grouped into 'non tree-based' and 'tree based'. As reported by Sites and Marshall (2004), tree-based methods are vary and been used for both morphological and molecular data to estimate phylogenetic relationships of individuals that may share their common evolutionary history. However, the non-tree based methods are traditionally relying on the concept of reproductive isolation between species. Occasionally, isolation could be tested directly but most methods infer reproductive isolation by indirectly estimating the gene flow within and between species (Knowles & Carstens, 2007).

Generally, species interact and associate with each other in their ecological environments. The interest of the ecologists is to understand the diversity, distribution, population, cooperation and competition between of the species, which may closely relate to evolutionary biology and genetics. Understanding the biodiversity that could affect the ecological function is important, as well as their cooperation and competition between the species; also within and among the ecosystems. Nowaday, both ecologists and evolutionary biologists using molecular marker as markers are readily available,

inexpensive and amplify closely related species (Sunnucks, 2000; Selkoe & Toonen, 2006).

According to Holttum (1953), the *Spathoglottis* species are ecologically could found in lowland and also in mountain which grow in poor grassland areas, roadsides and mountain foothills; hill forest, lower montane forest, roadside banks and ditches, margin of secondary vegetation, among mossy rock beside rivers. It is reported that the *S. plicata* is a lowland plant but their habitat could overlap with the mountain species as an example, the *S. aurea* at about 760m which a natural hybrid found by Comber (2001) in North Sumatra, Indonesia. It could be found in Peninsular Malaysia in a certain ecological location, example, the *S. plicata* and *S. microchilina* in Genting Highlands, Pahang; and *S. plicata* and *S. affinis* in Gunung Jerai, Kedah. Lacking of studies except by local knowledge information and taxonomist review, it is interesting to understand the species concepts and species delineation of *Spathoglottis*.

2.2.3 The *Spathoglottis* hybrid

Spathoglottis species is reported to be freely inter-fertile. Wood (1997) has reported of the cleistogamy where the *Spathoglottis* flowers have opened widely initially on a certain day but will close after pollination and remain closed. It is fresh for a long time. Cleistogamy is a type of automatic self-pollination by using non-opening and self-pollinating flowers. Pimonrat et al. (2012) reported that *Spathoglottis* is a crossed pollinated orchid but to induce new variation through cross breeding is difficult. Most hybrids either self or cross pollinated with parent or other species is nearly sterile (Holttum, 1953; Howcroft, 1992). *Spathoglottis* also has a long history of natural self-breeding. It has been cultivated long ago and several varieties have been selected, including the white flowered variety. Beltrame (2006) reported that there are 84 registered grex names for *Spathoglottis*. The first recorded *Spathoglottis* hybrid was *S.*

aurea-vieillardii made by Veitch in 1897, a cross between *S. aurea* and *S. viellardii* (syn. *S. plicata 'viellardii'*). This first *Spathoglottis* variety was registered in the Royal Horticultural Society (The International Orchid Register). *Spathoglottis* 'Rimba Ilmu' is a hybrid created by crossing between *S. lobbii* and *S. unguiculata* which was pollinated in October 2006; the first bloom was achieved in January 2008. The hybrid was registered by Lee Nam Fook with the Royal Horticultural Society on 28 January 2008. It was named after the botanical garden of the University of Malaya, where Malaysia's first conservatory highlighting rare indigenous plants and orchids was established in 2000. The *Spathoglottis* breeding continued until to date by orchid breeders in countries of origin. Till recently, the Royal Horticulture Society has received 110 registrations of *Spathoglottis* hybrids.

2.3 Economic importance of *Spathoglottis*

Spathoglottis is one of the most popular terrestrial orchids which were also known as ground orchid. It was also reported to be the most important horticultural cultivated species and has a potential as landscaping plants as well as potted plants and as cut flowers. There are species and hybrids of *Spathoglottis* with beautiful colours that are suitable to be marketed commercially as it is attractive, fast growing, easy to cultivate and maintain; and tend to bloom all year round. Since this genus is very well known for its wide range of beautiful flowers, classified by their flower colour, lip shape and lip colour, more breeding development is required. In Malaysia, breeding *Spathoglottis* has been done by Malaysian Agricultural Research and Development Institute (MARDI), private orchid companies and individuals. Most orchid seeds germinate on media *in vitro*. Some of the hybrid seeds are difficult to germinate and the seedlings growth not uniform. Due to this, *Spathoglottis* plant is sold at various prices based on the species, hybrid and variety in the orchid nurseries.

2.4 Taxonomic problem of *Spathoglottis*

Identification and relationships among the *Spathoglottis* species are challenging as the plants grow in the wild and are endangered due to natural disasters and orchid collectors. Therefore, there is a need to conserve the germplasm in natural habitat, *ex situ* or *in situ*. The horticultural collection and developmental activities are the cause of many orchid species being extinct in their natural habitat. Even the other inconspicuous orchids are also endangered because of their horticultural value as rare plants. Therefore, most orchid species are listed as endangered species (Reid & Miller, 1989) and protected under the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) including *Spathoglottis*.

Spathoglottis plicata is the first species established by Blume in 1825 under the *Spathoglottis* genus. Taxonomists then recognized approximately 40 described species under the genus *Spathoglottis* based on morphological classification from all across the Asia Pacific region (Howcroft, 1992; Beltrame, 2006). Schlechter (1912) observed that the main problems were the close similarity of some of the species such as those in *S. plicata* group (Holttum, 1964; Cribb & Tang, 1982) due to the lack of understanding of the most important characteristics and inadequate descriptions. *Spathoglottis* species has been mainly described and classified by their common phenotypic traits, including vegetative and floral characteristic; size and shape labellum; angle and shape lateral lobe and mid lobe. Nevertheless, the taxonomic differentiation of *Spathoglottis* in Malaysia has their limitation due to lack of studies information.

2.5 Morphological characterisation

Morphological is a first marker that used for determinants of a traits and indirect selection of a genetic determination. The traits available have obvious impact on the morphology of plant as an example the plant height, leaf and flower color etc. Plant

morphology is very useful in the identification of plants. Detailed morphological data for each species can be collected and documented together with notation of special characteristics for future reference. However, due to the lack of taxonomic expertise, identification of some of the orchid species is still not confirmed.

Morphological and phenotypic characteristics could be evaluated by the protocol and procedure that have been set up such as descriptors that have been developed by Bioversity International formerly known as International Plant Genetic Resources Institute (IPGRI) and International Union for the Protection of New Varieties of Plants (UPOV). However, there is yet a descriptor list to be developed for *Spathoglottis* and very little taxonomic work on Malaysian *Spathoglottis* has been documented.

In the previous study, Kartikaningrum et al. (2004) have characterized 21 phenotypic characters on Indonesian *Spathoglottis* collection. Kartikaningrum and Effendie (2005) have also conducted genetic variability and heritability study of germplasm collection of *Spathoglottis* orchid in Indonesian Ornamental Crops Research Institute (IOCRI). It has indicated a wide genetic variability related to length and width of leaf, number of shoot increment, length and width of flower, length and width of lip. There is high heritability value on the characters such as number of shoot increment, length and width of leaf, length and diameter of flower stalk, length and width of lip, ratio of lip length-width, length and width of flower. A study done recently by Ginibun et al. (2013) shows the morphological characteristics focusing on qualitative data of *Spathoglottis* species with the intention to develop a table of characteristics as guideline for *Spathoglottis* in the UPOV system or descriptor list based on format used by Bioversity International. A total of 47 qualitative characters were recorded and only 21 characters are recommended as useful characteristics, such as, colour of flower and shape of the lip to distinguish the *Spathoglottis* species and varieties in the future.

2.5.1 Colour charts for flower

Flower colour is one of the most important characteristic or descriptive traits that can be determined. The Royal Horticultural Society (RHS, 2007) colour charts were used to describe and determine the flower colours. It is a standard reference for plant colour identification as observation of colour by naked eye may compromise accuracy. The RHS colour charts were published in 1966 for the first time and since then revised four times (editions 1986, 1995, 2001 and 2007). The new RHS version 2007 has 12 new colours. This present version is printed with monochrome special colours for more precise and durable colour match.

UPOV document TGP/14/3 (UPOV, 2015) also provides guidance on names of the colours in RHS Colour Chart to harmonise the colour names for variety description. The RHS Colour Chart contains 96 colours which are divided into 23 "groups" to name the colours. UPOV has identified 50 colour groups consisting of the pure colour/colour hue, a combination of two pure colour/colour hues or a combination of the pure colour/colour hue with "light" or "dark".

2.6 Molecular markers

In recent years, molecular marker technologies have been introduced and has revolutionised the method of orchid genetic studies. Molecular markers have been used in plant germplasm characterization, finger printing, genetic analysis, linkage mapping and molecular breeding. It has also become a useful tool in developing and improving the orchid varieties.

There are a variety of molecular markers used as genetic marker in genetics studies where a fragment of DNA is associated with a certain location within the genome. A molecular marker used in molecular biology and biotechnology is to identify a particular sequence of DNA in a pool of unknown DNA. There are many types of

genetic markers which each have their limitations and strengths such as Inter-Retrotransposon Amplified Polymorphism (IRAP), Retrotransposon-Microsatellite Amplified Polymorphism (REMAP), Inter-Simple Sequence Repeat (ISSR), Sequence-Tagged Microsatellite (SSR), Chloroplast DNA, Random-Amplified Fragment Length Polymorphism (RAPD) and Amplified Fragment Length Polymorphism (AFLP). All markers have made molecular analysis and genotyping useful techniques in studying genetic diversity, genetic structure, species identification, phylogenetic and population genetic. However, choices of markers are dependent on the objectives of the research and the nature of the markers.

2.6.1 Inter Retrotransposon Amplified Polymorphism (IRAP)

Kalender and Schulman (2006) reported retrotransposons could be used as markers as their integration creates new joints between genomic DNA and their conserved ends. Marker systems generally rely on PCR amplification between these ends and some component of flanking genomic DNA to detect polymorphisms for retrotransposon insertion of elements hundreds to thousands of nucleotides long.

Inter-Retrotransposons Amplified Polymorphisms (IRAPs) were developed as an alternative valuable retrotransposon-based markers. It requires neither restriction enzyme digestion nor ligation to generate the marker bands. IRAP products are generated from two nearby retrotransposons using outward-facing primers. The IRAP protocol includes method for PCR amplification with a single or two primers. In this method, PCR oligonucleotide primers facing outwards from the LTR or other regions of retrotransposons are made and amplify between two retroelements inserted into the genome. The insertion of elements into the genome mean that the number of sites amplified and sizes of inter-retroelement fragments differ between different lines and

can be used as markers to detect genotypes, measure diversity or reconstruct phylogeny (Flavell et al., 1999; Kalender et al., 1999; Kumar & Hirochika, 2001).

Guo et al. (2006) reported that IRAP was an amplification of sequences between two retrotransposon with two retrotranposon primers which have been successfully applied in fingerprinting (Breto et al., 2001), analysis of genomic evolution (Kalender et al., 1999, Kalender et al., 2000) and genetic diversity (Yannic et al., 2004). Many studies have used IRAPs to investigate the genetic relationships and genetic diversity between the cultivar, varieties, related species and genus of plants such as for *Crocus* (Alavi-Kia et al., 2008); *Diospyros kaki* (Guo et al., 2006); Old Portuguese bread wheat (Carvalho et al., 2010); *Aegilops tauschii* (Saeidi et al., 2008); *Helianthus* (Vukich et al., 2009); *Pisum* (Smýkal, 2006); *Oryza* (Branco et al., 2007); *Citrus clementina* (Breto et al., 2001).

IRAP was also used to study the genome constitution and classification of the genus *Musa* cultivars (Teo et al., 2005); genome evolution of *Hordeum spontaneum* (Kalender et al., 1999; Kalender et al., 2000); and the genomic stability in population of the young allopolyploid species *Spartina anglica* (Baumel et al., 2002). Teo et al. (2005) used seven long terminal repeat (LTR) primers derived from the barley genome (*Hordeum vulgare*) and two primers (Reverse Ty1 and Reverse Ty2) facing outward from the highly conserved reverse transcriptase of the banana Ty1-copia-like retrotransposon designed by Teo et al. (2001) for characterizing genome constitutions and classifying cultivars of the genus *Musa*. The IRAP markers distinguished the A and B genomes of the banana species (*Musa acuminata* Colla and *Musa balbisiana* Colla) and between banana cultivars; and enabled phylogenetic analysis of the 16 Malaysian banana cultivars. Oliveira-Silva et al. (2014) selected eight long terminal repeat retrotransposons for the development of IRAP markers on Manioc, *Manihot esculent*. It

is recommended that molecular markers based on retrotransposons have good potential for analysis of genetic diversity given their abundance in the genome.

IRAP was also used in the orchids studies of *Phalaenopsis* which two Bacterial Artificial Chromosome (BAC) libraries were constructed using *Bam*HI and *Hind*III restriction enzymes to generate pair-end sequences and most of the repetitive DNA sequences were gypsy- and copia-like retrotransposons (Hsu et al., 2011). This work not only facilitates analysis of the *Phalaenopsis* genome but also helps to clarify similarities and differences in genome composition between orchids and other plant species.

2.6.2 DNA Barcoding

The whole organisms are normally needed by the taxonomists to identify a species. However, by using DNA barcoding method, only a minute sample is required to identify a species accurately just using DNA (Lahaye et al., 2008). Chase et al. (2005) and Newmaster et al. (2006) also used DNA barcoding as a method in the identification of new species.

According to Hebert et al. (2003), DNA barcoding is a taxonomic method that uses short genetic markers in an organism's DNA to identify it as belonging to a particular species. It's an effort to identify an unknown sample in term of pre-existing classification (Kress et al., 2005) or to access whether the species should be combined or separated (Koch, 2010). The most common barcode region used for animal is the mitochondrial gene, cytochrome oxidase I (COI) and has proven to be sufficiently informative for animals. However, the mitochondrial gene is not promising for plants as their nucleotide substitution rate is low (Newmaster et al., 2006; Fazekas, 2008). Therefore, researchers overcome this problem by using chloroplast regions as chloroplast can be found in all plants and have potential for barcoding (Kress & Erickson, 2007). Coding genes and non-coding in plastid gene regions such as *atpF*-

atpH spacer, *matK*, *rbcL*, *rpoB*, *rpoC1*, *psbK-psbI* spacer and *trnK-psbA* spacer were suggested as DNA Barcode for plants and been evaluated by The Consortium for the Barcode of Life (CBOL) Plant Working Group (2009).

2.6.3 Chloroplast DNA (cpDNA) marker

Chloroplasts are organelles that are found in the plant cells and they conduct photosynthesis process. According to Chumley (2006), the gene content of chloroplast genome is similar in all angiosperms. In the last few decades, many studies have been initiated to determine the complete DNA sequences for nuclear, mitochondrial and chloroplast genomes. The development of molecular technique using chloroplast genome had a great impact on understanding plant evolutions. The use of universal primers in chloroplast genome studies across plant genera has been recommended by Taberlet et al. (1991). The first fully sequenced chloroplast genome is *Nicotiana tabacum* was reported by Shinozaki et al. (1986). Since then numerous sequenced chloroplast genomes has been deposited in GenBank such as *Arabidopsis thaliana*, *Oryza sativa*, *Zea mays*, *Eucalyptus globulus*, *Medicago trancatula* and etc. (Heinze, 1986).

Clegg and Zurawski (1991) reported that chloroplast DNA (cpDNA) has been used to infer plant phylogenies at different taxonomic levels and direct sequencing of polymerase chain reaction (PCR) products expanded the plant systematics and evolution areas of studies. Chloroplast DNA is also a valuable marker for phylogenetic relationships studies between closely related species (Palmer, 1987; Palmer et al., 1988; Clegg et al., 1991). Many phylogenetic studies across plant families and in the Orchidaceae using chloroplast genes *rbcL* and *matK* have been reported (Cameron et al., 1999; Goldman et al., 2001; Hidayat et al., 2005; Barrett & Freudenstein, 2008; Freudenstein & Senyo, 2008; Cameron, 2009; CBOL Plant Working Group, 2009).

2.6.4 Amplified Fragment Length Polymorphism (AFLP)

AFLP is a PCR-based tool used in genetics research, DNA fingerprinting and in the practice of genetic engineering. The AFLP technique was developed in the early 1990's by Keygene N.V. Its technology is covered by patents and patent applications and a registered trademark of Keygene N.V. Vos et al. (1995) was the AFLP as a PCR-based molecular technique in which selected restriction fragments from the digestion of total DNA are amplified by polymerase chain reaction (PCR).

AFLP method basically uses restriction enzymes such as *Eco*RI and *Mse*I to digest genomic DNA, followed by ligation of adaptors to the sticky ends of the restriction fragments. A subset of the restriction fragments is then selected to be amplified. This selection is achieved by using primers complementary to the adaptor sequence, the restriction site sequence and a few nucleotides inside the restriction site fragments. The amplified fragments are visualized on denaturing polyacrylamide gels either through autoradiography or fluorescence methodologies.

There are many advantages to AFLP when compared to other marker technologies including randomly amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), and microsatellites. AFLP not only has higher reproducibility, resolution, and sensitivity at the whole genome level compared to other techniques, but it also has the capability to amplify between 50 and 100 fragments at one time. In addition, no prior sequence information is needed for amplification (Meudt & Clarke, 2007).

The AFLP technology has the capability to detect various polymorphisms in different genomic regions simultaneously. It has become widely used for the identification of genetic variation in strains or closely related species of plants, fungi, animals, and bacteria which the genomic makeup of various organisms is still unknown. The AFLP technology also has been used in criminal and paternity tests, in population genetics to

determine slight differences within populations and in linkage studies to generate maps for quantitative trait locus (QTL) analysis (https://en.wikipedia.org).

AFLP markers have been successfully used in the assessments of genetic diversity and genetic relationships in a wide range of orchid species and hybrids such as *Phalaenopsis* (Chang et al., 2009), *Diuris fragrantissima* (Smith et al., 2007), *Liparis loeselli* (Pillon et al., 2007) and *Dendrobium* (Xiang et al., 2003). Genetic diversity and genetic differentiation was studied between the populations using AFLP marker to orchids species such as *Liparis leoselli* (Pillon et al., 2007); *Neotinea maculata* (Duffy et al., 2009); *Orchis mascula* and *Orhis purpurea* (Jacquemyn et al., 2009 and 2007); *Neotinea ustulata* (Tali et al., 2006) and *Himantoglossum hircinum* (Pfeifer and Jetschke, 2006).

AFLP has been applied to study the distribution of genetic variation within and between wild populations of many plant taxa like *Populus* (Arens et al., 1998; Winfield et al., 1998; Fay et al., 1999), *Phylica* (Richardson, 1999); *Pedicularis* (Schmidt and Jensen, 2000), *Medusagyne* and *Rothmannia* (Fay et al., 2000); and *Dactylorhiza* (Hedrén et al., 2001).

It has been successfully used to determine the genetic diversity, population structure and phylogenetic inference among Italian orchid of the *Serapias* genus (Sardaro et al., 2012); among different *Crocus* genotypes (Erol et al., 2014) and to observe the pattern of population genetic structure in *Spiranthes romanzoffiana* (Forrest et al., 2004).

2.6.5 Molecular work on *Spathoglottis*

Spathoglottis is the genus represented by a number of terrestrial species. Molecular studies on *Spathoglottis* are very limited. However, in the past few years, researchers have started investigating and evaluating the *Spathoglottis* species using molecular method. Ginibun et al. (2010) successfully amplified and determined the chloroplast

DNA (cpDNA) regions (i.e. *accD*, *matK*, *ndhJ*, *rpoB*, *rpoC1*, *ycf5*, *rbcL-a*, and *trnH-psbA*) for the selected *Spathoglottis* species. Four of the cpDNA (i.e. *matK*, *rbcL-a*, *rpoB* and *rpoC1*) could be used as markers to identify the selected *Spathoglottis* species.

Pimonrat et al. (2012) successfully used AFLP technique to identify the mutation induced by gamma irradiation and investigate genetic diversity of the *S. plicata* Blume radiated clones with 1.89-29.85% polymorphism. Whereas, Romeida et al. (2012) used the inter-simple sequence repeat (ISSR) markers to identify genetic variations of orchids *S. plicata* and its mutants. They amplified 10 ISSR primers which produced 360 bands and 71 ISSR of the loci (90.14%) which were polymorphic. This study recommends the ISSR as a good marker for identification of *S. plicata* mutants.

Setiawan et al. (2013) used RAPD molecular method with OPAW11 primer in detection of polyploid *Spathoglottis* species. They also found that electrophorogram could be made as DNA barcoding for *Spathoglottis* species which could be used to trace the origin of orchids from Indonesia.

2.6.6 Molecular analysis

Genetic analysis commonly used to describe methods or overall process of studying and conducting research in sciences of genetic and molecular biology. There are various method used to transform molecular data to different kind of useful results e.g. estimate dis/similarities among samples, prepare summaries of the relationships using cluster analysis, ordination and multiple factor analyses. The results can be shown in both numerically and graphically patterns.

2.6.6.1 Clustering methods

There are two types of clustering methods that used to create a dendrogram or phylogenetic tree from a set of variables data; a similarity matrix or a distance matrix

namely; Distance-based methods and Model-based methods. Distance-based methods used to calculate a pairwise distance matrix which gives the distance entries to suitably define in between every pair of individuals. The matrix results represented using some convenient graphical (tree or multidimensional scalling plot) and clusters that could be identified by eye. This method are easy to apply and visually appealing. It has been commanly to adapt distance-based phylogenetic algorithms such as Unweighted Pair Group Method with Arithmetic mean (UPGMA) (Sneath & Sokal, 1973) and neighbor-joining (NJ) (Saitou & Nei, 1987). The Model-based methods are used by assuming that observations from each cluster are random draws from some parametric model. Inference for parameters corresponding to each cluster is then done jointly with inference for cluster membership of each individual using standard statistical method such as Bayesian methods (Pritchard et al., 2000).

UPGMA is the simplest algorithm used for tree construction. It was designed to produce single trees but they can sometimes derive more than one topology from the same data. Studies by Backeljau et al. (1996) found various computer packages could performed UPGMA tree with different efficiencies in finding ties such as Numerical Taxonomy System (NTSYS) and Molecular Evolutionary Genetics Analysis (MEGA).

Bayesian approach is uses Markov Chain Monte Carlo (MCMC) algorithms to detect the underlying genetic population among a set of individuals genotyped at multiple markers. Bayesian analysis seeking for a tree which maximizes the probability of the given data and the model of evolution. The Bayesian interference of phylogeny was performed using STRUCTURE is a model-based clustering method (Pritchard et al. 2000). It computes the proportion of the genome of an individual originating from each inferred population (quantitative clustering method).

2.6.6.2 Analysis of Molecular Variance

Analysis of Molecular Variance (AMOVA) is a method to detect population differentiation utilizing molecular markers (Excoffier et al., 1992). It is also a statistical model for the molecular variation in a single species and typically biological. AMOVA is a powerful tool that can help support hypotheses of population structure due to clonal reproduction or isolation without making assumptions about Hardy-Weinberg equilibrium.

2.6.6.3 Hardy-Weinberg Equilibrium

The Hardy-Weinberg Principle is a concept based on Mendel's principles of inheritance developed by Hardy and Weinberg. It is a model, theorem, or law, states that allele and genotype frequencies in a population will remain constant from one generation to next generation in the absence of other evolutionary influences. To discover the probable allele and genotype frequencies in a population and to track their changes from one generation to another, a simple equation was develop as formula:

$$p^2 + 2pq + q^2 = 1 \quad or \quad P + H + Q = 1$$

Where;

p = frequency of the dominant allele

q = frequecy of the recessive allele

$P = p^2$ = frequency of homozygous dominant

$H = 2pq$ = frequency of heterozygous

$Q = q^2$ = frequency of homozygous recessive

2.6.6.4 Allele frequencies

Allele or gene frequency is a measure of the relative frequency of a particular allele at a genetic locus in a population. It is usually expressed as a proportion or a percentage.

The formula is:

$$f(A_i) = [n(A_i A_i) + n(A_i A_j)] / N$$

Where;

$f(A_i)$ = frequency of allele A_i

$n(A_i A_i)$ = number of individuals homozygous for allele

$n(A_i A_j)$ = number of individuals heterozygous for A_i and A_j , $i \neq j$

N = total number of individuals in sample

2.6.6.5 Heterozygosity

Heterozygosity is a parameters present in data set. It is widely used measurement for quantifying genetic variation of polymorphic loci. The heterozygosity value ranges from zero to 1. The observed heterozygosity (H_o) for a locus means the proportion of individuals in a population which are heterozygous at the locus. It is estimated using the formula:

$$H_o = n(A_i A_j) / N$$

Where;

$n(A_i A_j)$ = number of individuals with genotype $A_i A_j$, $i \neq j$;

N = total number of individuals in sample

$A_i A_j$ = alleles at the locus

In some cases such as inbreeding, population persistence and genotypic fitness may cause low observed heterozygosity. Therefore, the expected heterozygosity (H_e) is

defined as the estimated fraction of all individuals who would be heterozygous based on the known allele frequency. Deviation observed from the expected hetegozygosity can be used as an indicator of important population dynamics. It is determined for a locus as follows:

$$H_e = N\,(1- \Sigma P_i\ )/N-1$$

Where;

P_i = the frequency of the *i*-th allele

N = the sample size.

2.6.6.6 Genetic differentiation (F_{st})

Fixation index ($F_{st)}$ is a measures or values that could help to understand the degree of population differentiation within species. It is developed as a special case of Wright's F-statistics as the most commonly used statistics in population genetics studies. The formula are;

$$F_{ST}= 1 – HS / HT$$

Where;

HS = is an average expected heterozygosity within Populations

HT = is an average expected heterozygosity in total population.

2.6.6.7 Genetic distance

Genetic distance is a measure of genetic divergence between species or between populations within a species. Populations with many similar alleles normally have small genetic distance. The genetic relationship between any two populations is a function of differences between them in allele's frequencies and this relationship is expressed in terms of a genetic distance. On the other hand, it also could be used to estimate the duration from the passed since the populations appeared as a single cohesive unit.

The estimated genetic distances may differ among loci, therefore the most accurate measurement would be obtained by averaging many loci. Several distance measurements are available in the literature, but Nei's standard genetic distance, *D*, (Nei, 1978) had been widely used in studies of many populations, species and genera. The Nei's standard genetic distance was used by the following formula:

$$D_N = ln\ I$$

Where;

$I = J_{XY} / (J_X J_Y)^{0.5}$, and J_X, J_Y, and J_{XY} are arithmetic means across all loci.

2.7 Cryopreservation

Cryopreservation is another important tool and an alternative long-term storage method of various plant germplasm including orchid materials where minimum space and maintenance is requires as compared to *ex situ* collections. It involves in vitro storage techniques at ultra-low temperature of -196^{o}C in liquid nitrogen with the presence of a cryoprotectant (Bajaj, 1995).

The late Professor Akira Sakai is the pioneer researcher of cryopreservation with his interest to develop a simple efficient and reliable method to replace the conventional method (Matsumoto & Niino, 2014). Sakai et al. (2008) has been developing several techniques of cryopreservation such as vitrification, encapsulation-dehydration and encapsulation-vitrification for conservation of endangered orchid species. Touchell and Dixon (1996); Thammasiri (2005) reported that cryopreservation offered a hope in enhancing the preservation of endangered species, rare plant and potential hybrids. Various techniques of cryopreservation have been developed and successfully cryopreserved a wide range of species and cultivars. Various cryopreservation techniques also have been published for application to various parts of orchid anatomy including zygotic embryos and immature seeds of *Bletilla striata* (Hirano et al., 2005a;

Ishikawa et al., 1997); seeds of *Doritis pulcherrima* (Thammasiri, 2000), *Ponerorchis graminifolia* (Hirano et al., 2005b), *Bratonia hybrid* (Popov, 2004), *Vanda coerulea* Griff. ex Lindl. (Thammasiri & Soamkul, 2007), *Cymbidium* species (Hirano et al., 2011); shoot tips of *Dendrobium* Walter Oumae (Lurswijidjarus & Thammasiri, 2004), in vitro grown shoot apices of *Arachnis* species (Gagliardi et al., 2003) and suspension culture cells of *Doritaenopsis* (Tsukazaki et al., 2000); protocorm and protocorm-like body of *Dendrobium candidum* (Brian et al., 2002).

2.7.1 Vitrification

Vitrification refers to a process that avoids ice formation in cell using highly concentrated cryoprotectants which increases viscosity to induce glass formation and by pass water crystallization (Pegg, 2010). Vitrification is a popular method in cryopreservation as it is quick, simple, reliable, low cost and practical for conserving many orchid seed (Vendrame et al., 2007). This method involves some degree of dehydration by exposing the samples to highly concentrated cryoprotectants solution before freezing (Engelmann, 2000). A cryoprotectant is a substance that is used to protect biological tissue from freezing damage (damage due to ice formation). Mixtures of cryoprotectants have less toxicity and are more effective than single-agent cryoprotectants. A mixture of formamide with DMSO, propylene glycol and a colloid was for many years the most effective of all artificially created cryoprotectants that have been used for vitrification.

Various research teams have developed different vitrification solutions and the most commonly used plant vitrification solution is designated as PVS2 (Sakai et al., 1990) which contains 30% glycerol, 15% ethylene glycol and 15 % DMSO together with 0.4 M sucrose. Sakai et al. (1990) developed the vitrification procedures for nucellar cells of the navel orange by determining the optimal time of exposure to PVS2. Since then,

vitrification techniques have been developed for a wide range of plant species including orchids for different type of materials such as shoot tip, embryos, protocorm, seed and etc. Sakai and Engelmann (2007) had listed the plants cryopreserved using vitrification techniques.

2.7.2 Encapsulation-vitrification

The encapsulation-vitrification is a modification of the vitrification method in which the plant cells are embedded in alginate gel and followed by dehydration with vitrification solution. Explants were encapsulated using sodium alginate beads, which provide enhanced protection of dried materials from mechanical and oxidative stress during storage (Niino & Sakai, 1992). This technique was established by Matsumoto et al. (1995) by combining the vitrification and encapsulation-dehydration methods. They studied this technique on wasabi shoot tips by encapsulating it in alginate bead (3-4 mm diameter) after preculturing with 0.3 M sucrose; followed by loading with a mixture of 2 M glycerol plus 0.4 M sucrose for 30 min and dehydrated with PVS2 solution in a 100 ml glass beaker on a rotary shaker (100 rpm) for 70-100 min at 0 ℃. The encapsulated dehydrated shoot tips are suspended in 0.7 ml PVS2 solution in 2.0 ml cryotube which is plunged in LN. Cryopreservation of the wasabi shoot tip using encapsulation-vitrification gave 30% higher that encapsulation-dehydration technique. Since the encapsulation-vitrification was established, it has been applied successfully to 22 genera of plant species also listed by Sakai and Engelmann (2007). This technique is user-friendly and reduces the time required for dehydration. However, studies to evaluate and develop specific parameters to different plant species, especially on the effect of various sucrose as loading solution, effect of PVS2 at different duration are required.

2.7.3 Histological study

Histology is a study of the microscopic anatomy of cells and tissues of plants by examination under a light microscope or electron microscope after being sectioned, stained and mounted on a microscope slide. The histological study was conducted using tissue culture where the tissue or cells are isolated and maintained in an artificial environment during research. The ability to visualize or differentially identify microscopic structures is frequently enhanced through the use of histological stains.

In cryopreservation studies, histological techniques was carried out to observe both the formation and development of any part of the plant cells; also the effect of any cryopreservation solution and cryoprotectant treatments. Engelmann (2014) have reported that the histological techniques have provided new information on the cryoprotectant effect where recent work used cellular imagery techniques to quantify cell polymolysis in hairy roots of *Rubia akane* after each step of a droplet-vitrification protocol. Results showed that the main plasmolysis effect occurred during the loading treatment for the root apical segments.

Histological analysis was carried by Antony et al. (2011) on cryopreserved PLBs of *Dendrobium* Bobby Messina orchid after 3 months of thawing. The cross section of non-cryopreserved PLBs showed presence of complete outer layer of the cell wall, visible nucleus and undamaged cell with a perfect polyhedral shape. Whereas, cryopreserved PLBs showed presence of breakage at the outer layer of cell wall, visible voluminous nucleus, denser cytoplasm and presence of more homogenous cell population; cells were also damaged and showed symptoms of plasmolysis, nuclear shrinkage, rupture in cell wall and cell membrane. Similar histological analysis results were obtained on the *Vanda* Kaseem's Delight orchid PLBs after vitrification treatment and cryopreservation (Poobathy et al., 2012); and *Dendrobium sonia*-28 PLBs after encapsulation-vitrification and cryopreservation (Ching et al., 2012). Thus, the

importance of histology now embraces the study of the structures of both tissue and cells and the relationship between these structures and physiological function after cryopreservation.

CHAPTER 3: MORPHOLOGICAL DESCRIPTION IN MALAYSIAN SPECIES OF *SPATHOGLOTTIS*

3.1 INTRODUCTION

Spathoglottis is a terrestrial orchid that reported to be a native and grow wild in Malaysia. Six species namely *S. plicata, S. aurea, S. affinis, S. gracilis, S. microchilina* and *S. hardingiana* have been identified by taxonomist occured in Peninsular Malaysia since 18th and 19th century. However, only five species are still found exist excluded the *S. hardingiana*. *Spathoglottis* is said to be facing extinction due to the changing environmental conditions along with human interference that involves horticulture and developmental activities. Therefore, there is a need to keep records of their morphological, biological and genetic information besides making efforts to conserve the germplasm through *in situ* or *ex situ* ways.

There is currently no established *Spathoglottis* morphological descriptor list although taxonomic descriptions have been reported. Considering the need for germplasm conservation and characterization of the various species of *Spathoglottis*, we have studied the morphological characteristics of this genus. The study focused on qualitative data, with the intention to develop a table of characteristics of test guidelines for *Spathoglottis* that could be recognised and used by the UPOV or by Bioversity International. The morphological identification will be part of the bigger initiative to have all germplasm of this species characterized and systematically documented.

3.2 LITERATURE REVIEW

Morphological markers are one of the earliest techniques applied to plant diversity characterisation and important in identifying/describing plant characteristics. Morphological characterization is description of an accession based on morphological markers taken at the various stages of growth and used in characterization of germplasm

that may include morphological/botanical features (Bioversity International, 2007). Descriptor lists are structured sets of individual descriptors used in the description of germplasm of a particular crop or species. Qualitative data, such as types of sample received, is not computable by arithmetic calculations and are expressed in discontinuous states. They are self-explanatory and independently meaningful labels or names that determine the class or category in which an individual, object or process falls. All possible states are necessary to describe the full range of a characteristic, and every form of expression can be described by a single descriptor state; the order of states is not important (Bioversity International, 2007, p.21).

The species of *Spathoglottis* are known for a wide range of flowers which can be differentiated or classified by their flower colour as well as their lip shape and lip colour. Taxonomists recognized approximately 40 described species under the genus *Spathoglottis* based on morphological classification from all across Asia Pacific region (Howcroft, 1992; Beltrame, 2006). Holttum (1953); and Seidenfaden and Wood (1992).They have described the Malaysian *Spathoglottis* with illustrations. As reported, Peninsular Malaysia and Borneo have six species of *Spathoglottis* wherein *S. plicata* is found in lowlands and the most common species distributed almost throughout Malaysia with shades of flower colour in purple, mauve and white hues (Holttum, 1953; Teo et al., 1985; Comber, 2001). *Spathoglottis affinis*, *S. aurea*, *S. gracilis* and *S. microchilina* are mountain species with beautiful yellow flowers (Seidenfaden & Wood, 1992). The *S. hardingiana* species was reported to be restricted to Langkawi Island that found growing on limestone near the sea with light purple, pale mauve or crimson flower colour but this species is very rare and is almost extinct.

3.3 MATERIALS AND METHODS

3.3.1 Plant materials

Five *Spathoglottis* species of S. *plicata, S. affinis, S. aurea, S. gracilis* and S. *microchilina* native to Peninsular Malaysia were used in this study and five samples were collected from the wild plants as shown in the location map in Figure 3.1. They are *S. affinis* from Gunung Jerai, Kedah, *S. aurea* from the Cameron Highlands, Pahang, *S. gracilis* from Endau Rompin, Pahang, *S. microchilina* from Genting Highlands, Pahang and *S. plicata* collected from Kuala Klawang, Negeri Sembilan. The *S. hardingiana* was left out from this study as the sample was not available.

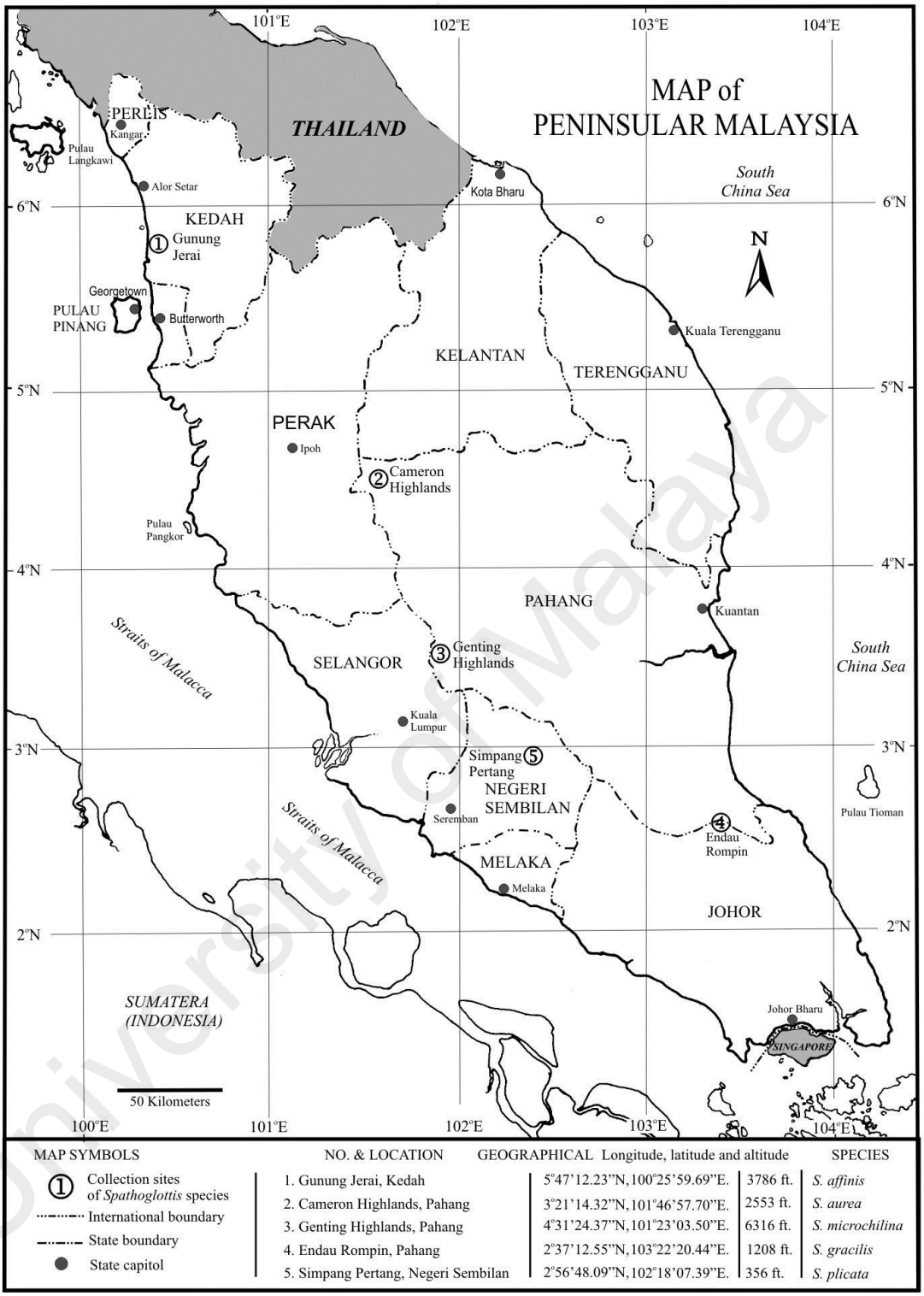


Figure 3.1: Location map of *Spathoglottis* species sampling in Peninsular Malaysia

3.3.2 Morphological characterisation

The phenotypic characteristics of the *Spathoglottis* species were observed from the live plant specimens *in situ*. Photographs of the specimen were taken (Figure 3.2) and the observed qualitative characteristic data were recorded. Documentation of data was mainly based on the *in situ* living material supported by taxonomy references, key for the species, herbarium collection, orchid experts, plant identification terminology, crop descriptor list from Bioversity International (2007) and test guidelines of orchids for UPOV (1999, 2002, 2003, 2004 and 2009).

a) S. aurea *b) S. affinis* *c) S. microchilina*

d) S. gracilis *e) S. plicata*

Figure 3.2: Photograph of Malaysian native *Spathoglottis* species. The samples were collected from a) Cameron Highlands, Pahang; b) Gunung Jerai, Kedah (picture by Dr. Choong Chieh Wean); c) Genting Highlands, Pahang; d) Endau Rompin, Pahang; e) Kuala Klawang, Negeri Sembilan.

3.3.3 Colour chart

The different colours of flowers in this study were observed, described and determined using the Royal Horticultural Society (RHS, 2007) colour charts. Observing flower colour using visual aids may compromise the accuracy in determining the exact colour. Therefore, RHS colour chart was a useful tool in determining the colour variations occuring inside the flower species. It is a standard reference for plant colour identification and developed for determination of colour of flowers. Each colour chart patch with central porthole was used over the object to obtain the closest match. UPOV document TGP/14 was described the way to use the RHS colour chart. The RHS colour chart is shown as Appendix B.

3.4 RESULTS

The flower colour and the shape of the lip are important characteristics in resolving the morphological difference in *Spathoglottis* species. Although the different colours and colour patterns are important, the colour differences are sometimes difficult to differentiate. Therefore, other qualitative characteristics of the flower were also observed, especially on the morphology of the bract, sepals, petals and lip of the flowers. The morphology characteristics of *S. plicata* flower are shown in Figure 3.3. From our observation, there were four characteristics that could distinguish the species from shape of the bract, lip side lobes, lip mid-lobe and the callus of lip. The bract shape was categorized into two types as shown in Figure 3.4. Whereas, the shape of lip side lobes was categorized into four types (Figure 3.5). Both, the shape of lip mid-lobe (Figure 3.6) and the callus of lip (Figure 3.6) were categorized into two types. In general, the leaf colour in all *Spathoglottis* species varied from green to dark green and some may have red pigmentation. Almost all the cross sections of the leaf are plicate.

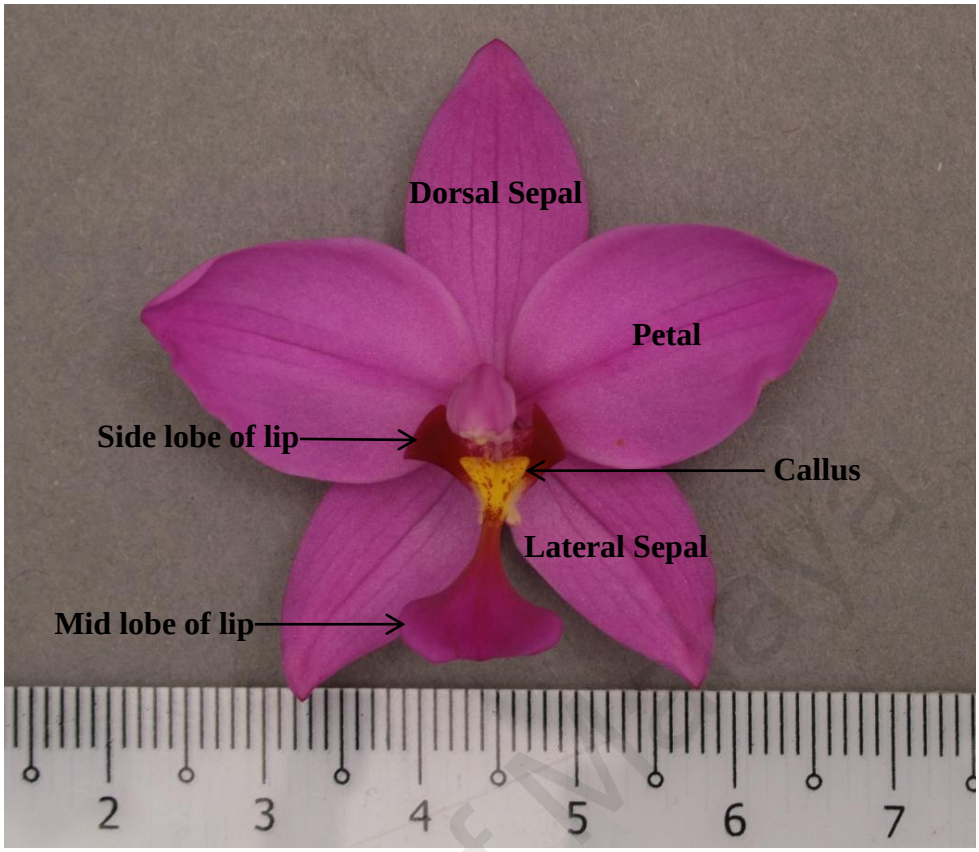


Figure 3.3: The flower of *Spathoglottis plicata*

Spathoglottis plicata could be recognised by its purple flower using the Royal Horticultural Society (RHS, 2007) colour chart numbers N81D of purple-violet group. The bract shape was categorized as Type 1 (Figure 3.4). The petals are elliptic, distinctly larger than the sepals, which are oblanceolate oblong. The lip has an upward curve and has rectangular side lobes. Lip callus is present on the either side at the base of the side lobes and the shape was as Type 1 (Figure 3.5 and Figure 3.6). The mid-lobe has a long claw and spreads out to a broad spatular 2-lobed apex (as Type 1 in Figure 3.6). Whereas, *S. affinis*, *S. aurea*, *S. gracilis* and *S. microchilina* have yellow as the dominant flower colour with each of them displaying different shades of yellow that could be identified by the RHS colour chart. *Spathoglottis affinis*, *S. aurea*, *S. gracilis* and *S. microchilina* have almost similar characteristics when visualized by naked eye. Their bract shapes are grouped as Type 2 (Figure 3.4). *Spathoglottis aurea* has golden

yellow flowers (RHS no. 17C of yellow orange group). The petals and sepals are similar. The lip side lobes and calluses are more or less flushed with spotted or are mottled with purple or crimson colour. The lip mid lobe is more swords shaped, with small magenta spots in streaks. *Spathoglottis microchilina* has bright yellow flowers (RHS no. 15A of yellow-orange group). Their characteristics are almost similar to *S. aurea* but the lip differs in having scattered purple markings. The sword shaped mid-lobe is a bit wider. However, both *S. aurea* and *S. microchilina* lip mid-lobes and its calluses were grouped as Type 2 (Figures 3.6) and the lip side lobes categorized as Type 2 and 3 (Figure 3.5). *Spathoglottis affinis* have small flowers with yellow colour (RHS no. 6A of yellow group). The dorsal sepal is narrower than the lateral sepals. The reddish brown streaks on the lateral sepals make this species distinguishable. The lip is clawed and has broad and notched apex. The flowers of *S. gracilis* are pale yellow (RHS no. 7C of yellow group). The lip is quite similar to *S. affinis* but the lip midlobe claw is wider (Type 1, Figure 3.6). Both *S. affinis* and *S. gracilis* share the same shape of lip side lobes (Type 4, Figure 3.5), spathulate with reddish spots.

Type 1 Type 2

Figure 3.4: Shape of bract for *Spathoglottis* species. Type 1 shown was from *S. plicata* and Type 2 from *S. microchilina*

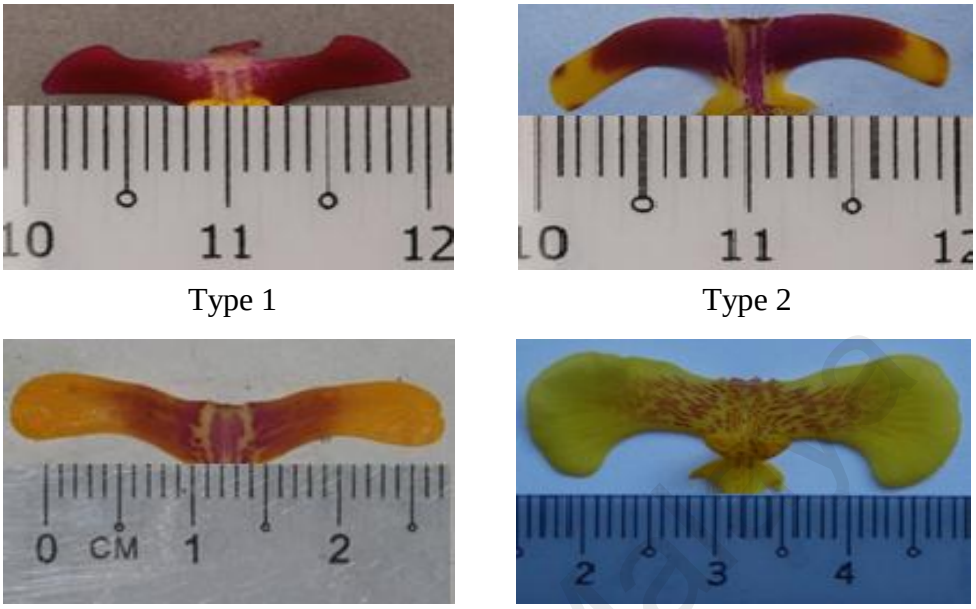


Figure 3.5: Shape of lip side lobe (SL) for *Spathoglottis* species. Type 1 shown was from *S. plicata*, Type 2 from *S. aurea*, Type 3 from *S. microchilina* and Type 4 from *S. gracilis*.

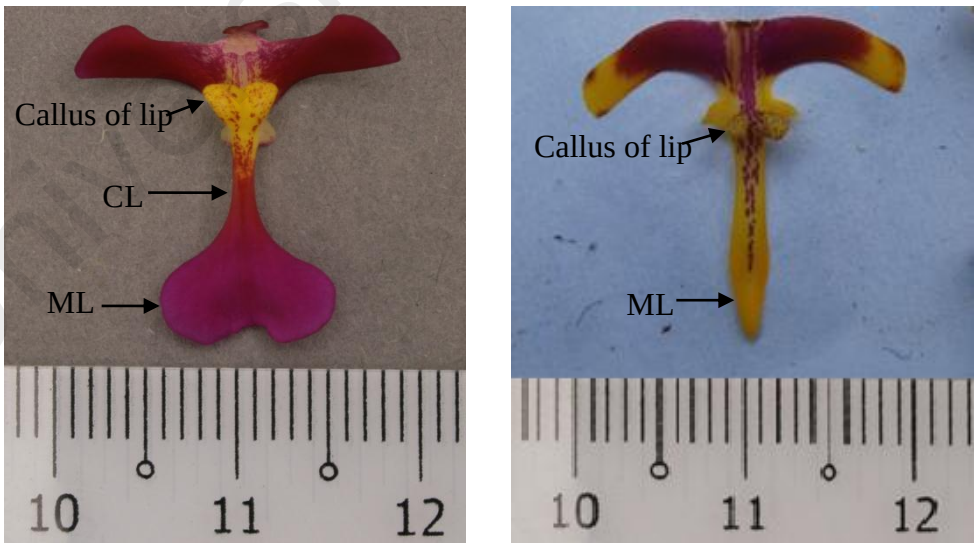


Figure 3.6: Shape of lip mid lobe (ML) and thickness of lip mid-lobe claw (CL); and callus of lip for *Spathoglottis* species. Type 1 shown was from *S. plicata* and Type 2 from *S. microchilina*

After detailed observation on the five wild *Spathoglottis* species of *in situ* living plant materials, a total of 47 qualitative characters (see Appendix B) were recorded for leaf (2), peduncle (1), bract (5), flower (3), dorsal sepal (6), lateral sepal (6), petal (6), lip (1), lip side lobes(5), lip mid-lobe claw (2), lip mid-lobe (5), lip callus (3) and column (1). Table 3.1 shows the 21 qualitative characteristics that distinguished the different species and could be recommended as useful grouping characteristics for *Spathoglottis* species or varieties in the future.

Table 3.1: Morphological qualitative characteristics observed from the *Spathoglottis* species of Malaysia

	Plant characteristic	***S. plicata***	***S. aurea***	***S. microchilina***	***S. affinis***	***S. gracilis***
1 PQ	Bract: shape (Figure 3.4).1-Type 1, 2-Type 2	1	2	2	2	2
2 PQ	Bract: shape of apex. 1-acuminate, 2-Obtuse	1	2	2	2	2
3 PQ	Flower: main colour (RHS)	N81D	17C	15A	6A	7C
4 PQ	Dorsal Sepal: shape.1-Oblanceolate oblong, 2- Elliptic	1	2	2	1	1
5 PQ	Dorsal Sepal: shape of apex.1- Acute, 2-Obtuse	1	2	2	1	1
6 PQ	Dorsal Sepal: main colour (RHS)	N81D	17C	15A	6A	7C
7 PQ	Lateral sepal: shape. 1-Oblanceolate oblong, 2- Elliptic	1	1	1	2	1
8 PQ	Lateral sepal: shape of apex. 1- Acute, 2-Obtuse	1	1	1	2	1
9 PQ	Lateral sepal: main and pattern colour (RHS)	N81D N80C	17C	15A	6A 60B	7C 7A
10 QL	Lateral sepal: colour pattern. 1-Self coloured, 2- Striped	2	1	1	2	2
11 PQ	Petal: shape.1-Elliptic , 2-Ovate	2	1	1	2	2
12 PQ	Petal: shape of apex. 1-Acute, 2-Obtuse	1	1	2	2	2
13 PQ	Petal: main colour (RHS)	N81D	17C	15A	6A	7C
14 PQ	Side lobe of lip: shape (Figure 3.5). 1-Type 1, 2-Type 2, 3-Type 3, 4-Type 4	1	2	3	4	4
15 PQ	Side lobe of Lip: main and pattern colour (RHS)	59C N80B	60C 17B	21A 31A	9A 60A	7A 34A
16 QL	Side lobe of Lip: colour pattern. 1-Shaded, 2-Spotted	1	1	1	2	1
17 PQ	Mid lobe of lip: shape (Figure 3.6). 1-Type 1, 2-Type 2	1	2	2	1	1
18 PQ	Mid lobe of Lip: main and pattern colour (RHS)	71D	17B N80B	23A 60D	9A	12A 34A
19 QL	Mid lobe of Lip: colour pattern.1-Self coloured, 2-Spotted	1	2	2	1	2
20 PQ	Mid lobe claw of lip: thickness at the narrowest point. 1-thin, 2-broad (Figure 3.6)	1	2	2	1	2
21 PQ	Callus of lip: Shape (Figure 3.6). 1-Type 1, 2-Type 2	1	2	2	1	1

Note: QL: Qualitative and PQ: Pseudo-qualitative

3.5 DISCUSSION

Malaysia has six *Spathoglottis* species that grow wild in their natural habitat. In this study, only five *Spathoglottis* species, *S. affinis*, *S. aurea*, *S. microchilina*, *S. gracilis* and *S. plicata* were used for morphological characterization. The *S. hardingiana* was not included in this study as there were no samples available. The collected data were categorized as either qualitative or quantitative. However, in this study, qualitative data obtained through visual observation on the plant species was used to determine the *Spathoglottis* morphological characteristic. The major characteristics observed were the leaf, bract, flower, side lobe of lip, apical lobe of lip, callus and column. These were developed as keys following the guidelines for developing crop descriptors (Bioversity International, 2007; UPOV, 2002) and for the conduct of tests for distinctness, uniformity and stability (UPOV, 2004) in other orchid genera: *Cymbidium* (UPOV, 1999), *Dendrobium* (UPOV, 2009) and *Phaleonopsis* (UPOV, 2003). Qualitative characteristics were also recorded as pseudo-qualitative where each individual state of expression needs to be identified to adequately describe the range of the characteristic. The characteristic that could group the species or varieties is important to use as a guide for distinguishing the differences in variation of *Spathoglottis*.

The morphological characteristics studied by Kartikaningrum et al. (2004) on the *Spathoglottis* leaf and flower showed variations on the characters of the sepal and petal shape; and also the colour of the flowers. It has dominant colour of purple, yellow, pink and white with gradation of colour and large diversity. Similar observation on the Malaysian *Spathoglottis* species that show the purple and yellow flower colour appeared to be diverged by adaptation to their environmental habitat. The observation found that *S. plicata* has a dominant flower colour of purple. Visually, *S. affinis*, *S. aurea*, *S. gracilis* and *S. microchilina* have similar characteristics and each of them have

dominant flower colour of yellow with different shades of yellow identified through the RHS colour chart.

The morphology study on the plant of *Spathoglottis* species especially on the flowers revealed that the traits on the shape of bract; shape of lip side lobe; shape lip mid lobe and thickness of mid lobe claw; and shape of callus of lip are some of the characters that may have been inherited and pooled into the species. However, the number of sample size need to be more then one to get an accurate results in differentiating the species for future study.

3.6 CONCLUSION

Taking into account of the growing threat to the diversity of the species due to loss of habitat and conversely its increasing popularity as an ornamental ground orchid plant, there is a need to establish a structured database with accurate information on their morphological characteristic. In this study it has been shown that the colour of the flower and the shape of the lips are important characteristics that can be used to resolve the morphological difference within the *Spathoglottis* species. The data presents a clear description of the collected Malaysian *Spathoglottis* species using the descriptors chosen.

CHAPTER 4: GENETIC RELATIONSHIPS AMONG *SPATHOGLOTTIS* SPECIES IN PENINSULAR MALAYSIA BASED ON SIMPLE DNA MARKER ANALYSIS (IRAP)

4.1 INTRODUCTION

Taxonomists generally differentiate or classify the *Spathoglottis* species by their flower morphological characteristics. However, it is difficult to differentiate the *Spathoglottis* species by morphology unless it is flowering. Unfortunately, this approach poses some limitations as the few morphological characteristics could be influenced by environmental factors and some species is flowers seasonally. Therefore, to complement the morphological approaches, the application of molecular genetics for species differentiation would be a valuable addition for genetic diversity, phylogenetic relationship and evolutionary research. Molecular marker technology playing an essential role in plant biology to study all aspects of genetics including DNA fingerprinting and phylogenetic relationship; modern plant breeding; and for map-based cloning of genes, ranging from the identification of genes responsible for the desired traits to the management of backcrossing programs. It is important to use simple method for genetic investigation of the *Spathoglottis* species. Kalender et al. (1999; 2010) has developed a universal retrotansposons-based method of Inter-Retrotransposon Amplified Polymorphism (IRAP) and Retrotransposons are well suited as molecular markers (Kalender, 2011). It has been successfully used for the investigation of genetic diversity for several species (Vukich et al., 2009; Kalender et al., 2010; Smykal et al., 2011).

Information on the genetic relationships of *Spathoglottis* species using molecular markers is limited. Only a few reports have been published on chloroplast DNA Barcoding and RAPD (Ginibun et al., 2010; Setiawan et al., 2013). Additionally, data is not available for the genus studied. Therefore, in this study, we have optimized and

investigated the utility of barley based LTR-RTNs and conserved reverse transcriptase (RT) priming sites of the banana Ty1-copia-like retrotransposons for generating an IRAP molecular marker system for *Spathoglottis* genus which can contribute to the evaluation of the genetic relationships of its species.

4.2 LITERATURE REVIEW

Retrotransposons (RTNs) are mobile genetic elements that can amplify themselves in a genome and ubiquitous components throughout all plant species that have been examined (Flavell et al., 1992; Voytos et al., 1992; Kumar et al., 1997) including the orchid genus *Phalaenopsis* (Hsu et al., 2011). They are present in high copy numbers, widely dispersed within the euchromatic regions of chromosomes and showing all the extensive insertional polymorphism in plants (Kumar et al., 1997). Retrotransposons provide an excellent basis for the development of DNA-based markers based on polymerase chain reaction (PCR) techniques for polymorphism detection. RTNs flanked by long-terminal repeats (LTR) are abundant in plants (Pearce et al., 1996).

An inter-retrotransposon amplified polymorphism (IRAP) method is require simple PCR techniques, using retrotransposon primer(s) to detect level of polymorphism followed by gel analysis (Kalender et al., 1999; Smykal, 2006). IRAP method using the barley BARE-1 and other retrotransposon have been studied and demonstrated to provide suitable polymorphic markers for variety identification or breeding purposes (Kalender et al., 1999; Manninen et al., 2000; Vicient et al., 2001; Boyko et al., 2002). IRAP also reported has been used either alone or combination with REMAP in the studies of genetic diversity, genetic variability, phylogenetic relationships and genome evolution within several plant genera of *Hordeum* (Kalender et al., 1999, Kalender et al., 2000), *Musa* (Teo et al., 2005), *Pisum* (Smykal, 2006), *Oryza* (Branco et al., 2007), *Crocus* (Alavi-Kia et al., 2008) and *Triticum* (Carvalho et al., 2010). The distribution

range of a particular RTN family has been routinely suggested to be limited to the species in the same genus (Vicient et al., 1999), however the barley-derived RTN markers proved to be transferable to closet (Breto et al., 2008) and distant plant species (Teo et al., 2005). The molecular characterisation of species with RTN-based markers could distribute a deepest knowledge of the diversity, evolution and phylogenies among individuals, species or populations.

4.3 MATERIALS AND METHODS

4.3.1 Plant materials

Five native *Spathoglottis* species in Peninsular Malaysia were selected for this study and one leaf sample per species collected from the wild. The samples were collected as describe in Chapter 3.3.1 and Figure 3.1. Other five additional species were selected to be used in these studies (see Figure 4.1) which literaturely are native to other regions. Only one sample per species available is purchased from orchid nurseries: *S. plicata* var *alba* - a native species of Thailand, *S. unguiculata* (Labill.) Rchb.f. - a native species of New Caledonia, Vanuatu, Fiji, *S. kimballiana* Hook.f.- a native species of Borneo, *S. vanoverberghii* Ames - a native species of the Island of Luzon, Philippines, *S. lobbii* Rchb.f.- a native species to Burma, a *Spathoglottis* hybrid (unknown origin) and the *Dendrobium affine* (as an outgroup).

transferred into a DNeasy Mini spin column in a 2 ml collection tube. It was centrifuged for 1 min at ≥ 6000 x g (≥8000 rpm) and the flow-through discarded. The spin column was placed into a new 2 ml collection tube and about 500 µl of Buffer AW was added followed by centrifuging for 2 min at 20,000 x g. The spin column was removed from the collection tube carefully so that the column does not come into contact with the flow-through. The spin column was then transferred into a new 1.5ml or 2ml microcentrifuge tube and 100 µl Buffer AE added for elution. The column was incubated for 5 min at room temperature and centrifuged for 1 min at ≥6000 x g. This step was repeated.

4.3.3 Quantification of DNA

The presence of DNA after extraction was detected by using both gel electrophoresis and spectrophotometry. Agarose gel electrophoresis confirmed the presence of the DNA extracted from the samples. For electrophoresis, 5µl of the DNA sample was mixed with 2µl of loading dye (Vivantis, USA) and loaded into 1% agarose gels. These were electrophoresed for 60 minutes at 75 volt and were viewed under UV illumination for bands.

The concentration and purity of the DNA extracted was determined by spectrophotometric measurement of UV absorbance using a spectrophotometer (Eppendorf, Germany) at a dilution ratio of 5:45, in which 5µl of the sample was diluted with 45µl of autoclaved dH_2O. The optical density (OD) ratio was read and the range was recorded.

4.3.4 Inter-Retrotransposon Amplified Polymorphism (IRAP) analysis

The IRAP method was carried out as described by Teo et al. (2005) using seven long terminal repeat (LTR) primers derived from the barley genome (*Hordeum vulgare*); and

two primers (Reverse Ty1 and Reverse Ty2) facing outward from the highly conserved reverse transcriptase of the banana Ty1-copia-like retrotransposon designed by Teo et al. (2002). The primer sequences, retrotransposon sources and their orientation are shown in Table 4.1.

Table 4.1: The IRAP primers, RTNs sources (accession number), orientation (base pair positions) and sequences (Teo et al. 2005)

Name	**RTN sources, Orientation**	**Sequence, accession, position**
LTR 6149	BARE-1→	CTCGCTCGCCCACTACATCAACCGCGTTTATT Z17327,1993-2012
LTR 6150	BARE-1←	CTGGTTCGGCCCATGTCTATGTATCCACACATG TA Z17327, 418-439
5'LTR1	BARE-1←	TTGCCTCTAGGGCATATTTCCAACA Z17327, 1-26
5'LTR2	BARE-1←	ATCATTCCCTCTAGGGCATAATTC Z17327, 314-338
3'LTR	BARE-1→	TGTTTCCCATGCGACGTTCCCCAACA Z17327, 2112-2138
Sukkula	Sukkula →	GATAGGGTCGCATCTTGGGCGTGAC AYO5437, 4301-4326
Nikita	Nikita →	CGCATTTGTTCAAGCCTAAACC AYO78073, AYO78074, AYO78075, 1-22
Reverse Ty1	W1,W3, W7, W8 ←	CCYTGNAYYAANGCNCT AF416815, AF416816, AF416817, AF416818, 1-17
Reverse Ty2	W1,W3, W7, W8 →	TRGTARAGRAGNTGRAT AF416815, AF416816, AF416817, AF416818, 252-269

The IRAP Polymerase Chain Reaction (PCR) was performed in a 20µl reaction mixture containing 50 ng of DNA, 1X PCR buffer (Promega), 2mM $MgCl_2$, 0.2 mM dNTP mix, 10µM of each primer and 1 U of Taq Polymerase (Promega) in 0.2 ml thin-walled PCR tubes. Amplification was performed using an Eppendorf AG Thermocycler. The amplification program consisted of 95℃ for 5 min, 30 cycles of 95℃ for 60 seconds, annealing at T_a (specified in Table 4.2) for 60 seconds, ramp of 0.5℃ s^{-1} to 72℃ and 72℃ for 2 min with a 3 seconds increment per cycle and a final extension of

72 ℃ for 10 min. PCR products were analysed by electrophoresis on 2% (w/v) agarose gels with Ethidium Bromide (EtBr) staining. A 100 bp DNA ladder (Vivantis, USA) was used as a molecular weight standard in each run at left and right positions in the gel for band designation. The gel was viewed under UV light using an Alpha Imager™ 2000 transilluminator.

Table 4.2: The respective annealing temperatures (℃) of IRAP primer combinations; Ø indicates unsuccessful primer combinations for amplification; *indicates optimum temperature primers combination amplified in all samples

	R TY1	R TY2	LTR 6149	LTR 6150	5'LTR 1	5'LTR 2	3'LTR	Sukkula	Nikita
R TY1	Ø	Ø	Ø	Ø	Ø	Ø	49.8	49.8	46.7
R TY2		Ø	Ø	Ø	Ø	Ø	49.8	49.8	41.0
LTR 6149			Ø	Ø	Ø	Ø	41.9*	45.4*	42.9*
LTR 6150				40.4*	40.2	Ø	41.9*	41.9	41.9*
5'LTR1					Ø	Ø	45.4*	45.4	45.4
5'LTR2						Ø	46.7*	46.7	41.9*
3'LTR							42.9*	48.8	44.2*
Sukkula								46.7*	47.8
Nikita									49.5

4.3.5 Data analyses

The IRAP banding pattern was scored visually directly from the agarose gel picture (Appendix C). The presence or absence of each single fragment was coded by 1 or 0. Only clearly distinguishable bands were scored. PAST (Paleontological Statistics) version 2.03 software (Hammer et al., 2001) was used to analyse the binary data matrix with all polymorphic scorable IRAP bands. The similarity and distance indices were calculated using the Jaccard coefficient (Jaccard, 1908) with the formula of $d_{jk}=M/(M+N)$, where M for the number of matches and N for the total number of column with present in just one row. Cluster analysis was a hierarchical clustering routine to produce a 'dendrogram' based on the average distance between all species with 1000 bootstraps replicates. The unweighted pair-group method with arithmetic means (UPGMA) were the algorithms used to cluster the species data. Principal

Coordinate Analysis (PCoA) is another ordination method also known as Metric Multidimensional Scaling to find the eigenvalues and eigenvectors of a matrix containing distances or similarities between all the data. The association among the species was assessed by PCoA.

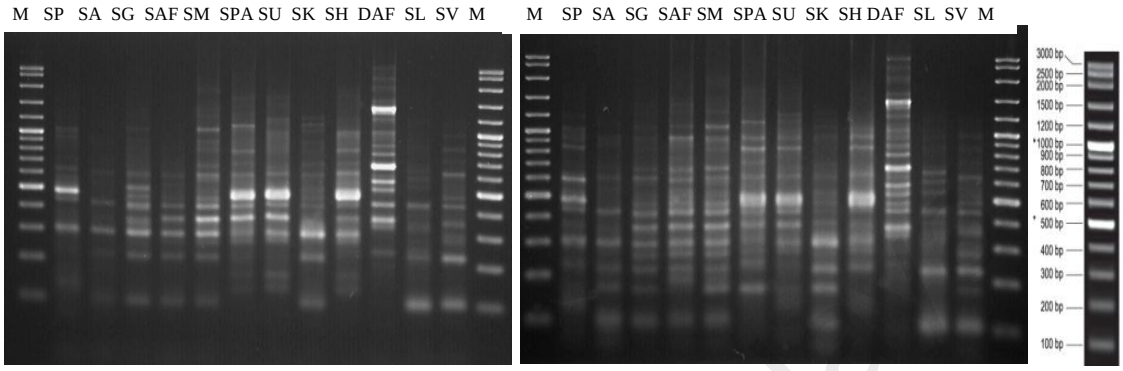
4.4 RESULTS

4.4.1 Amplification of IRAP primers

Twenty six IRAP primers comprising four singled out of nine and 22 combinations out of 36 possible LTRs and RT primers combinations were successfully used to amplify the target fragments of *S. plicata* DNA samples for annealing temperature optimization. The optimized PCR conditions were then applied across the five Malaysian native *Spathoglottis* species, five additional selected species, a hybrid of *Spathoglottis* and an outgroup of *Dendrobium affine* for further amplification in order to establish the IRAP profiles. Twelve out of twenty six amplified IRAP primers which consisted of three single primers (LTR 6150, 3'LTR and Sukkula) and nine IRAP primer combinations (LTR 6149 + Sukkula, LTR 6149 + Nikita, LTR 6149 + 3'LTR, LTR 6150 + Nikita, LTR6150 + 3'LTR, 5'LTR1 + 3'LTR, 5'LTR2 + 3'LTR, 5'LTR2 + Nikita and 3'LTR + Nikita) were able to generate clear multiple fragments in all *Spathoglottis* samples using the optimal temperatures (Table 4.2).

The amplified fragments were highly polymorphic between the *Spathoglottis* species tested while the number of polymorphic bands varied from 22 to 34 per primer combination (Table 4.3). In total, the IRAP analysis generated 341 polymorphic bands observed from the twelve amplified primer combinations ranging in size from 100 to 3000 bp. Overall the IRAP band patterns of the *Spathoglottis* species exhibited high level of polymorphism (100 %). The scorable bands and the reproducibility of the amplification patterns were consistent for each primer combination. The representative

gel picture of polymorphism pattern from five Malaysian native *Spathoglottis* species, five additional selected species, a hybrid of *Spathoglottis* and an outgroup of *Dendrobium affine* show in Figure 4.2 and the other picture was in Appendix C.



(1) Sukkula (2) LTR 6149+Sukkula

Figure 4.2: The IRAP Polymorphism pattern of five Malaysian native *Spathoglottis* species, five additional selected species, a hybrid of *Spathoglottis* and an outgroup of *Dendrobium affine* from the agarose gel picture

Table 4.3: IRAP optimum temperature with polymorphism detected using single and paired primers, polymorphic band and band size range of the IRAP products in five Malaysian native *Spathoglottis* species, five additional selected species, a hybrid of *Spathoglottis* and the outgroup *Dendrobium affine*

Primer combination	**Optimum Temperature (℃)**	**Polymorphic bands**	**Band size (bp)**
LTR 6150	40.4	28	100-1800
3'LTR	42.9	33	150-3000
Sukkula	46.7	26	100-3000
LTR 6149 + 3'LTR	41.9	28	100-1400
LTR 6149 + Sukkula	45.4	24	100-3000
LTR 6149 + Nikita	42.9	22	130-2700
LTR 6150 + 3'LTR	41.9	28	100-1600
LTR 6150 + Nikita	41.9	32	100-1800
5'LTR1+3'LTR	45.4	32	100-3000
5'LTR2 + 3'LTR	46.7	27	220-1700
5'LTR2 + Nikita	41.9	27	140-2400
3'LTR + Nikita	44.2	34	100-1500
Total		341	-

4.4.2 Genetic relationships among *Spathoglottis* species

The analysis was based on 341 polymorphic bands amplified using the 12 selected IRAP primers combinations (Table 4.3). Jaccard coefficient commonly used in species similarity and distance indices was utilised to study the relatedness of the *Spathoglottis* using the fragments generated in this study. The similarity matrix of the five Malaysian native *Spathoglottis* species, the other five additional species, a hybrid of *Spathoglottis* and an outgroup of *Dendrobium affine* species are shown in Table 4.4 which ranged from 0.090 to 0.655. The results of this study indicated that there is a 17-65% similarity among the Malaysian native *Spathoglottis* species. There are genetic differences in between the *S. plicata* with the other four Malaysian *Spathoglottis* species of *S. aurea*, *S. gracilis*, *S. affinis* and *S. microchilina* with 17-29%. However, *S. affinis* and *S. microchilina* are closely related with 65% similarity. Whereas, 53% similarity seen between *S. gracilis* and *S. affinis* and 57% with *S. microchilina*. However, only about 30% similarity between *S. aurea* and the *S. gracilis*, *S. affinis* and *S. microchilina* species was recorded. The genetic similarity between the Malaysian *Spathoglottis* species with other five selected species of *S. plicata* var *alba*, *S. unguiculata*, *S. kimballiana*, *S. vanoverberghii*, *S. lobbii* and the hybrids are low (9-48%). In term of their genetic distance, *S. vanoverberghii*, *S. lobbii* and *S. kimballiana* show higher distance from the other species.

Table 4.4: Similarity and distance indices between the five Malaysian native *Spathoglottis* species [i.e. *S. plicata* (SP), *S. aurea* (SA), *S. gracilis* (SG), *S. affinis* (SAF), *S. microchilina* (SM)]; five additional selected species [*S. plicata alba* (SPA), *S. unguiculata* (SU), *S. kimballiana* (SK), *S. vanoverberghii* (SV), *S. lobbii* (SL)] and a hybrid of *Spathoglottis* (SH) and a *Dendrobium affine* (DAF) as outgroup by Jaccard

	SP	**SA**	**SG**	**SAF**	**SM**	**SPA**	**SU**	**SK**	**SH**	**DAF**	**SL**	**SV**
SP	1											
SA	0.178	1										
SG	0.297	0.356	1									
SAF	0.263	0.270	0.539	1								
SM	0.225	0.285	0.574	0.655	1							
SPA	0.246	0.144	0.258	0.281	0.308	1						
SU	0.297	0.198	0.301	0.314	0.328	0.487	1					
SK	0.105	0.138	0.195	0.168	0.218	0.169	0.119	1				
SH	0.386	0.168	0.272	0.279	0.299	0.429	0.475	0.139	1			
DAF	0.176	0.094	0.179	0.190	0.189	0.187	0.252	0.107	0.212	1		
SL	0.099	0.178	0.193	0.193	0.185	0.127	0.152	0.185	0.139	0.139	1	
SV	0.090	0.208	0.238	0.197	0.205	0.156	0.127	0.239	0.127	0.135	0.197	1

A dendrogram based on the Jaccard similarity coefficients of the five native Malaysian *Spathoglottis* species, five additional selected species, a hybrid and the outgroup *Dendrobium affine* was constructed with bootstrap values shown in Figure 4.3. The cophenetic correlation between the similarities coefficients derived from the dendrogram produced by UPGMA was 0.93 which corresponds to a very good fit. The *Spathoglottis* species were divided into two clusters at a similarity level of between 0.1 and 0.2 with 100% bootstrap value. Cluster 1 was divided into two groups, where Group 1 included *S. plicata alba, S. unguiculata,* the *Spathoglotis* hybrid and *S. plicata* (Malaysian species). Whereas, Group 2 included all the native Malaysian species of *S. aurea, S. gracilis, S. affinis* and *S. microchilina*. Literature report that the *S. plicata* exhibit purple flowers and *S. aurea, S. gracilis, S. affinis* and *S. microchilina* exhibit yellow flowers. The IRAP results clearly differentiated the samples by their flower colour hue. Cluster 2 included *S. kimballiana, S. vanoverberghii* and *S. lobii* where all species are not native to the Peninsular Malaysia. *Dendrobium affine* was on its own as

the outgroup for this study. This analysis is also supported by the results of the Principal Coordinate Analysis (PCoA) based on Jaccard similarity matrix as shown in Figure 4.4.

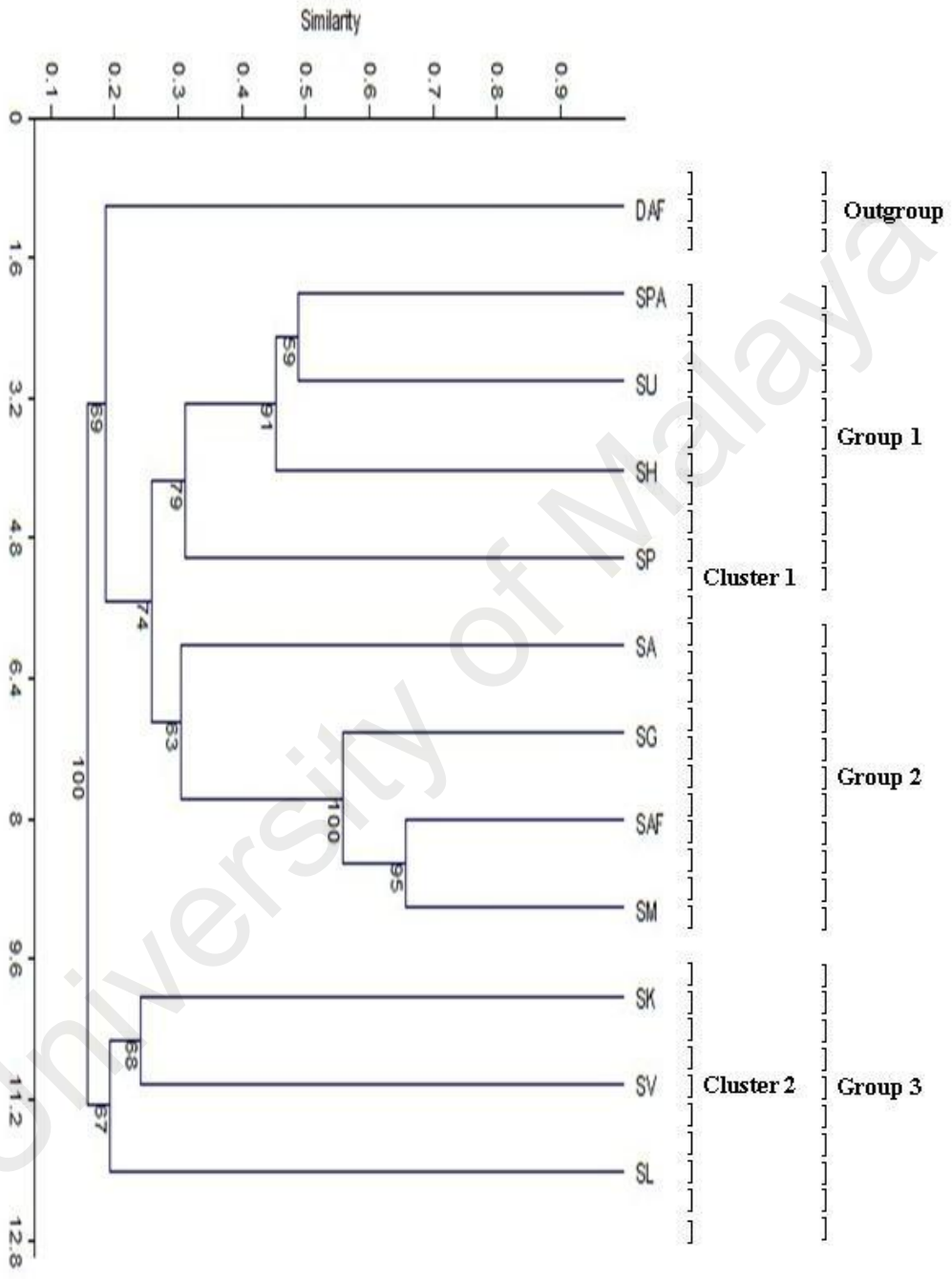


Figure 4.3: An unweighted pair-group method with arithmetic means (UPGMA) dendrogram of genetic relationship among five Malaysian native *Spathoglottis* species, five additional selected species, a hybrid and *Dendrobium affine* as outgroup based on Jaccard similarity coefficients from the IRAP data. Number next to branches is bootstrap value. Note: DAF: *Dendrobium affines*; SPA: *S. plicata alba*; SU: *S. unguiculata*, SH:*Spathoglottis* hybrid; SP: *S. plicata*; SA: *S. aurea*; SG: *S. gracilis*, SAF: *S. affinis*; SM: *S. microchilina*; SK: *S. kimballiana*; SV: *S. vanoverberghii*; SL: *S. lobbii*

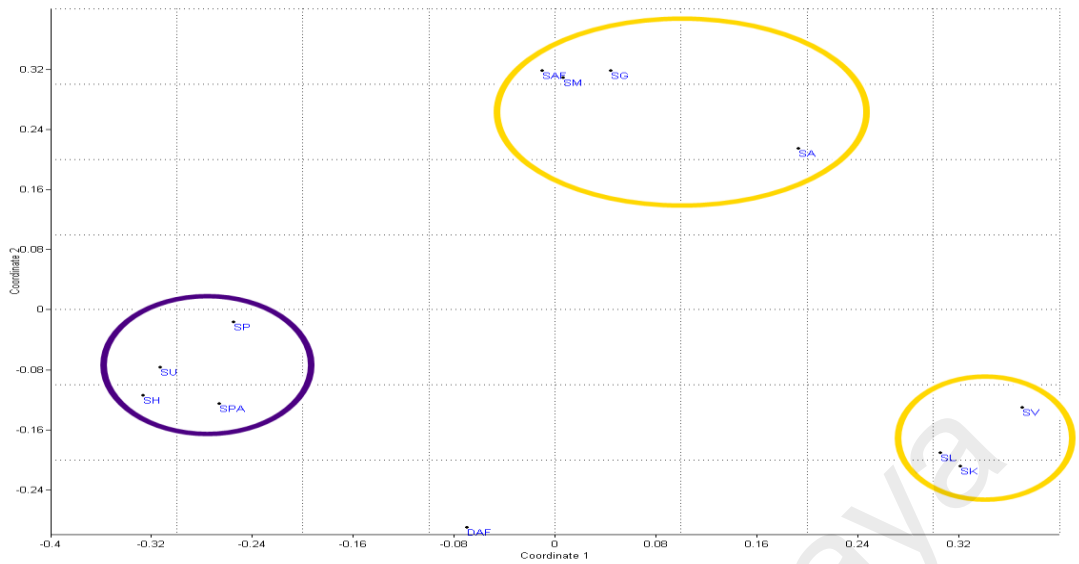


Figure 4.4: Relationships among the five Malaysian native *Spathoglottis* species, five additional selected species, a hybrid and a *Dendrobium affine* as outgroup visualized by Principal Coordinate analysis (PCoA) obtained by IRAP marker analyses.

4.5 DISCUSSION

There are about 40 known species of *Spathoglottis* in the world and these are widely distributed in Asia Pacific Regions. In Malaysia, particularly the Peninsular has only 6 recorded species. Differentiating the *Spathoglottis* species by morphology is quite difficult unless it has flowered. In this study, molecular markers were used to study the phylogenetic relationship between the five native Malaysian species, other selected species and one hybrid. IRAP techniques are chosen in this study as they are extremely polymorphic which could facilitate in the evaluation of inter and intra specific relationships, linkages, evolution and species or varietal determination.

The seven LTR-RTN primers derived from barley and two primers Reverse TY1 and Reverse TY2 designed facing outwards from the highly conserved RT priming sites of the banana Ty1-copia-like retrotransposon (Teo et al., 2005) were utilized in forty five combinations. Only twelve primer combinations gave successful amplification at annealing temperatures ranging from 40.4 ℃ to 46.7 ℃ (Table 4.2). This was within the temperature range of their use in other plant genomes such as *Musa* cultivars (40.0 ℃

and 48.4 ℃) (Teo et al., 2002; 2005) and *Crocus* species (42 ℃ and 53 ℃) (Alavi-Kia, 2008) and reiterated the transferability of IRAP primers across genus and the conserved nature of these sequences (Teo et al., 2005). The degree of polymorphism of the IRAP products using IRAP primer in *Spathoglottis* was high with 100% polymorphism. Similarly high levels of polymorphism was also reported in *Musa* cultivars using LTR6149 + Nikita and LTR6150 + Nikita primer combination (100%) and 3LTR + Nikita and LTR 6150 + 3'LTR (90%) (Teo et al., 2005). Analysis using Sukkula primer and LTR6149 + Sukkula of Old Portuguese bread wheat also revealed high polymorphism of approximately 95.5% and 96.3 % respectively (Carvalho et al., 2010).

Both BARE-1 and Sukkula, whether as single primers, or in combinations (BARE-1 and BARE-1, BARE-1 and Sukkula or BARE-1 and Nikita) produced clear fragments in *Spathoglottis* species. Less satisfactory amplification was observed with Nikita, Reverse TY 1 and Reverse TY 2 primers alone or with LTR primers combined with degenerate RT primers (data not shown). Kalender et al. (1999) reported that high numbers of amplified fragments revealed by retrotransposon markers are likely to be due to their high copy number in the genome. In *Crocus*, BARE-1 and Sukkula RTNs showed higher number of bands compared to Nikita (Alavi-Kia et al., 2008). However, in this study, BARE-1 (3'LTR) showed higher number of bands compared to Sukkula which may indicate a high copy number within the *Spathoglottis* genus. However, not all of the studied *Spathoglottis* species amplified high numbers of fragments using the same primer combinations. Analysis of *S. aurea* and *S. plicata* in many primers combination (e.g. 5'LTR2 + Nikita) showed fewer and less clear amplified bands compared to the other species. Interestingly the outgroup *Dendrobium affine* chosen for this study had the highest number of fragments for many of the primers tested.

Among the LTR primers, BARE-1, the 3'LTR and LTR6149, primers that were designed based on the 3' region of RTN-LTR were shown to independently amplify

several fragments indicating sufficient prevalence of related priming sites (Kalendar et al., 1999; 2000) but the LTR6149 primer did not amplify any fragments on *Spathoglottis*. The reverse primers of LTR 6150 which were designed based on the LTR of 5' region of the RTN could be amplified in *Spathoglottis*. However, primers 5'LTR1 and 5'LTR2 could not amplify any fragment. This may be due to the different frequencies of insertion of end-to-end or head-to-head RTNs and the difference sequence diversity and fitting of primers within the *Spathoglottis* genome. Nikita and Sukkula primers in combination with BARE-1 produced larger numbers of fragments in end-to-end and end-to-head position except for Nikita primer in combination with BARE-1 (LTR6150) which amplified lower number of fragments in the end to head position. The results generally showed that both single and combination of IRAP primers exhibited potential to be used for species differentiation of *Spathoglottis* species. It also confirmed the applicability of barley and banana RTNs for genome analysis for the *Spathoglottis* genus.

The dendrogram (Figure 4.3) and PCoA (Figure 4.4) results suggested a distinct grouping of the *Spathoglottis* tested into two colour groupings. The *Spathoglottis* of *S. plicata* and *S. unguiculata* was grouped into Group 1 both exhibit purple flowers. However, *S. plicata alba* has white flowers and the hybrid a mixture of purple colours. *S. plicata* and *S. plicata alba* in fact have similar floral characteristic with flower colour as the only observable morphological difference. It has been suggested that the latter is a variety derived from *S. plicata* Blume.

Group 2 clustered the species which had flowers in a range of yellow hues, all of which are wild natives of Peninsular Malaysia. Despite having the same yellow coloured flowers *S. aurea*, *S. gracilis*, *S. affinis* and *S. microchilina*, differ in other morphological characteristics in particular with respect to their lip structure (Ginibun et al., 2013). Interestingly Cluster 2 included *S. kimballiana, S. vanoverberghii* and *S. lobii*

which also had yellow flower colours but are located in different geographical locations not within Peninsular Malaysia. *Spathoglottis kimballiana* is a native species of Borneo, *S. vanoverberghii* a native species of the Island of Luzon, Philippines and *S. lobbii* a species native to Burma.

Not surprisingly, the results suggest that genetic relationships among species from Peninsular Malaysia and perhaps the neighbouring country of Thailand possess the same colour and are closer to that from different regions. The Principal Coordinate Analysis (PCoA) also supported the results shown in Figure 4.4.

Recent reports have reaffirmed that transposable elements play a significant role in shaping many plant species evolution and that both genomic as well as environmental influences can play a role in the proliferation of these plants (Kawakami et al., 2010; Kawakami et al., 2011). This could therefore also present an opportunity for further study using the IRAP markers to differentiate geographic origins. However, more samples from these areas would need to be analysed before confirmation of any association can be made. For a more detailed study on genetic relatedness and breeding analysis would also be useful to develop a native IRAP or other retrotransposon system based on the *Spathoglottis* genome.

Markers based on retrotransposons (IRAP-inter-retrotransposon-amplified polymorphism) generate great quantities of information, making them good tools for detecting genomic changes associated with their activity, because they create large and stable insertions in the genome; they are highly reproducible, show abundant polymorphism, and are easily viewed in a single gel (Kalendar et al., 2011).

4.6 CONCLUSION

This study confirmed the utility of Barley (*Hordeum vulgare*) and Banana *(Musa acuminata*) RTNs based markers for the analysis of the *Spathoglottis* species. Twelve single and combined IRAP primers: LTR 6150, 3'LTR, Sukkula, LTR 6149+Sukkula, LTR 6149+Nikita, LTR 6149+3'LTR, LTR 6150+Nikita, LTR 6150+3'LTR, 5'LTR1+3'LTR, 5'LTR2+3'LTR, 5'LTR2+Nikita and 3'LTR+Nikita had been successfully amplified and were able to be utilised for genetic analysis of the selected *Spathoglottis* species. This represents a useful first step towards elucidating the evolutionary relationships within this diverse genus and the utility of retrotransposons for this purpose. Due to the differences between LTR sequences of barley and genus *Spathoglottis*, cloning and sequencing of *Spathoglottis* RTNs for future RTN-marker based genome analysis will potentially add even more information for the characterization of this genus in addition to the optimized single and combination IRAP primers established in this study.

The study reaffirms the advantage of this marker system for use in species diversity studies for which no prior genetic information is available. Thus it will be a useful aid for future conservation work in tandem with the use of other PCR based markers such as REMAP and AFLP to determine the extent of biodiversity and the evolutionary relationships of the *Spathoglottis*. The current sample size per species is not sufficient enough to confirm the results. Therefore, in future studies, increasing the sample size is really important.

CHAPTER 5: PHYLOGENETIC ANALYSIS OF *SPATHOGLOTTIS* SPECIES USING CHLOROPLAST DNA

5.1 INTRODUCTION

The *Spathoglottis* species have been characterized and identified based on morphological characteristics in Chapter 3. However, a morphological feature alone is not always possible and sometimes even unreliable for taxonomic identification. This limitation has been one of the problems for taxonomist in differentiating between two or more species in the orchid genus. To overcome this problem, researchers have investigated alternative methods that can be used to identify different species in orchids. Molecular genetic techniques have facilitated in the study of orchid taxonomy and genetic relationships. The IRAP technique was studied in Chapter 4 and those markers were able to differentiate the *Spathoglottis* species.

To further advance the studies on characterisation of this species, the work described in this chapter is to evaluate DNA based identification using DNA barcoding to provide an alternative method for species identification. DNA barcoding is a molecular technique for identifying and describing species using a standard short genomic region. It has a universal application with a defined length of a standardized DNA sequence. Ginibun et al. (2010) has determined that chloroplast DNA (cpDNA) regions (i.e. *accD, matK, ndhJ, rpoB, rpoC1, ycf*5, *rbcL-a*, and *trnH-psbA*) in selected *Spathoglottis* species and has defined the DNA barcodes using the chloroplast regions. Four cpDNA regions (*matK, rpoB, rpoC1 and rbcL-a*) could be used as markers to identify the selected *Spathoglottis* species.

Chloroplast DNA sequences contain useful primary source of data which is widely utilized in plant molecular systemic studies. Therefore, in this study we aim to test the universality of the combinations cpDNA regions of *rpoB, rpoC1 and rbcL-a* to analyse

and assess the phylogenetic relationships among five native Malaysian *Spathoglottis* species, five species from other countries, a hybrid of *Spathoglottis* and an outgroup of *Dendrobium* by utilizing direct sequencing of the cpDNA regions.

5.2 LITERATURE REVIEW

There are many studies that have reported the complete DNA sequences for nuclear, mitochondrial and chloroplast genomes. The mitochondrial DNA region (Cytochrome Oxidase I gene) has been successfully used by researchers to develop the DNA barcoding system for animal diversity. However, it cannot be used in plants as their barcodes do not show sufficient variation (Saunders, 2005). Therefore, researchers have proposed the use of the chloroplast DNA markers as DNA barcodes (Kress & Erickson, 2007) since chloroplast can be found in all land plants.

Chloroplasts are organelles found in plant cells that conduct photosynthesis and is member of a class of the organelles known as plastids. Chloroplasts also contain one or more molecules of the small circular DNA. DNA barcoding can be rapidly and accurately used to identify a species just by using DNA sequences from a small fragment (Lahaye et al., 2008).

Chloroplast DNA sequence plays an important role as a genetic marker especially for use in molecular phylogenetic analysis. It is a primary source of data for plant molecular systematic studies and has been utilized for systematic studies in constructing plant phylogenetic models. This is because of their cpDNA and its ease of amplification and sequencing; and it has also a range in variability which provides useful phylogenetic characters (Soltis & Soltis, 1998). Shaw et al. (2005, 2007) reported that a few chloroplast regions are commonly used for phylogenetic studies and efforts have been made to determine the most variable domains. There are many different coding and non-coding regions in the chloroplast genome that have been used for molecular research,

particularly for phylogenetic analysis. Taberlet et al. (1991) recommended the use of universal primers in chloroplast genome across plant genera to study the species intra-specific variation. Since then, the approach has been widely adopted particularly the coding regions (such as *ycf*1, *trnl*, m*atK, rpoB, rpoC1 and rbcL-a*) and its combination have been successfully applied in many plant species such as Oryzeae tribe (Ge et al., 2002); *Oryza sativa* & *Oryza rufipogon* (Li et al., 2012); *Ligustrum* L. (*Oleaceae*) (Gu et al., 2011); *Tulipa* (Yanagisawa et al., 2012); and Orchidaceae (Neubig et al., 2009); and *Dendrobium* (Singh et al., 2012).

5.3 MATERIALS AND METHODS

5.3.1 Plant materials

Five *Spathoglottis* species native to Peninsular Malaysia, five additional species, a *Spathoglottis* hybrid (unknown origin) and the *Dendrobium affine* (as an outgroup) with five accessions of each plant species were used in this study. Collection of sample of said species are as described in Chapter 4.3.1.

5.3.2 DNA extraction

Genomic DNA of *Spathoglottis* was extracted from fresh leaf material according to the extraction method using Qiagen DNeasy plant mini kit (Qiagen, Germany) as described in Chapter 4.2.2.

5.3.3 Polymerase Chain Reaction (PCR) amplification

Polymerase Chain Reaction (PCR) was performed in a 10 µl mixture reaction containing 50 ng DNA, 1X PCR Buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs mix, 1 U Taq Polymerase (Promega, USA) and 10 µM of each primer. Bio Rad Thermal Cycler was used for the amplification. The PCR parameters consisted of 95 ℃, 3 min; 40 cycles of

94 ℃, 1 min; annealing at 50 to 55 ℃ (depending on the primer) for 30s; 72 ℃, 30s and the final extension was at 72 ℃ for 7 min. The annealing temperatures applied are shown in Table 5.1. The PCR products were analysed by means of electrophoresis on 1% (w/v) agarose gel and detected by Ethidium bromide (EtBr) staining. The gel was viewed under UV light using Alpha Imager$_{TM}$ 2200 transilluminator and the cpDNA banding pattern was viewed under the UV illuminator in a dark room in order to excise the selected band.

The excised gel with selected band was then transferred into 1.5ml microcentrifuge tube and sliced into smaller parts in the 1.5ml tube. The excised gel was extracted and purified using Gel Extraction Kit (AXYGEN, USA). The tube containing excised gel was centrifuged for 30 seconds at 12000x g. About 300µl of DE-A buffer was added and vortexes for a few seconds. The tube was then incubated at the 75 ℃ in the water bath for six to eight minutes until the gel dissolved. After incubation, 150µl DE-B buffer and 100µl isopropanol were added to the tube. The mixture was then transferred into the Axyprep column with new 2ml tubes and centrifuged at 12000 x g for 1 minute. The mixture was discarded and 500µl of W1 buffer was added and centrifuged again at 12000 x g for 30 seconds. The W1 buffer was discarded and 500µl W2 buffer was added, followed by centrifuging for 30 seconds at 12000 x g. The step was repeated with 700µl of W2 buffer and centrifuged at 12000 x g for 1 minute. The W2 buffer was discarded and the tube was centrifuged again for 1 minute (12000 x g). The Axyprep column was transfered into clean and new 1.5ml tube and finally 30µl of eluent was added to the centre of the membrane. The tube was incubated overnight at 4 ℃ and centrifuged at 12000 x g for 1 minute to obtain the extracted DNA fragment. The extracted DNA fragments were then analysed by electrophoresis on 1% (w/v) agarose gel and stained by EtBr.

Table 5.1: List of the cpDNA primers with the respective annealing temperatures and product sizes after amplification across the *Spathoglottis* species

Region	Name of primer	Sequence 5' – 3'	Annealing Temperature	Product Size (bp)
matK	matKF	CCTATCCATCTGGAAATCTTAG	50.0 ℃	891
	matKR	GTTCTAGCACAAGAAAGTCG		
RpoB	rpoBF	ATGCAACGTCAAGCAGTTCC	54.0 ℃	585
	rpoBR	GATCCCAGCATCACAATTCC		
rpoC1	rpoC1F	GTGGATACACTTCTTGATAATGG	51.7 ℃	589
	rpoC1R	TGAGAAAACATAAGTAAACGGGC		
rbcL-a	rbcL-aF	ATGTCACCACAAACAGAGACTAAAGC	54.6 ℃	698
	rbcL-aR	CTTCTGCTACAAATAAGAATCGATCTC		

5.3.4 Chloroplast DNA sequencing and analysis

After purification, the extracted DNA fragments from gel were sequenced using ABI 3730XL Automated sequencer. All sequences were checked and revised against chromatograph data using Chromas software (Technelysium Pty Ltd) to ensure high quality sequences and to remove uncertain and ambiguous bases. Successful amplified cpDNA sequences were then analysed using National Centre for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) program to confirm the identity of the amplified products from the cpDNA full sequence. After successfully analysing all the partial sequences, they were aligned using ClustaW (Thompson et al., 1997) with default parameters and trimmed at both ends to exclude any unsure sequences using MEGA 4 software version 4.1 (Tamura et al., 2007). The sequences were checked for any signs of unusual amino acid substitutions, stop codons and length mutations in protein-coding regions (Sorenson et al., 1998). After cleaning all the sequences, the software DnaSP v5 (Librado & Rozas, 2009) was used to summarise haplotype information. All sequences were then deposited and registered in GenBank databases in the NCBI under the accession numbers HM193249-HM193295 (Table 5.2).

The genetic distance matrix was calculated by the two-parameter method (Kimura, 1980) using MEGA based on the alignment sequences. Maximum likelihood model (Felsentein, 1981) was used to construct a phylogenetic tree. The interior branch tests were 1,000 replicates (Sitnikova et al., 1995) to test relative node support for the resulting phylogenetic tree using p-distance in the program MEGA.

Table 5.2: List of species, cpDNA regions and GenBank accession numbers (nucleotide sequences)

	Accession Number GenBank (NCBI)			
Species	***matK***	***rbcL-a***	***rpoB***	***rpoC1***
Dendrobium affine	HM193249	HM193260	HM193272	HM193284
Spathoglottis affinis	HM193250	HM193261	HM193273	HM193285
Spathoglottis aurea	HM193251	HM193262	HM193274	HM193286
Spathoglottis gracilis	HM193252	HM193263	HM193275	HM193287
Spathoglottis hybrid	HM193253	HM193264	HM193276	HM193288
Spathoglottis kimballiana	HM193254	HM193265	HM193277	HM193289
Spathoglottis lobbii	HM193255	HM193266	HM193278	HM193290
Spathoglottis microchilina	HM193256	HM193267	HM193279	HM193291
Spathoglottis plicata	HM193257	HM193268	HM193280	HM193292
Spathoglottis plicata alba	HM193258	HM193269	HM193281	HM193293
Spathoglottis unguiculata	HM193259	HM193270	HM193282	HM193294
Spathoglottis vanoverberghii		HM193271	HM193283	HM193295

Note: matK -maturase_K; rbcL-a - ribulose-1,5-bisphosphate_carboxylase; rpoB - RNA_polymerase_B; rpoC1 - RNA_polymerase_Beta_Subunit

5.4 RESULTS

5.4.1. Chloroplast DNA PCR amplification for *Spathoglottis* species

Ginibun et al., (2010) had successfully optimised the cpDNA primers and based on the result, further amplification was conducted across the five *Spathoglottis* species native to Peninsular Malaysia, five additional species, a *Spathoglottis* hybrid and the *Dendrobium affine* (as an outgroup) using only the four amplified primer pairs (i.e. *matK, rbcL-a, rpoC1* and *rpoB*). The four primer pairs were found to amplify across all the species by producing single bands and constructing DNA barcode for the species. The estimated product size of the tested PCR amplified cpDNA in *Spathoglottis* was scored by viewing on 1% agarose gel using the Alpha Imager™ 2200 (Appendix D). The obtained product size followed the standardized short sequences of DNA (400-800 bp).

Each *Spathoglottis* species was tested on the four primers pairs to ensure reproducible results. NCBI (http://www.ncbi.nlm.nih.gov) Basic Local Alignment Search Tool (BLAST) programme was used to analyze the obtained sequences and to confirm the four primers amplified on the target gene. The sequences of the amplified product of *S. plicata* using *matK, rbcL-a, rpoB* and *rpoC1* regions were analysed using BLAST. It gave 99% maximum identity on *matK* and *rbcL-a* regions, whereas the maximum identity scores for *rpoB* and *rpoC* were 98 and 97%, respectively. The results of the homology search using this program is shown in Appendix D. New sequence generated in the present study included 11 *matK* sequences, 12 *rbcl_a*, 12 *rpoB* and 12 *rpoC1*. In total 47 sequences were submitted to GenBank as listed in Table 5.2.

For subsequent analysis we used the three regions of *rbcL-a*, *rpoB* and *rpoC1* and its combinations. m*atK* was excluded due to inconsistent sequence quality and lack of species discrimination.

5.4.2 Haplotype analysis

The three regions of *rbcL-a*, *rpoB* and *rpoC1* were analysed individually and combined, where the haplotypes were constructed. In total, 28 intraspecific variable sites were detected within these three coding regions; *rbcL-a* (5), *rpoB* (11) and *rpoC1* (12). The variable site and the maximum number of cpDNA haplotypes constructed from the combination of three regions shown in Table 5.3. The haplotypes, a combination of nucleotide sites selected in a region was constructed to differentiate between species. A total of 8 haplotype groups were defined from the variable sites which were identified from the five *Spathoglottis* species native to Peninsular Malaysia, five additional species, a *Spathoglottis* hybrid and the *Dendrobium affine* (as an outgroup). The eight haplotypes formed the proposed structure for the DNA barcodes for the *Spathoglottis* species. *S. plicata* was designated as the reference haplotype and annotated as Haplotype 1. The *S. plicata var alba* and *S. unguiculata* share the same Haplotype 1 with *S. plicata*. The *S. gracilis* were solely found as Haplotype 2; whereas, *S. aurea* and *S. vanoverberghii* share the same Haplotype 3. The *S. microchilina* and *S. affinis* share the same Haplotype 6. However, no haplotype was shared among the other species. The *S. kimballiana* (Haplotype 4), *S. lobbii* (Haplotype 5), the hybrid (Haplotype 7) and the outgroup (Haplotype 8). The haplotype groups from the combination of cpDNA regions could clearly verify the results of the phylogenetic tree.

Table 5.3: The variable sites and the maximum number of chloroplast DNA haplotypes that can be constructed for the combination three regions using software DnaSP v5 (Librado & Rozas, 2009).

Species	rbcl_a					rpoB														rpoC1											Haplotype
	135	276	298	324	342	5	26	51	60	79	139	140	176	222	243	381	384	387	21	48	137	213	240	256	271	280	312	321	366	369	
SP1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1
SP2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1
SP3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1
SP4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1
SP5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1
SG1	.	T	.	.	G	.	.	.	T	.	.	.	.	C	A	G	.	.	.	.	.	.	.	.	T	C	.	.	.	.	2
SG2	.	T	.	.	G	.	.	.	T	.	.	.	.	C	A	G	.	.	.	.	.	.	.	.	T	C	.	.	.	.	2
SG3	.	T	.	.	G	.	.	.	T	.	.	.	.	C	A	G	.	.	.	.	.	.	.	.	T	C	.	.	.	.	2
SG4	.	T	.	.	G	.	.	.	T	.	.	.	.	C	A	G	.	.	.	.	.	.	.	.	T	C	.	.	.	.	2
SG5	.	T	.	.	G	.	.	.	T	.	.	.	.	C	A	G	.	.	.	.	.	.	.	.	T	C	.	.	.	.	2
SA1	.	T	.	.	G	.	.	.	T	.	.	.	.	C	A	G	.	.	.	.	G	.	.	.	T	C	.	.	.	.	3
SA2	.	T	.	.	G	.	.	.	T	.	.	.	.	C	A	G	.	.	.	.	G	.	.	.	T	C	.	.	.	.	3
SA3	.	T	.	.	G	.	.	.	T	.	.	.	.	C	A	G	.	.	.	.	G	.	.	.	T	C	.	.	.	.	3
SA4	.	T	.	.	G	.	.	.	T	.	.	.	.	C	A	G	.	.	.	.	G	.	.	.	T	C	.	.	.	.	3
SA5	.	T	.	.	G	.	.	.	T	.	.	.	.	C	A	G	.	.	.	.	G	.	.	.	T	C	.	.	.	.	3
SPA1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1
SPA2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1
SPA3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1
SPA4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1
SPA5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1
SK1	.	T	.	.	G	A	.	.	T	.	.	.	.	C	A	G	.	.	.	.	.	.	.	.	.	C	.	.	.	T	4
SK2	.	T	.	.	G	A	.	.	T	.	.	.	.	C	A	G	.	.	.	.	.	.	.	.	.	C	.	.	.	T	4
SK3	.	T	.	.	G	A	.	.	T	.	.	.	.	C	A	G	.	.	.	.	.	.	.	.	.	C	.	.	.	T	4
SK4	.	T	.	.	G	A	.	.	T	.	.	.	.	C	A	G	.	.	.	.	.	.	.	.	.	C	.	.	.	T	4
SK5	.	T	.	.	G	A	.	.	T	.	.	.	.	C	A	G	.	.	.	.	.	.	.	.	.	C	.	.	.	T	4
SU1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1
SU2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1
SU3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1
SU4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1
SU5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1
SL1	A	T	.	.	.	.	.	A	T	.	.	.	.	.	A	G	.	.	G	.	.	.	.	T	.	.	.	.	.	.	5
SL2	A	T	.	.	.	.	.	A	T	.	.	.	.	.	A	G	.	.	G	.	.	.	.	T	.	.	.	.	.	.	5
SL3	A	T	.	.	.	.	.	A	T	.	.	.	.	.	A	G	.	.	G	.	.	.	.	T	.	.	.	.	.	.	5
SL4	A	T	.	.	.	.	.	A	T	.	.	.	.	.	A	G	.	.	G	.	.	.	.	T	.	.	.	.	.	.	5
SL5	A	T	.	.	.	.	.	A	T	.	.	.	.	.	A	G	.	.	G	.	.	.	.	T	.	.	.	.	.	.	5
SM1	.	T	.	.	G	.	.	.	T	.	.	.	.	C	A	G	.	.	.	.	.	.	.	.	.	C	.	.	.	.	6
SM2	.	T	.	.	G	.	.	.	T	.	.	.	.	C	A	G	.	.	.	.	.	.	.	.	.	C	.	.	.	.	6
SM3	.	T	.	.	G	.	.	.	T	.	.	.	.	C	A	G	.	.	.	.	.	.	.	.	.	C	.	.	.	.	6
SM4	.	T	.	.	G	.	.	.	T	.	.	.	.	C	A	G	.	.	.	.	.	.	.	.	.	C	.	.	.	.	6
SM5	.	T	.	.	G	.	.	.	T	.	.	.	.	C	A	G	.	.	.	.	.	.	.	.	.	C	.	.	.	.	6
SAF1	.	T	.	.	G	.	.	.	T	.	.	.	.	C	A	G	.	.	.	.	.	.	.	.	.	C	.	.	.	.	6
SAF2	.	T	.	.	G	.	.	.	T	.	.	.	.	C	A	G	.	.	.	.	.	.	.	.	.	C	.	.	.	.	6
SAF3	.	T	.	.	G	.	.	.	T	.	.	.	.	C	A	G	.	.	.	.	.	.	.	.	.	C	.	.	.	.	6
SAF4	.	T	.	.	G	.	.	.	T	.	.	.	.	C	A	G	.	.	.	.	.	.	.	.	.	C	.	.	.	.	6
SAF5	.	T	.	.	G	.	.	.	T	.	.	.	.	C	A	G	.	.	.	.	.	.	.	.	.	C	.	.	.	.	6
SH1	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	7
SH2	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	7
SH3	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	7
SH4	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	7
SH5	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	7
DAF1	.	T	T	.	.	.	A	.	T	C	A	.	.	.	A	G	T	T	G	T	.	C	T	.	.	C	C	A	C	.	8
DAF2	.	T	T	.	.	.	A	.	T	C	A	.	.	.	A	G	T	T	G	T	.	C	T	.	.	C	C	A	C	.	8
DAF3	.	T	T	.	.	.	A	.	T	C	A	.	.	.	A	G	T	T	G	T	.	C	T	.	.	C	C	A	C	.	8
DAF4	.	T	T	.	.	.	A	.	T	C	A	.	.	.	A	G	T	T	G	T	.	C	T	.	.	C	C	A	C	.	8
DAF5	.	T	T	.	.	.	A	.	T	C	A	.	.	.	A	G	T	T	G	T	.	C	T	.	.	C	C	A	C	.	8
SV1	.	T	.	.	G	.	.	.	T	.	.	.	.	C	A	G	.	.	.	.	G	.	.	.	T	C	.	.	.	.	3
SV2	.	T	.	.	G	.	.	.	T	.	.	.	.	C	A	G	.	.	.	.	G	.	.	.	T	C	.	.	.	.	3
SV3	.	T	.	.	G	.	.	.	T	.	.	.	.	C	A	G	.	.	.	.	G	.	.	.	T	C	.	.	.	.	3
SV4	.	T	.	.	G	.	.	.	T	.	.	.	.	C	A	G	.	.	.	.	G	.	.	.	T	C	.	.	.	.	3
SV5	.	T	.	.	G	.	.	.	T	.	.	.	.	C	A	G	.	.	.	.	G	.	.	.	T	C	.	.	.	.	3

5.4.3 Phylogenetic analysis in *Spathoglottis* species

Individual data analyses of *rbcL-a*, *rpoB* and *rpoC1* regions were not strongly supported and incongruent pattern relationships were detected. Therefore, a combined analysis of all data was performed. However, only 44 data sequences were used to construct the phylogenetic tree, the other 16 sequences was excluded due to poor sequence quality while conducting the multiple alignments. The phylogenetic tree inferred from the combination regions sequences to reconstructed Maximum Likelihood method is shown in Figure 5.1. The dendrogram using UPGMA (shown in Appendix D) was also constructed using the combination of *rbcL-a*, *rpoB* and *rpoC1* regions for comparison. The maximum likelihood tree showed that all five native *Spathoglottis* species to Peninsular Malaysia, five additional species, a *Spathoglottis* hybrid except *S.lobbii* were belong to one monophyletic clades as Group 1. The combination regions separated the native species of Peninsular Malaysia clustered in Group 1 into 2 Sub Groups with 81% of bootstrap. Sub Group 1 included the *S. microchilina*, *S. affinis*, *S. aurea*, *S. gracilis*, *S. kimballiana* and *S. vanoverberghii*. The said species may closely related species where some of the accessions are located in the same clade as morphological characteristic belong to yellow colour of flower group. Wheare as, the *S. plicata*, *S. plicata alba*, *S. unguiculata* and the hybrid clades in Sub Group 2 belong to the purple colour group. The molecular data analysed show that the S*pathoglottis* species have a close relationship based on the morphological characteristic. However, there are some species accessions located in distant clades for example the *S. aurea* located in Sub Group 2, suggesting that there might be mixtures sample. The *S. lobbii* and *Dendrobium affine* (outgroup) stood alone in Groups 2 and 3 which was shown more distant related.

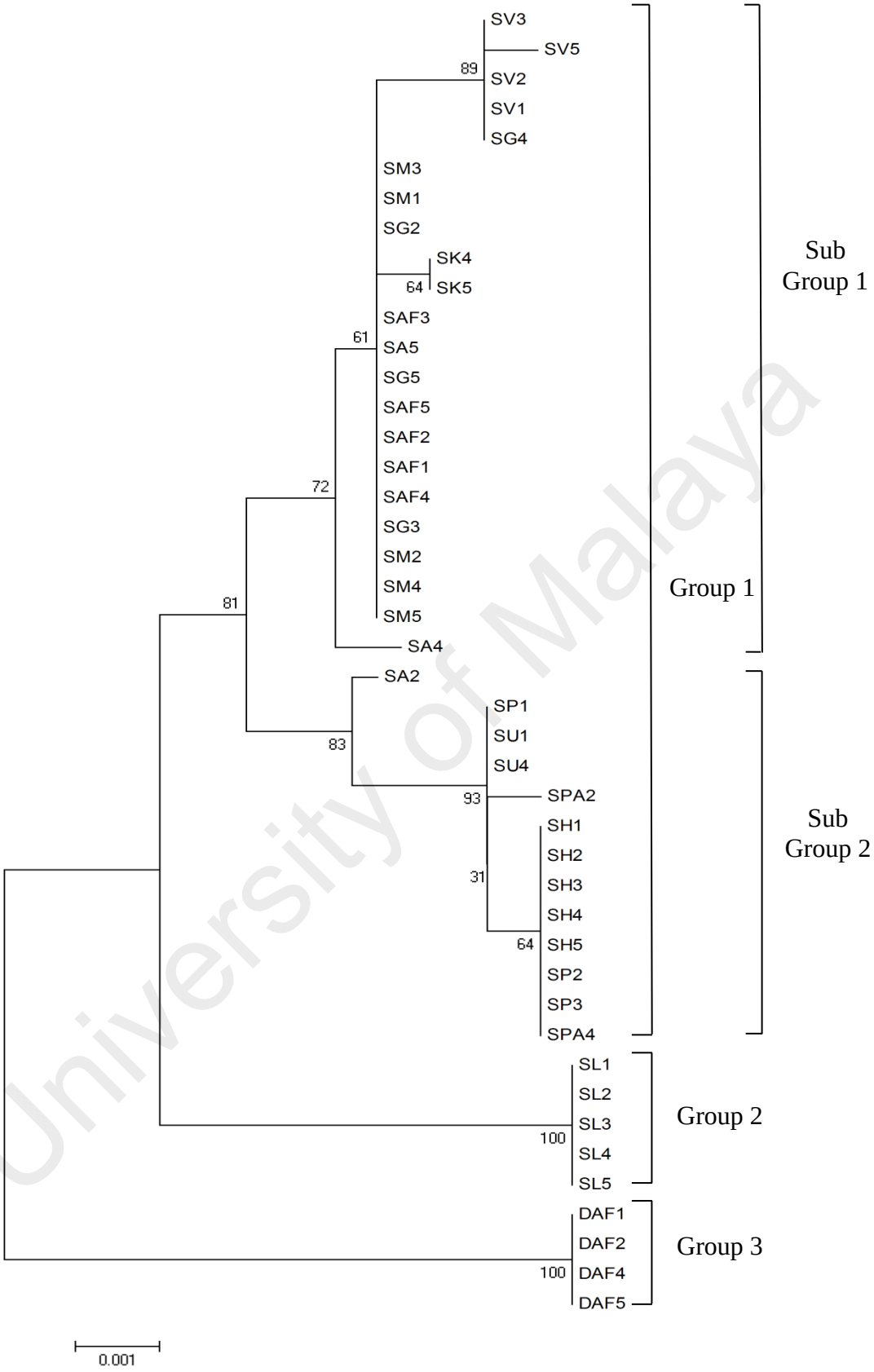


Figure 5.1: Phylogenetic tree contructed from the combination of *rbcL-a*, *rpoB* and *rpoC1* sequences of native *Spathoglottis* species, additional species, hybrid and *Dendrobium affine* (outgroup) using maximum likelihood. Numbers above nodes indicate bootstrap estimate for 1000 replications.

5.5 DISCUSSION

Chloroplast regions have been reported to have good potential to be used as DNA barcodes in plants (Chase et al., 2005). Chloroplast genomes of more than a hundred species have been sequenced and characterized and they have their own advantages in research. Its application on new plant species is much needed to search a suitable cpDNA region. Ginibun et al. (2010) had successfully amplified four coding genes (*matK*, *rbcL-a*, *rpoB* and *rpoC1*) to develop a chloroplast marker in *Spathoglottis* species with specific annealing temperatures, working concentrations and protocols for PCR amplification. According to Brown (1995), PCR reaction mixture and protocols are very important for amplification. Besides, the annealing temperatures, PCR can affect the specificity of the reaction because DNA-DNA hybridization is a temperature-dependent phenomenon.

In the CBOL Plant Working Group (2009) study, they have also utilised *matK*, *rbcL*, *rpoB* and *rpoC1* to identify standards for DNA Barcoding for land plants. Other research groups have proposed a combination of the loci for their preferred plant's barcodes (Lahaye et al., 2008, Neubig et al., 2009 and Singh et al., 2012). In this study, we combine the data from the rbcL-*a*, *rpoB* and *rpoC1* regions to conduct further analysis on phylogenetics across the five Malaysian native species and additional five selected species, a hybrid and an outgroup (*Dendrobium affine*).

In the previous study by Ginibun et al. (2010), it was found that the *matK* region was able to differentiate all the seven selected *Spathoglottis* species using the selected nucleotide sites (fifteen nucleotide positions) more than the other three regions. It reinforced the results of reports by Johnson and Soltis (1994) as well as Olmstead and Palmer (1994) where they found the rate of substitution in *matK* was three times higher at the nucleotide level and six times higher at the amino acid level than *rbcL*. Lahaye et al. (2008) also suggested that *rpoC1* exhibits the lowest intraspecific divergence

barcode compared to *matK*. A potential region that can be used as a barcode should have higher variable nucleotide sites. Therefore, *matK* showed the most potential among all the four selected regions, followed by *rpoB, rpoC1* and *rbcL-a*.

However, according to Neubig et al. (2009) it is a challenge with respect to plant molecular systematics to find DNA markers that are variable enough to provide resolution among genera and species. Although, *matK* was the most commonly used as DNA markers it is often not variable enough to provide a satisfactory outcome. Therefore, in this study we did not use *matK* regions due to inconsistency in sequence quality and they were found to be not variable enough for some of the *Spathoglottis* species. As reaffirmed by a few study, researcher suggested that *matk* may not be functional in some plant due to the rapid rate of substitution, rare presence of frameshift indels and a few cases of premature stop codons (Kores et al., 2000; Whitten et al., 2000; Kugita et al., 2003; Hidalgo et al., 2004; Janakowiak et al., 2004). Therefore, an important aspect in DNA barcoding is that each region should be able to discriminate species by their own individual resolution.

In single region analysis in this study, there were five nucleotide sites selected in the *rbcL-a* region, eleven for *rpoB* and twelve for *rpoC1*. We found that the individual resolution in each of the region was poor. The single region analysis was unable to distinctively separate the selected species. DNA barcodes could also be built by combining the multiple regions; hence, the three analysed single regions were combined with a total of 28 nucleotides sites for haplotype analysis. Haplotype is a particular combination of nucleotide sites selected in a region. The species differentiation in *Spathoglottis* could be seen clearly when the haplotype was constructed. The haplotypes of five *Spathoglottis* species native to Peninsular Malaysia, five additional species, a *Spathoglottis* hybrid (unknown origin) and the *Dendrobium affine* (as an outgroup) were constructed to clearly differentiate between the species. Fazekas et al. (2008) also

suggested that the combination of these multiple regions in other plants had improved the resolution compared to the individual resolution. Although, this study formed eight haplotypes but not all haplotypes could be proposed structure for the DNA barcodes of *Spathoglottis* species. Some of the species e.g. *S. aurea* and *S. vanoverberghii* are sharing their haplotype 3; and *S. microchilina* and *S. affinis* in haplotype 6. It shows that this species have their similarity and close related species although morphologically both have different shape of lip. Therefore, there is a need to further clarify this species in future.

Chloroplast DNA has been used extensively to infer plant phylogenies at different taxonomic levels. Direct sequencing of polymerase chain reaction (PCR) products is now becoming a rapidly expanding area of plant systematics and evolution (Clegg and Zurawski, 1991). A phylogenetic tree was obtained from comparisons of the combination regions sequences using maximum likelihood and UPGMA. Individual dendrogram tree for phylogenetic analysis using *rbcL-a* region, *rpoB* and for *rpoC1* cpDNA region was not able to show clear species differentiation. However, with the combination of the three cpDNA regions, more information on the genetic variability in the individual species needs to be clearly observed.

Studies by Gu et al. (2011) on species identification of *Ligustrum* L, reported that the used of the combination of *rbcL* and *mat*K showed the ability to improved in identify the species. The overview reported by Berg et al. (2005) on the phylogenetic relationship within the epidendroid orchids with emphasis on tribes Epidendreae and Arethuseae were assessed with parsimony and model-based analyses of individual and combined DNA sequence data by ITS, *trnL*, *matK* and *rbcL* regions. Several studies by Cameron et al. (1999); Cameron and Chase (1999); Freudenstein and Chase (2001); Freudenstein et al. (2004) have also obtained good resolution of relationships among orchid subfamilies with the combination of the cpDNA regions.

This study is very important in providing a possible tool that could be used in an effort to conserve the *Spathoglottis* species in Malaysia. The DNA barcoding for this species could be applied to distinguish its look-alikes and reduce ambiguity for identification of the species especially in trading. In the future, the system may play an important role in varietal protection for new hybrids and also for the protection of endangered species. For future research, more regions in the cpDNA should be studied to find the most unique region for each species. More *Spathoglottis* species should also be studied and the number of individuals for each species should be increased to make the results more precise and valuable. In addition, barcoding on other orchid genera should be studied as well. This study has shown that the DNA barcoding using the chloroplast region could be developed for species level identification and similarity in this orchid.

5.6 CONCLUSION

The coding regions of *matK*, *rbcL-a*, *rpoB* and *rpoC1* has successfully amplified the chloroplast DNA in the *Spathoglottis* species. This research data analysis for the four regions has proven that the chloroplast DNA markers have significant potential for DNA barcoding in the selected orchid species. The combination of *rbcL-a*, *rpoB* and *rpoC1* sequence had produced a higher resolution for identification and classification between the species. The DNA barcodes with present of 8 haplotypes numbers data arrangement define that some species are shared haplotype. It shows that the species maybe share a similar genetic relationship. However still we can conclude that coding sequences of chloroplast DNA are useful as genetic markers for phylogenetic analysis at species level of *Spathoglottis*. The phylogenetic analysis clearly revealed that the five Malaysian native species are in a similar group and where *S. plicata* is separated from

the *S. microchilina*, *S. affinis*, *S. aurea* and *S. gracilis*. The phylogenetic tree results revealed were similar with the IRAP analysis.

Nevertheless, to achieve a conclusive result, further studies have to be conducted with an extended number of samples of various species and more individuals of *Spathoglottis* species. This study can also help to establish the use of chloroplast DNA markers as a molecular tool to help in species identification in taxonomy at molecular level for applications in conservation, breeding and varietal protection.

CHAPTER 6: GENETIC ANALYSIS OF *SPATHOGLOTTIS* IN PENINSULAR MALAYSIA USING AMPLIFIED FRAGMENT LENGTH POLYMORPHISM (AFLP) MARKERS

6.1 INTRODUCTION

The uses of DNA as markers in elucidating the structural analysis of *Spathoglottis* species have an advantage in comparison to morphological markers. Agarwal et al. (2008) suggested that molecular markers, which are based on DNA polymorphism, could replace morphology markers, as molecular markers are more informative and independent of environmental condition. There are severeal molecular markers such as ISSR, RAPD, DNA barcode and AFLP that have been used in a broad range of plant species including *Spathoglottis* species (Romeida et al., 2012; Setiawan et al., 2013; Ginibun et al., 2010; Pimonrat et al., 2012). However, the usage of molecular markers types is depending on the interest that would benefit in identifying and clustering the *Spathoglottis* species based on their morphological information.

In Chapter 4 and 5, the IRAP and Chloroplast DNA markers were used to study the genetic relationship on *Spathoglottis* species with significant outcomes. Amplified fragment Length Polymorphism (AFLP) is one of the popular molecular marker techniques was studied in this Chapter 6. AFLP has its own advantages such as high reproducibility and high level of polymorphism which is widely distributed across the genome with no prior DNA sequence information needed compared to other DNA-based markers (Jones et al., 1997). It covers a broad area of the plants genome and produces more independent polymorphic loci in a single analysis compared to other molecular marker systems (Vos et al., 1995; Sathyanarayana et al., 2011).

AFLP markers have been successfully used in the assessments and determination of genetic diversity and genetic relationships between and within species and wild populations of a wide range of orchid species and hybrids such as *Phalaenopsis* (Chang

et al., 2009); *Diuris fragrantissima* (Smith et al., 2007); *Liparis loeselli* (Pillon et al., 2007); *Dendrobium* (Xiang et al., 2003); *Orchis mascula* and *Orchis purpurea* (Jacquemyn et al., 2009 and 2007); *Neotinea maculata* (Duffy et al., 2009); *Neotinea ustulata* (Tali et al., 2006) and *Himantoglossum hircinum* (Pfeifer and Jetschlee, 2006). However, there are only a few reports on molecular markers used to studies genetic relationship, genetic differentiation or genetic structure in the *Spathoglottis* species. Therefore, the aims of this study were to determine the level of genetic variability, as well as to elucidate and understand the genetic relationships between and within the *Spathoglottis* species based on AFLP markers. It will also enhance the understanding of the genetic variation that may occur in the existing wild *S. plicata* found distributed in different geographical regions in Peninsular Malaysia. This study will provide better insights on factors which contribute to the genetic variation and conservation of wild *S. plicata* population in future.

6.2 LITERATURE REVIEW

AFLP was developed and introduced by Vos et al. (1995) as a PCR-based molecular technique where selected restriction fragments from the digestion of total DNA are amplified by polymerase chain reaction (PCR). AFLP analysis uses selective amplification of a subset of restriction enzyme-digested DNA fragments to generate a unique fingerprint of a particular genome (Mueller & Wolfenbarger, 1999).

The procedure as originally described by Vos and Zabeau in 1993 was divided into three steps; (1) Digestion of total genomic DNA with one or more restriction enzymes of which the most often used was *Eco*RI and *Mse*I and ligation of restriction half-site specific adaptors to all restriction fragments; (2) Selective amplification of some of these fragments with two PCR primers that have corresponding adaptor and restriction

site specific sequences and (3) Electrophoretic separation of amplifications on a gel matrix, followed by visualisation of the band pattern.

AFLP is a dominant marker system like RAPDs and ISSRs expected to be scored as present and absent which could be an attractive alternative as co-dominant markers such as microsatellite/SSRs and SNPs (Jones et al., 1997; Belaj et al., 2003; Campbell et al., 2003; Bensch and Akesson, 2005). This marker has been widely applied to numerous genetic analyses in orchid species such as genetic variation, population structures and phylogenetic relationships.

AFLP was successfully determined the genomic variations among different cultivars of the Vandaceous orchid hybrids (Chen et al., 1999) and infer the relationships between closely related orchid species in *Dactylorhiza* (Hedren et al., 2001). It has also been applied to study the distribution of genetic variation within and between wild populations of many plant taxa like *Populus* (Arens et al., 1998; Winfield et al., 1998; Fay et al., 1999), *Phylica* (Richardson, 1999); *Pedicularis* (Schmidt and Jensen, 2000), *Medusagyne* and *Rothmannia* (Fay et al., 2000) and *Dactylorhiza* (Hedrén et al., 2001). Hence, AFLP was used to determine the genetic diversity, population structure and phylogenetic inference among Italian orchids of the *Serapias* genus (Sardaro et al., 2012) and to observe the pattern of population genetic structure in *Spiranthes romanzoffiana* (Forrest et al., 2004). This technique was also successfully used to identify and investigate genetic diversity the mutation induced by gamma irradiation of the *Spathoglottis plicata* Blume radiated clones with 1.89-29.85% polymorphism (Pimonrat et al., 2012).

6.3 MATERIALS AND METHODS

6.3.1 Plant materials

6.3.1.1 Sampling of *Spathoglottis* species

Sample of fresh leaves derived from five *Spathoglottis* species native to Peninsular Malaysia, five additional species, a hybrid of *Spathoglottis*, as well as an out group of *Dendrobium affine* with five individuals each and a total of 60 samples were used in this study as described in Chapter 5.2.1.

6.3.1.2 Sampling of *Spathoglottis plicata*

Our sampling consists of five wild populations of *Spathoglottis plicata* located in Peninsular Malaysia (Figure 6.1) and one population that is a vegetatively propagated *S. plicata* obtained from the Selangor Orchid Nursery as a control samples. A total of 25 to 30 individuals were sampled in each location by collecting leaf material. In total 172 accessions from the six populations were sampled (Table 6.1).

Table 6.1: Sampling location and number of accession of *Spathoglottis plicata* populations in Peninsular Malaysia

Location	Geographical Location	Altitude	Habitat	Accession
Simpang Pertang, Kuala Kelawang, Negeri Sembilan	2º56’48.09”N 102º18’07.39”E	356 ft	Roadside	30
Sekayu, Terengganu	4º58’07.19”N 102º57’ 18.96”E	247 ft	Open fields	29
Gunung Jerai, Kedah	5º47’12.23”N 100º25’ 59.69” E	3786 ft	Mountain foothills and edges of rocks	29
Ulu Sedeli, Johor	1º48’20.04”N 103º56’ 59.15”E	127 ft	Open field with loam soil	29
Genting Highland, Pahang	3º21’14.32”N 101º46’57.70”E	2553 ft	Mountain foothills	25
Selangor	3º05’0.9”N 101º40’46.61”E	134 ft	Propagated/cultivated	30

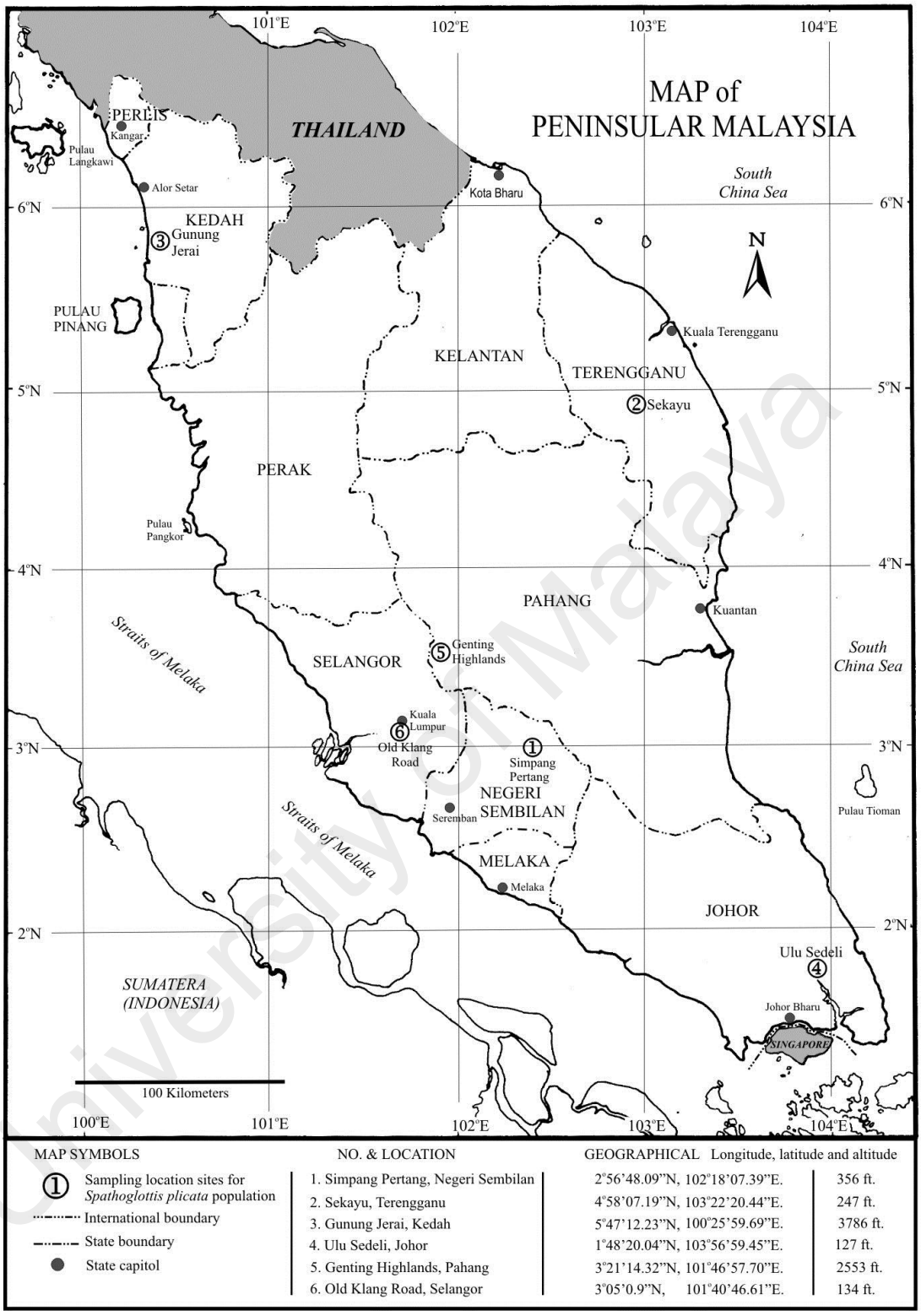


Figure 6.1: Map of sampling location for *Spathoglottis plicata* populations in Peninsular Malaysia

6.3.2 DNA extraction

Genomic DNA of the *Spathoglottis* was extracted from fresh leaf material according to the extraction method using Qiagen DNeasy plant mini kit (Qiagen, Germany) as described in Chapter 4.2.2. DNA concentration was measured using NanoDrop (ND-1000 Spectrophotometer, ISOGEN Lifescience). The quality was checked by electrophoresis on a 0.8% agarose gel in tris-borate-EDTA (TBE) buffer.

6.3.3 Amplified Fragment Length Polymorphism (AFLP) analysis

The AFLP method was conducted according to Vos et al. (1995) with some modifications. Genomic DNA (300ng) was digested with restriction enzymes *Eco*RI and *Mse*I (Biolegio B, Nijmegen, The Netherlands). *Eco*RI and *Mse*I adapters were ligated in a final volume of 50 µl. The reaction contained 0.05 RL-buffer, 10 mM ATP, 50 pMol MseI adapter, 5 pMol EcoR1 adapter, 5 U *Mse*I, 5 U *Eco*R1 and 1 U T4 DNA Ligase (Fermentas, USA). The reactions were gently mixed and incubated at 37 ℃ overnight. After restriction-ligation, the reaction mixture was diluted 10 times with TE buffer (10 mM Tris-HCl; 0.1 mM EDTA pH 8.0).

Pre-amplification was carried out in a final volume of 20 µl consisting of 30 ng unlabeled Eco-primer (*Eco*RI+A and *Eco*RI+C), 30 ng unlabeled Mse-primer (*Mse*I+C), 5 mM dNTPs (Invitrogen Life Technologies, Breda, The Netherlands), 1x Dream Taq Buffer, 0.4 U Dream Taq (Fermentas, USA) and 5 µl diluted restriction-ligation product. Polymerase Chain Reaction (PCR) was carried out in a Gene Amp PCR System 2700 (AB Applied Biosystems, USA) programmed at 94 ℃ for 5 min, followed by 24 cycles denaturation at 94 ℃ for 30 seconds, 56 ℃ annealing for 30 seconds, 72 ℃ extension for 60 seconds and final incubation at 72 ℃ for 7 min. The pre-amplification products were diluted 20 times in TE buffer and used as a template for selective amplification.

Selective amplification was carried out in 10 µl reaction volume containing 50 ng unlabeled Mse-primer, 1 pmol/µl IRD (700 or 800)-labelled Eco-primer, 5 mM dNTPs, 1 X Dream Taq buffer, 0.2 U/µl Dream Taq and 5 µl of diluted pre-amplification product.

The PCR profile was a touchdown PCR carried out in a Gene Amp PCR System 2700 (AB Applied Biosystems, USA) starting at 94 ℃ for 5 min, followed by 12 cycles denaturation at 94 ℃ for 30 seconds, 65-56 ℃ annealing for 30 seconds, with a decrease of 0.7 ℃ for each cycle, 72 ℃ extension for 60 seconds, and continued with 24 cycles at 94 ℃ for 30 seconds, 56 ℃ annealing for 30 seconds, 72 ℃ extension for 60 seconds and a final incubation at 72 ℃ for 7 min.

Meanwhile, the amplified fragments were separated on a 6.0% denaturing polyacrylamide gel (Long Ranger) mix with Tetramethylethylenediamine (TEMED) and 10% APS, followed by analysing them on a Li-Cor 4300 DNA analyzer (Li-Cor Biosciences, Lincoln, NE, USA).

A total of 56 AFLP primers were initially screened (Appendix E) and eight *Eco*RI + 3 bases/*Mse*I + 3 bases primer sets (namely, IRD700 E+CAC/M+CCG, IRD800 E+ACA/M+CAG, IRD800 E+ACA/M+CAT, IRD700 E+ACT/M+CAC, IRD700 E+ACT/M+CAG, IRD800 E+ACA/M+CTG, IRD700 E+ACT/M+CAA and IRD700 E+CAG/M+CCA) which showed clear scorable and highly polymorphic fragments (Table 6.2a) were selected for the fluorescent AFLP reactions to all the 60 samples of 10 *Spathoglottis* species, a hybrid of *Spathoglottis* and the outgroup of *Dendrobium affine*.

Whereas, about eleven *Eco*RI + 3 bases/*Mse*I + 3 bases primer sets (namely IRD800 E+AAG/M+CAA, IRD700 E+AAG/M+CCG, IRD800 E+ACA/M+CAG, IRD800 E+ACA/M+CAT, IRD700 E+ACA/M+CCT, IRD800 E+ACA/M+CTG, IRD700 E+ACT/M+CAA, IRD700 E+ACT/M+CAC, IRD700 E+ACT/M+CAG, IRD700

E+CAC/M+CCG and IRD700 E+CAG/M+CCA) which showed clear scorable and highly polymorphic fragments (Table 6.2b) were selected for the fluorescent AFLP reactions to all the 172 samples of six *Spathoglottis plicata* populations.

Table 6.2a: List of the eight *Eco*RI + 3 bases/*Mse*I + 3 bases primer sets generated for *Spathoglotttis* species

No	Fluorchrome label of Li-cor PCR	Selective Primer	
		***Eco*RI**	***Mse*I**
1	IRD800	ACA	CAG
2	IRD800	ACA	CAT
3	IRD800	ACA	CTG
4	IRD700	ACT	CAA
5	IRD700	ACT	CAC
6	IRD700	ACT	CAG
7	IRD700	CAC	CCG
8	IRD700	CAG	CCA

Table 6.2b: List of eleven *Eco*RI + 3 bases/*Mse*I + 3 bases primer sets generated for *Spathoglottis plicata* population

No.	Fluorchrome label of Li-Cor PCR	Selected Primer	
		***Eco*RI**	***Mse*I**
1	800	AAG	CAA
2	700	AAG	CCG
3	800	ACA	CAG
4	800	ACA	CAT
5	700	ACA	CCT
6	800	ACA	CTG
7	700	ACT	CAA
8	700	ACT	CAC
9	700	ACT	CAG
10	700	CAC	CCG
11	700	CAG	CCA

6.3.4 Data analysis

The AFLP profiles (polymorphic bands) were manually scored from Li-Cor Tiff images as presence (1) and absence (0) as band (binary) data ranging from 40 to 300 bp. The analysis of AFLP markers were based on the assumptions that the AFLP is a dominant marker, with dominant alleles coding at the present band at the given locus and a recessive null-allele coding for the absence of a band; and population at the hardy-Weinberg Equilibrium (HWE).

6.3.4.1 *Spathoglottis* species

The level of genetic distance and similarity between the *Spathoglottis* species samples were calculated using Paleontological Statistics (PAST) version 2.03 (Hammer et al., 2001). The results were further used to construct a dendrogram of the unweighted pair group method with arithmetic means (UPGMA) based on Dice index (Nei & Li, 1979) with 1000 bootstrapping values to estimate the reliability of the clustering pattern. The Dice similarities were used to compare two rows where a match is counted for all columns which presences in both rows. *M* used for the number of matches and *N* for the total number of columns with presence in just one row; $djk = 2M / (2M+N)$.

Meanwhile, analysis of molecular variance (AMOVA) was used to partition genetic variability within and among population the each species. AMOVA-PREP 1.01 (Miller, 1998) was used to prepare an AMOVA input file, while AMOVA 1.55 (Excoffier, 1995, 1993) was utilized for the analysis of the population genetic structure at the molecular level of the *Spathoglottis* species. The hierarchical genetic structure was assessed using AMOVA, where the total genetic diversity was partitioned between and among species, population and individuals within population in order to evaluate the differentiation.

As an alternative to AMOVA, the Bayesian approach from STRUCTURE 2.3.3 (Pritchard et al., 2010) program was used to examine whether species form a separate

clusters or species group, or whether hybrids are classified within or between these groups. STRUCTURE was also developed to study populations in which the individual samples are assumed to be able to exchange genetic material. Most likely during the STRUCTURE simulation, the individuals sample is assigned to populations or more population are combine together as if their genotypes indicate that they are admixtures (mixed ancestry). In the case of population structure configuration, estimations were obtained under the model of admixture using the correlated allele frequencies option of F model (analogous to $F_{ST,}$ Falush et al., 2003).

A pilot analysis was carried out on the AFLP data sets to portioning the genetic variation within and among the *Spathoglottis* species groups using STRUCTURE to test whether it was suitable to analyse the AFLP data. The number of inferred groups was evaluated at value of *K* ranging from 1 to 12, in which the maximum of *K*=12 corresponds to the number of samples species in *Spathoglottis* (including hybrid and outgroup). Three replicate runs were conducted for each value of *K*. The STRUCTURE computation was carried out with a length of the burn-in of 30,000 cycles and Markov Chain Monte Carlo (MCMC) data run of 100,000 cycles. The admixture model estimates the proportion of each accession genome that descended from each of the K inferred groups.

The model choice criterion that implemented in STRUCTURE was to detect the true *K* by estimate the posterior probability of the data for a given *K*, *Pr (X|K)* (Pritchard et al., 2000). In structure output, this value called 'Ln P(D)' which obtained by first computing the log likelihood of the data at each step of the MCMC. The value average was computed and half of their variance was subtracted to the mean. This gives 'Ln P(D)', the model choice criterion to which then refer as *L*(*K*). True number of populations (*K*) is often identified using the maximal value of *L*(*K*) returned by structure (Zeisset & Beebee, 2001; Ciofi et al., 2002; Vernesi et al., 2003; Hampton et al., 2004).

However, in most cases, simulation observed once the real *K* is reached, *L*(*K*) at larger *K*s plateaus or continues increasing slightly (a phenomenon mentioned in the structure's manual, Pritchard & Wen, 2010) and the variance between runs increases.

Estimating the *K* number of the ancestral genetic populations and the ancestry membership proportions of each individual in these clusters was based on Evanno method (Evanno et al., 2005). Different runs could produce different likelihood values and each data set was carried out to quantify the amount of variation of the likelihood for each *K*. The true number of *K* groups was determined by plotting the mean likelihood of *K* [*L(K)(±SD)*]; the rate of change of the likelihood distribution (mean ±SD) calculated as $L'(K) = L(K) - L(K-1)$; absolute values of the second order rate change of the likelihood distribution (mean ±SD) calculated according to the formula $|L''(K)| = |L'(K+1) - L'(K)|$; and Delta *K* calculated as $\Delta K = m|L''(K)|/s|L(K)|$.

6.3.4.2 *Spathoglottis plicata* population

The statistical analysis among and within six investigated *Spathoglottis plicata* population was also carried out using AMOVA, PAST version 2.03 and STRUCTURE 2.3.3 as describe in 6.3.4.1. The UPGMA dendrogram performed are based on Jaccard's coefficient of similarity for pairwise of all individuals of the six populations. The Structure computation was carried out in 5 replicate for each user-defined *K* value from 1 to 6 with a burn in period of 60,000 iterations and Markov Chain Monte Carlo (MCMC) sampling run of 300,000 iterations. The *K* value statistics analysis also explained in 6.4.3.1.

6.4 RESULTS

6.4.1 *Spathoglottis* species

6.4.1.1 Optimization AFLP primers combination for *Spathoglottis*

A total of 891 polymorphic bands out of 905 present bands ranging in sizes of 40 to 300 bp were scored using the eight selected primer combinations. Each AFLP profile of the *Spathoglottis* species, a hybrid and an outgroup of *Dendrobium affine* that generated by AFLP primer combinations of IRD800 *Eco*RI+ACA/*Mse*I+CAG are shown in Appendix E. The number of polymorphic bands scored for each primer pairs varied from 81 (IRD700 E+CAC/M+CCG) to 146 (IRD700 E+CAG/M+CCA) as shown in Table 6.3. The average number of the polymorphic bands detected was 111 per primer combination. Meanwhile, the average percentage of polymorphism was 98% and the range among the primer combinations was from 94% (IRD800 E+ACA/M+CAT) to 100% (IRD700 E+ACT/M+CAC).

Table 6.3: Polymorphic bands generated by eight AFLP primer combinations on *Spathoglotttis* species.

No	Fluorchrome label of Li-cor PCR	Selective Primer		Total present bands	No. of Variable bands	% Variable bands
		***Eco*RI**	***Mse*I**			
1	IRD800	*ACA	CAG	98	96	**97.95**
2	IRD800	ACA	CAT	107	101	**94.39**
3	IRD800	ACA	CTG	117	117	**100**
4	IRD700	ACT	CAA	125	123	**98.4**
5	IRD700	ACT	CAC	111	111	**100**
6	IRD700	ACT	CAG	117	116	**99.15**
7	IRD700	CAC	CCG	82	81	**98.78**
8	IRD700	CAG	CCA	148	146	**98.64**
Total				**905**	**891**	
Mean				**113.13**	**111.38**	**98.41**

6.4.1.2 Genetic variation among different species of *Spathoglottis*

A dendrogram for the five *Spathoglottis* species, five additional species, a hybrid of *Spathoglottis* and a *Dendrobium affine* (outgroup) was constructed according to the UPGMA analysis. The UPGMA dendrogram was generated from the AFLP data based on the Dice coefficient of similarity measures fitted the observed data well, as indicated by the high and significant cophenetic correlation coefficient (0.985). The dendrogram (Figure 6.2) revealed that the *Spathoglottis* species were clustered into two groups with a bootstrap value of 91% and forming at 34% of similarity level between groups. Group 1 contained *S. kimballiana*, *S. vanoverberghii*, *S. gracilis*, *S. affinis*, *S. microchilina* and *S. aurea* and further partitioned into two subgroups which were labelled as Subgroups A and Subgroup B. Subgroup A contained *S. kimballiana* and *S. vanoverberghii*, forming at 60% similarity level. Whereas Subgroup B contained *S. gracilis*, *S. affinis*, *S. microchilina* and *S. aurea* which the four species were reported to be natives to Peninsular Malaysia. The dendrogram show 72% similarities between the *S. aurea* with the three species of *S. gracilis*, *S. affinis*, *S. microchilina* which form 80% similarities.

Group 2 contained *S. plicata alba*, the hybrid of *Spathoglottis* (unknown), *S. plicata* and *S. unguiculata* in Subgroup C, forming of 60% similarity level, whereas *S. lobii* alone in Subgroup D. Subgroups C and D are supported by a 75% bootstrap value of the clustering. The *S. plicata alba*, the hybrid of *Spathoglottis* (unknown) and *S. plicata* are grouped at 64% similarities level but *S. plicata alba* separated from the hybrid of *Spathoglottis* (unknown) and *S. plicata* which both form 70% similarities. The *Dendrobium affinis* (outgroup) samples remains alone which it is not belonging to any of the groups.

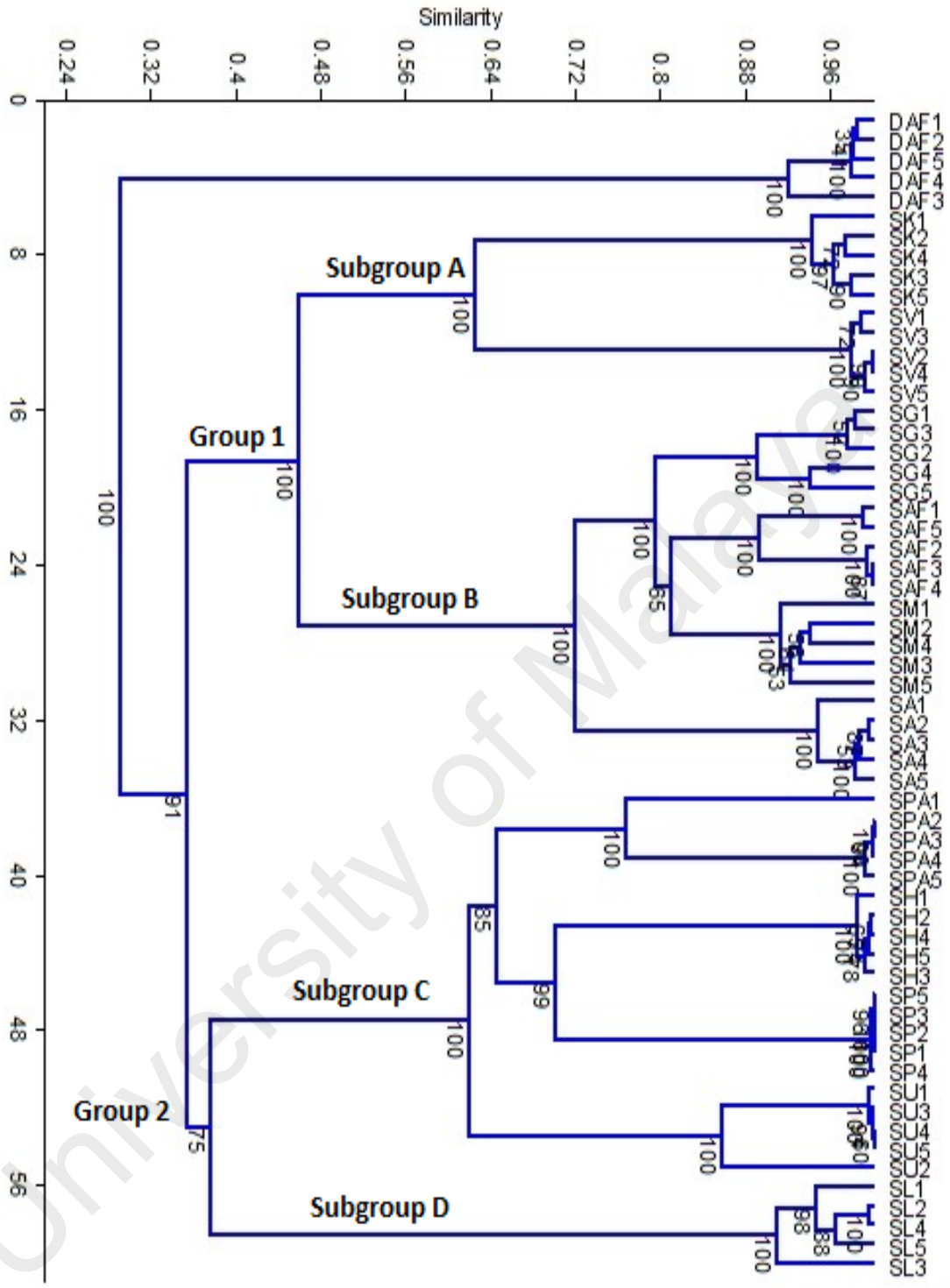


Figure 6.2: Dendrogram for the five Malaysian native *Spathoglottis* species, five additional selected species, a hybrid and *Dendrobium affine* (outgroup) generated by UPGMA based on AFLP data using the Dice coefficient of genetic similarity estimates (Nei & Li, 1979). Number on the branches is bootstrap value. (SP) *S. plicata*, (SA) *S. aurea*, (SG) *S. gracilis*, (SAF) *S. affinis*, (SM) *S. microchilina*, (SPA) *S. plicata alba*, (SU) *S.unguiculata*, (SK) *S. kimballiana*, (SL) *S. lobbii*, (SV) *S. vanoverberghii*, (SH) S. hybrid and (DAF) *D. affine.*

The total genetic diversity in *Spathoglottis* species was partitioned into two hierarchical levels, i.e., between species population and between individuals within populations (5 accessions of each species). The AMOVA results (Table 6.4) revealed that 91.1% of the molecular variance between the species population and 8.7% of the molecular variance was within the (species) populations. The molecular variance between (species) population detected was high (91.1%) and highly significant ($p<0.001$). It may indicate that the *Spathoglottis* species are relatively different in their genetic makeup. The genetic differentiation averaged over all loci amounted PHIst = 0.911. All the pair-wise genetic distances among the (species) population show values ranging from 0.600 to 0.988 (Table 6.5).

Table 6.4: Results of AMOVA for the AFLP data set based on eight primer combinations

Source	d.f	SS	MS	Variance	% variance	*P*
Among population	11	9190.58	835.51	163.92	91.14	P<0.001
Within population	48	764.40	15.93	15.93	8.86	P<0.001
Total	59	9954.98				

Table 6.5: Pairwise distances of eleven *Spathoglottis* species populations based on 891 loci

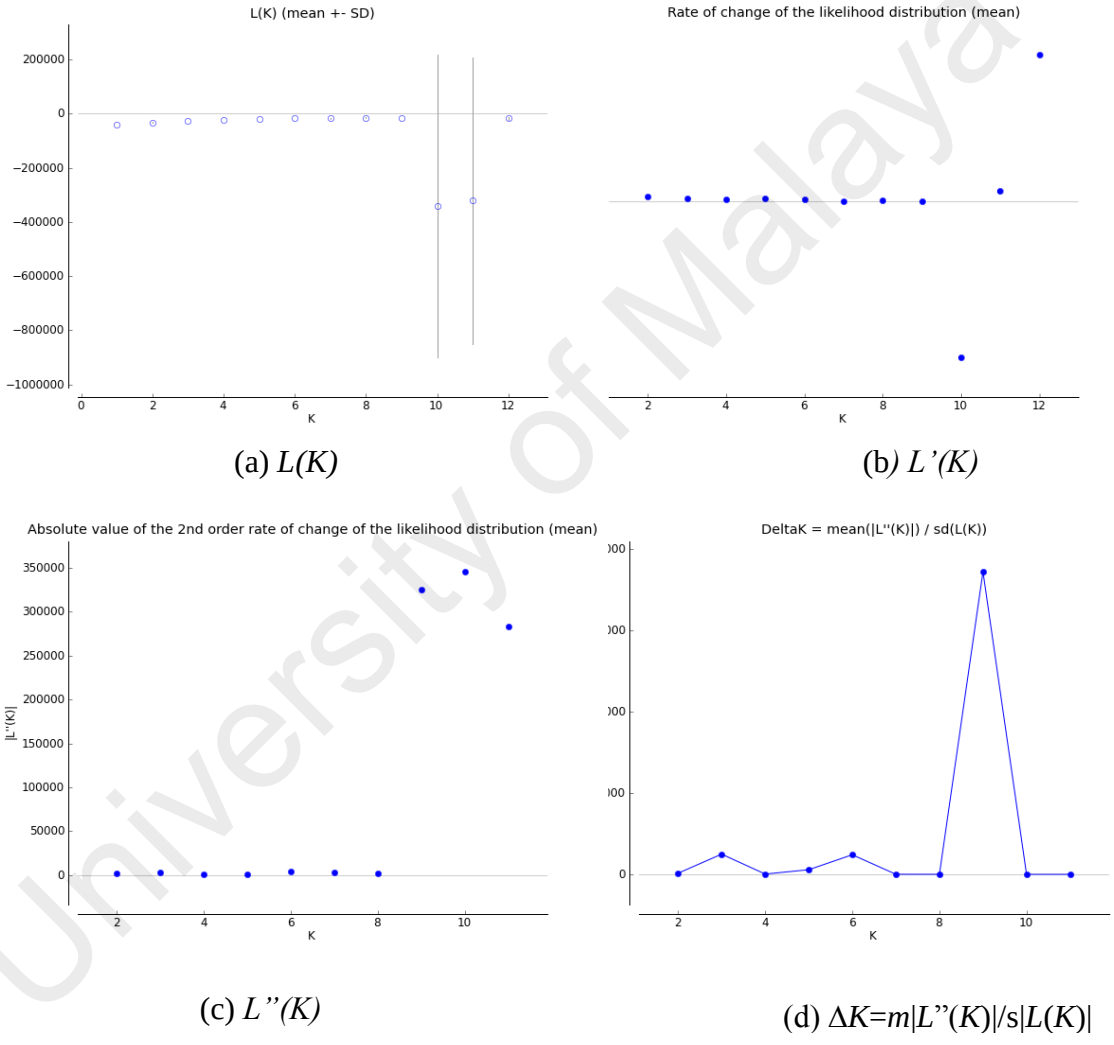
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
1. *S. plicata*		***	***	***	***	***	***	***	***	***	***	***
2. *S. aurea*	0.978		***	***	***	***	***	***	***	***	***	***
3. *S. gracilis*	0.942	0.797		***	***	***	***	***	***	***	***	***
4. *S. affinis*	0.953	0.824	0.661		***	***	***	***	***	***	***	***
5. *S. microchilina*	0.943	0.805	0.600	0.619		***	***	***	***	***	***	***
6. *S. plicata alba*	0.854	0.892	0.847	0.861	0.853		***	***	***	***	***	***
7. *S. unguiculata*	0.912	0.929	0.889	0.902	0.892	0.805		***	***	***	***	***
8. *S. kimballiana*	0.965	0.936	0.887	0.901	0.887	0.877	0.918		***	***	***	***
9. *S. lobii*	0.952	0.932	0.893	0.904	0.895	0.869	0.907	0.918		***	***	***
10. *S. vanoverberghii*	0.988	0.954	0.902	0.914	0.901	0.899	0.937	0.916	0.941		***	***
11. *S.* hybrid (unknown)	0.979	0.969	0.928	0.940	0.930	0.846	0.902	0.956	0.948	0.979		***
12. *Dend. affine* (outgroup)	0.971	0.949	0.910	0.921	0.913	0.885	0.923	0.937	0.920	0.957	0.965	

6.4.1.3 Bayesian structure analysis in *Spathoglottis* species

A Bayesian population clustering approach implemented in the STRUCTURE program was used to test separation into groups and the presence of admixture in accessions within *Spathoglottis* species. In this study, we used Evanno et al. (2005) method by plotting the graph to observe the optimal number of *K*. The four steps of graphical method allow the detection through number of group *K* for *Spathoglottis* species over three runs show in Figure 6.3a-d. Although, the Figure 6.3d show an obvious peak of *K*=9 but it may not the optimal point for this study. This is because the calculation of delta*K* for *K*=9 involve subtracting to *K*=10 which show high standard deviation (Table E1 in Appendix E). The standard deviation causes inflation of delta*K* for *K*=9, therefore it shown a false signal. In order to inference the true *K*, we used the nonparametric test. The true number population (*K*) identified by using the maximal value *L(K)* returned by STRUCTURE. According to Rosenberg et al. (2001), when *K* is approaching a true value, *L(K)* plateus (or continues increasing slightly) and has high variation between runs. Through observation on graph Figure 6.3a, we could estimate that the best *K* value was at *K*=6. The estimation of the probability function of Ln P(D) for runs with *K*=6 show the consistency improvement provide strong support for the species group. However, K>6 did not run consistently. At *K*=6, the clustering results were stable and most clusters had the same composition in all the three replicate runs. Therefore, in this study, *K*=6 [Ln P(D)] = -16158.4) was taken as the optimal *K*.

The estimated population structure of the three run at K=6 is shown in Figure 6.4. The species group were distinguished largely in line with the species group in dendrogram. Each individual accession is presented by a thin vertical line. The line shows coloured segments that represent the relative percentage of membership to the *K* clusters. *Spathoglottis aurea*, *S. affinis*, S. *gracilis* and *S. microchilina* which are clustered as one, with *S. kimballiana* and *S. vanoverberghii* in one cluster, while *S.*

plicata, *S. unguiculata* and the hybrid in another cluster. *Spathoglottis lobbii* and *S. plicata alba* and also the *Dendrobium affine* (outgroup) are assigned to one STRUCTURE cluster each.

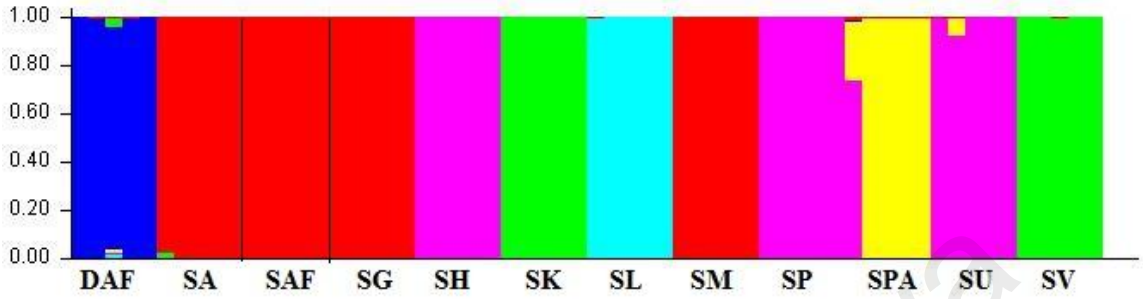


(a) *L(K)* (b) *L'(K)*

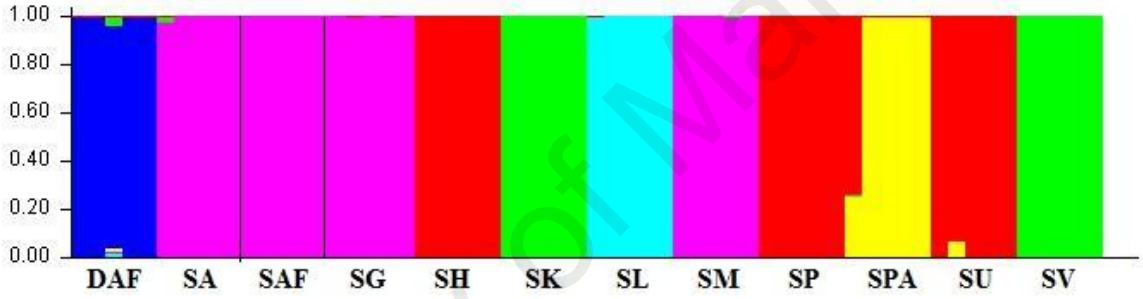
(c) *L''(K)* (d) $\Delta K=m|L''(K)|/s|L(K)|$

Figure 6.3a-d: Description of the four steps for the graphical method allowing detection of the true number of group *K*=6 for *Spathoglottis* species. (a) Mean [*L(K)*(±SD)] over three runs for each *K* value. (Hierarchical Island Model). (b) Rate of change of the likelihood distribution (mean ±SD) *L'(K)*. (c) Absolute values of the second order rate change of the likelihood distribution (mean ±SD) |*L''(K)*|. (d) Delta *K* calculated as $\Delta K=m|L''(K)|/s|L(K)|$. The model value of this distribution is the true K or the uppermost level of structure here is six clusters.

Run 1



Run 2



Run 3

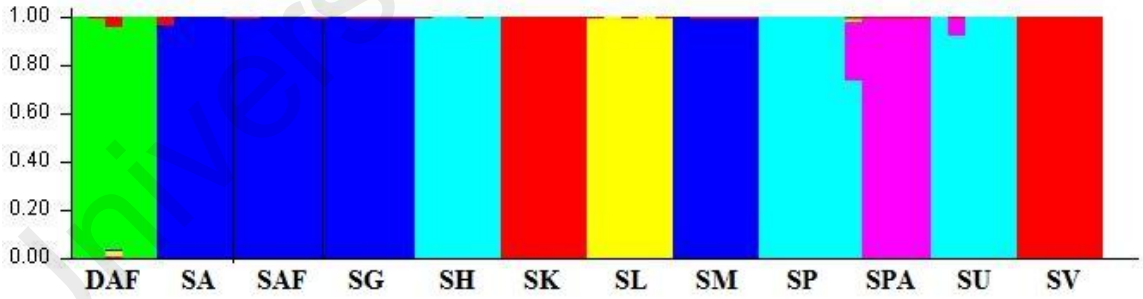


Figure 6.4: STUCTURE analysis of *Spathoglottis* species population. Estimated population structure at *K*= 6. Each accession is represented by thin line which is partitioned in *K* coloured segment that represents the membership to *K* clusters. Label below indicates the species. (DAF) *D. affine*, (SA) *S. aurea*, (SAF) *S. affinis*, (SG) *S. gracilis*, (SH) S. hybrid, (SK) *S. kimballiana*, (SL) *S. lobbii*, (SM) *S. microchilina*, (SP) *S. plicata*, (SPA) *S. plicata alba*, (SU) *S.unguiculata*, (SV) *S. vanoverberghii.*

6.4.2 *Spathoglottis plicata* population

6.4.2.1 Genetic diversity and differentiation analyses

The eleven AFLP primer combinations screened have the potential to capture the genetic variation within and between populations; and sufficient to distinguish clearly all the individuals from the six *S. plicata* populations of Peninsular Malaysia. It resulted in a total of 279 unambiguous scorable polymorphic bands among the 172 *S. plicata* individuals from 691 total bands (Table 6.6a) which provide clear amplification profiles. The polymorphic band either within or among populations was scored in between 40 to 300 bp for each primer pairs which varied from 16 (IRD 700 E+CAC/M+CCG) to 30 (IRD 700 E+CAG/M+CCA).

AMOVA was conducted on the *Spathoglottis plicata* population at two hierarchical levels, the proportions of genetic variation attributable to among population higher that within population. The AMOVA results (Table 6.6b) revealed that 78.5% of molecular variance among populations, but within population was 21.5%. The genetic differentiation between the population averaged overall loci amounted to (PHIst = 0.785) and was highly significant ($P<0.001$). All pairwise genetic distances between populations were significant (P value) and values ranged from 0.3975 to 0.9953 as shown in Table 6.6c. It was indicated that the six populations are relatively different and partly similar in their genetic makeup observed.

Table 6.6a**:** Primer combination (only showing the three 3'selective nucleotides) used in AFLP and the scored number of polymorphic bands in five wild and one propagated *S. plicata* populations

No.	Fluorchrome label of Li-Cor PCR	Selected Primer		Variable bands	Total present bands	% variable bands
		*Eco*RI	*Mse*I			
1	800	AAG	CAA	26	58	44.83
2	700	AAG	CCG	26	40	65
3	800	ACA	CAG	18	50	36
4	800	ACA	CAT	27	80	33.75
5	700	ACA	CCT	26	58	44.83
6	800	ACA	CTG	24	67	35.82
7	700	ACT	CAA	29	94	30.20
8	700	ACT	CAC	29	75	38.67
9	700	ACT	CAG	28	67	41.79
10	700	CAC	CCG	16	34	47.06
11	700	CAG	CCA	30	68	44.11
Total variable bands				279	691	

Table 6.6b: Analysis of Molecular Variance (AMOVA) based on 279 AFLP loci in five wild and one propagated *S. plicata* populations

Source	df	SS	MS	Est. Var.	% var	*P*
Among populations	5	6885.396	1377.079	47.616	78.51%	<0.001
Within populations	166	2163.552	13.033	13.033	21.49%	<0.001
Total	171	9048.948				

Table 6.6c: Pairwise genetic distances (PHIst) among *S. plicata* populations

	SPN9	SPT	SPK	SPJ	SPP	SPS
SPN9	0.0000					
SPT	0.5178	0.0000				
SPK	0.9753	0.5774	0.0000			
SPJ	0.7161	0.5180	0.9884	0.0000		
SPP	0.6991	0.3975	0.7953	0.7197	0.0000	
SPS	0.9779	0.6608	0.9862	0.9953	0.7807	0.0000

Note: SPN9, *S. plicata* Negeri Sembilan; SPT, *S. plicata* Terengganu; SPK, *S. plicata* Kedah; SPJ, *S. plicata* Johor; SPP, *S. plicata* Pahang; SPS, *S. plicata* Selangor.

6.4.2.2 Genetic structure of selected *S. plicata* populations

The AMOVA statistical analyses showed significant differences between the six populations. The pairwise genetic distance between population are recorded in Table 6.6c. The UPGMA dendrogram generated with Nei's (1978) unbiased distance values (Appendix E). UPGMA results indicated three possible subclusters in which Negeri Sembilan and Johor populations are grouped together; whereas Kedah clustered as one subgroup together with the Selangor population and Terengganu and Pahang clustered as a group with possible genetic admixture. The AFLP molecular data were further analyse with constructing the UPGMA dendrogram between individuals within populations for the six *Spathoglottis plicata* populations based on Jaccard coefficient similarity for pairwise using PAST software. The corphenetic correlation based on Jaccard similarity measures is 0.9835. The individuals are names according to their State location. The results used UPGMA dendrogram revealed that there are two distinct clades that shows a possibility which clades arise due to the genetic distribution between the populations (Figure 6.5). Clades 1 consist of Selangor population; Kedah, Terengganu and Pahang populations that are grouped together. Clades 2 consist of Negeri Sembilan, Johor, Terengganu and Pahang. These could probably due to geographically separation by the Titiwangsa Range; with the Kedah population representing the Northern population and Terengganu and Pahang representing the Eastern population. One of the clades is further subdivided into two distinct clusters that show the populations could have diverged recently, for which Negeri Sembilan and Johor is clustered together, representing the southern region, whereas Terengganu and Pahang are representing the eastern region.

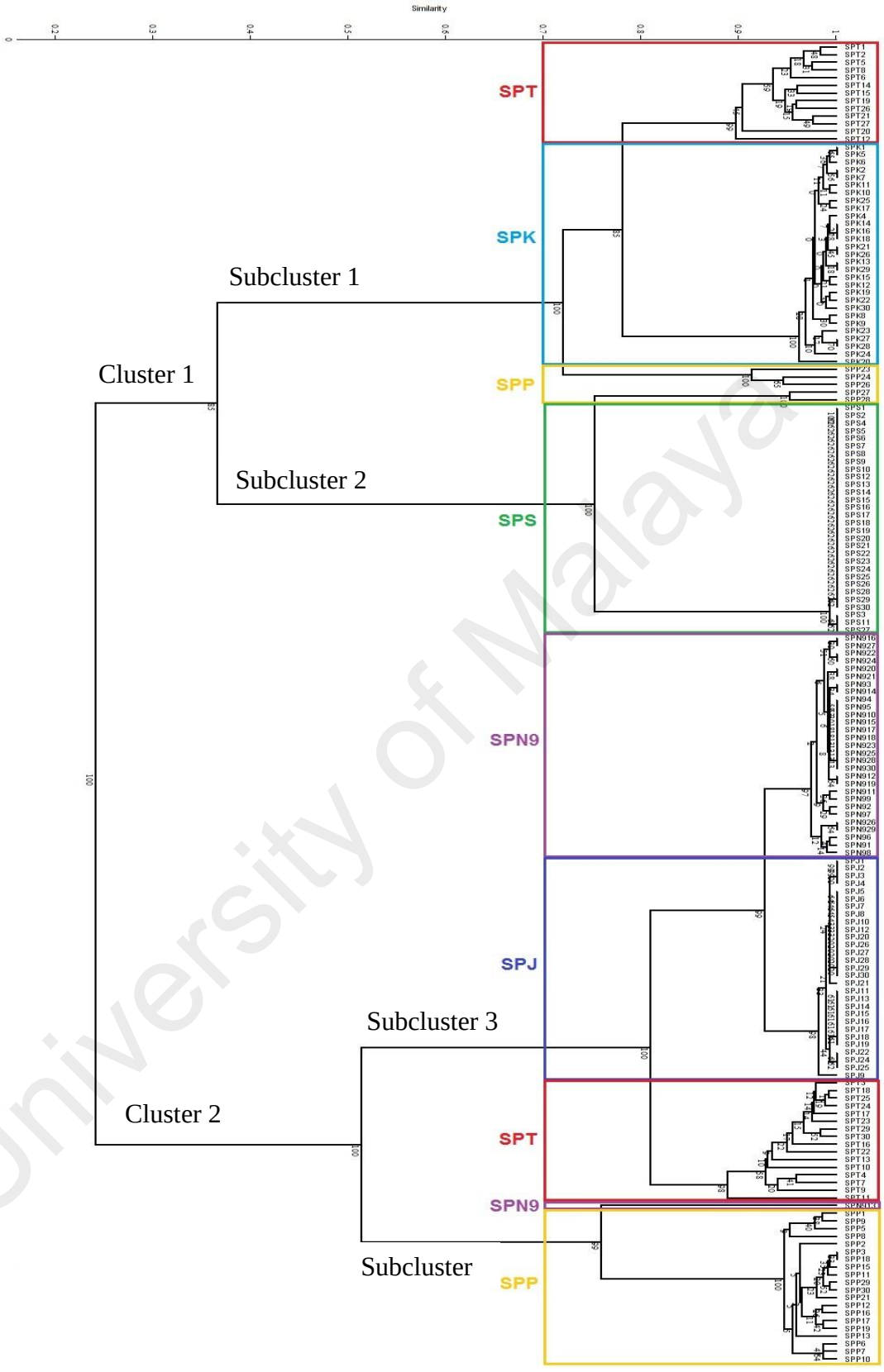


Figure 6.5: UPGMA dendrogram are based on Jaccard's coeffient of similarity for pairwise of all individuals of the six *Spathoglottis plicata* populations. The individuals are names according to their State localization.

Inspiringly, both eastern region (Pahang and Terengganu) populations were present in both clusters. Therefore, a further analysis with AMOVA was conducted to observe the variation between the populations. The detailed analyses of AMOVA and cluster analysis showed in Table 6.7. These populations also show significantly different with P<0.001. The molecular variance between the two populations of Terengganu and Pahang populations was 39.75%, but within populations the molecular variance variation was 60.25%. The genetic differentiation averaged overall loci amounted to PHIst = 0.397. It indicate that the variation were high within Terengganu and Pahang populations if compare between these two populations. Thus, in the aspect of conservation planning, it is important to understand the genetic variation in detail between individuals of these two populations as there are high variations within Terengganu and Pahang populations.

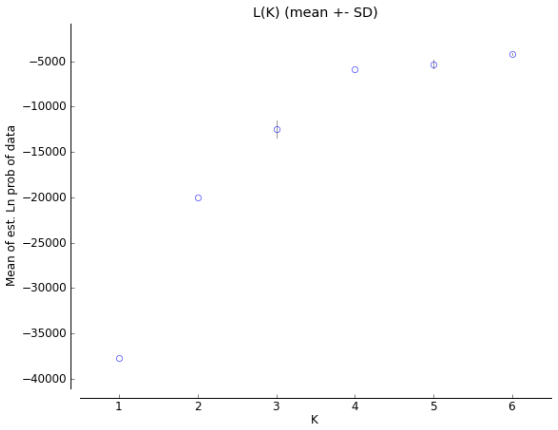
Table 6.7. Analysis of molecular variance (AMOVA) based on 53 AFLP loci in two variable *S. plicata* populations of Terengganu and Pahang.

Source	df	SS	MS	Est. Var.	% var	*P*
among populations	1	725.9446	725.945	25.59057707900	39.75%	<0.0010
within populations	52	2017.0924	38.790	38.79023872700	60.25%	<0.0010
Total	53	2743.0370				

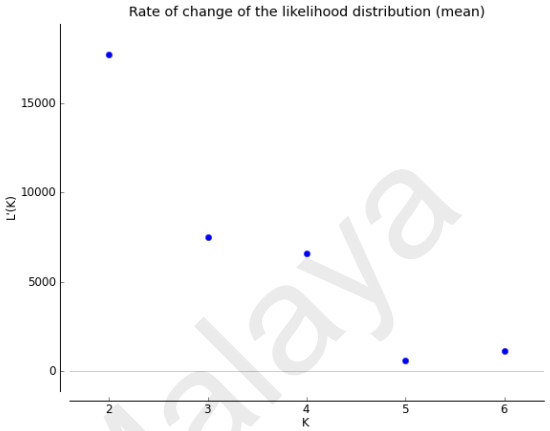
To further describe their genetic relationships among populations the computer program STRUCTURE could be the most frequently used tools. Detailed classifications between the six populations of *S. plicata* population accession were investigated in Bayesian analysis using STRUCTURE 2.3.3. The number of inferred group was evaluated at value *K* ranging from 1 to 6, which correspond with number of population. The STRUCTURE analysis describe the four steps graphical Evanno method which allowing detection of the true number of group K shown in Figure 6.6a-d. Results show

two higher peak in the graph which indicate the structure at *K*=2 and substructure at *K*=4.

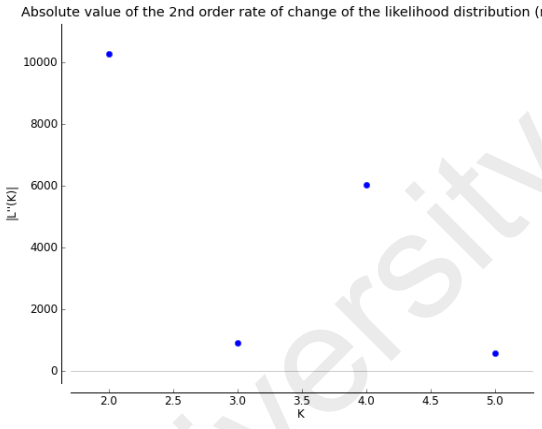
Although the Evanno structure output highlighted the *K* value is 2 and delta*K* graph pattern in Figure 6.6d show that *K*=2 was the highest peak but true *K* value of 4 was selected. According to Evanno et al. (2005), the distribution of delta*K* almost always showed a mode at the real *K* but this in the case of *Spathoglottis plicata* population analysis the graph pattern plot the peak of *K*=4. Their distribution of *L*(*K*) may show an accurate mode for the true *K* (Figure 6.6a). The estimation of the probability function of Ln P(D) for runs with *K*=4 show the consistency improvement provide strong support for the population group. However, *K*>4 did not run consistently. At *K*=4, the clustering results were stable and most clusters had the same composition in all the five replicate runs. The run with the highest probability for *K*=4 [Ln P(D)]= -5900.6] was most cluster defined by STRUCTURE for *K*=4 are the same in all 5 runs and taken as the optimal *K*. The estimated population structure at *K*=4 and its three replicate shown in Figure 6.7 and the other two in Appendix E. It is possible to detect *S. plicata* population using 279 AFLP loci in this study. The Negeri Sembilan and Johor populations indicated the same genetic groups. The Kedah and Selangor populations represent two difference genetic groups. Interestingly, the Terengganu have a mixture of genetic groups from both the Northen and southern region. Whereas, Pahang populations show three mixtures of genotype of it own individual and shared with Kedah and Selangor genotypes. The results show that there are four genotypes occurred from the population sample that sampling from a different geographical region which is Northen (Kedah), Southern (Negeri Sembilan and Johor), Central (Selangor) and Eastern (Pahang) region.



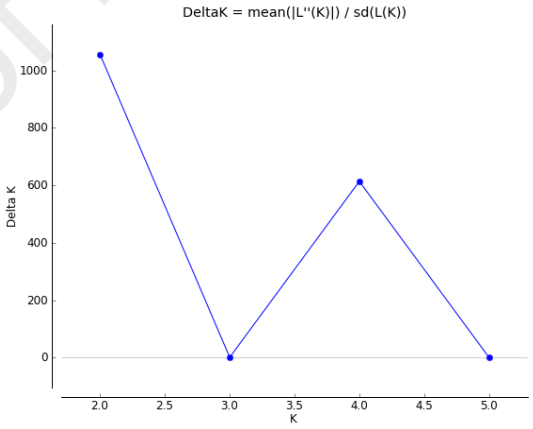
(a) *L*(*K*)



(b) *L'*(*K*)



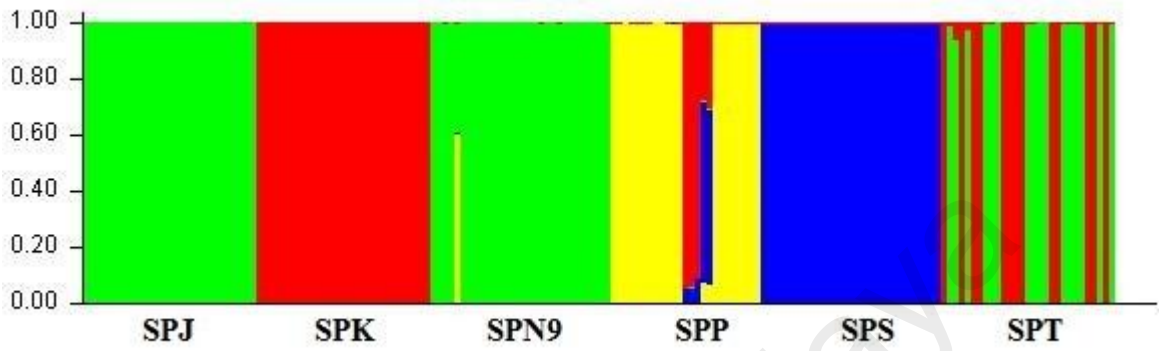
(c) *L''(K)*



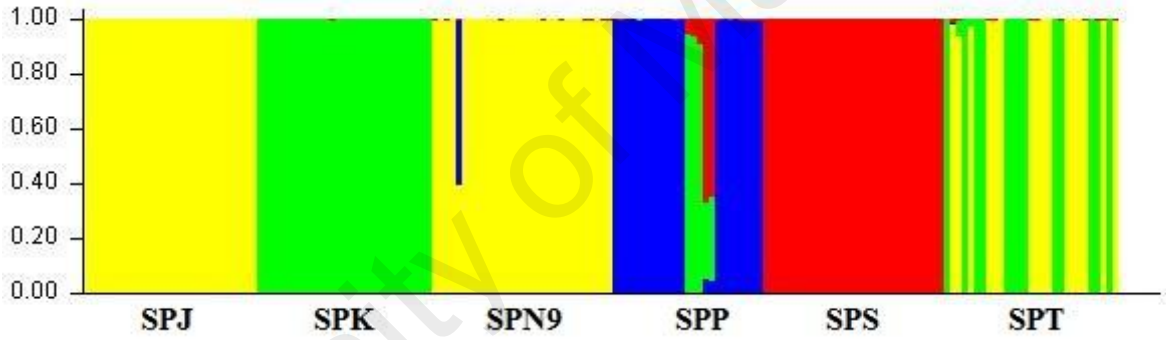
(d) Δ*K*=*m*|*L*''(*K*)|/s|*L*(*K*)|

Figure 6.6a-d: Description of the four steps for the graphical method allowing detection of the true number of group *K*=4 for *S. plicata* populations. (a) Mean [L(*K*)(±SD)] over five runs for each K value. (Hierarchical island model). (b) Rate of change of the likelihood distribution (mean ±SD) L'(*K*). (c) Absolute values of the second order rate change of the likelihood distribution (mean ±SD) |L''(*K*)| d) Delta *K* (Δ*K*). The model value of this distribution is the true K or the uppermost level of structure here is four clusters.

Run 1



Run 2



Run 3

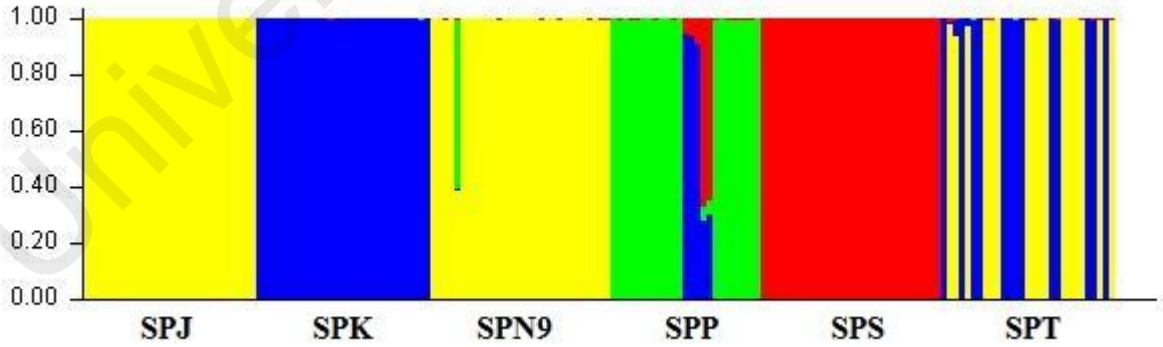


Figure 6.7: STRUCTURE analysis of Malaysian *S. plicata* population. Estimated population structure results of admixture analysis at *K*= 4. *K* coloured segment (Colours) represent different genetic groups and the membership to *K* cluster.

6.5 DISCUSSION

6.5.1 *Spathoglottis* species

AFLP primer combinations were screened and determined for data analysis of *Spathoglottis* species. In this study, fifty six (56) primer combinations with three selective bases at the 3' end were tested on *Spathoglottis plicata*. A good primer combination should give a large number of well-separated polymorphic bands of similar signal intensity. However, evaluation was subjective based on the number, distribution and uniformity intensity of bands was used to evaluate the primer combinations (Xiang et al., 2003). The number of bands obtained per primer ranged from 10 to about 110 in the 40 and 500 bp size ranges. Based on this criteria, eight primer pairs were selected for genotyping all the available plant materials. The eight selected AFLP primer combinations were successfully amplified in the *Spathoglottis* species, in which some of the primer combinations were also tested in other orchid studies. An average of 98% of the AFLP profile generated 891 polymorphic bands among and within the five native *Spathoglottis* species, five additional species, a hybrid of *Spathoglottis* and the outgroup of *Dendrobium affine* of 60 individuals in total. High polymorphism occurred in *Spathoglottis* accession and similar to another orchid genus of *Phaleonopsis* with 95% of the AFLP generated loci were polymorphic among 16 *Phaleonopsis* species and hybrids from 10 primer combinations (Chang et al., 2009). The orchid genus of *Dendrobium* generated 83% polymorphic among 43 hybrids from 12 primer combinations (Xiang et al., 2003), while *Spiranthes romanzoffiana* generated 62% polymorphic within and among the populations (Forrest et al., 2004).

The AFLP molecular analysis in this study most probably supported the taxonomists' examination treatment on the *Spathoglottis* species on their morphological traits and is potential to cluster the species according to their colours of flowers. The dendrogram obtained by UPGMA using Dice coefficient of genetic similarity shows a distances

(0.34) between clusters among the species. High bootstrap values (91%) supported the major clades in the *Spathoglottis* species was obtained in this study. The result also attempts to reconstruct the relationship of the species using morphological characteristic guide to produce a reliable and accurate classification. Regardless, the other morphological characteristics and limitation of molecular studies to differentiate in the species, AFLP markers application generates an informative data to distinguish the five native *Spathoglottis* species and the five additional species and a hybrid. Chang et al. (2009) reported that the *Phalaenopsis* species and hybrids were classified by using the AFLP clustering, in which its dendrogram was revealed through simple classification that is based on the colour of the flowers, although the accuracy of their genetic relatedness may be reflected.

As reported by Teo (1985), Seidenfaden and Wood (1992), morphologically the *S. gracilis*, *S. affinis*, *S. microchilina* and *S. aurea* were characterised in various ranges of yellow colour flower whereas *S. plicata* having purple colour flower. Although the yellow and purple colours were variable found native in Malaysia but the taxonomy of this genus are still controversial as similar species could be due to origin from other countries. The geographic location also defined the *Spathoglottis* genus into two sections of yellow flowered species. Cootes (2001) reported that *S. kimballiana* (var. angustifolia) has been identified from Borneo and *S. vanoverberghii* as a native species in the central Luzon of the Philippines, also in a group of yellow flowers.

The purple flower colour clustered from the dendrogram into Group 2, whereby the flowers of *S. plicata* and *S unguiculata* are purple in colour, whereas *S. plicata alba* is white. The unknown *Spathoglottis* hybrid has a combination mixture of pale purple, white and yellow. Holttum (1953) describes that *S. plicata* has been cultivated for a long period and several varieties may have been selected, including the white ones. Therefore, *S. plicata alba* could be a variety of *S. plicata* and taxonomists describe of both their

flowers and labellum characteristics as similar except for the fact that the colour is white. This could also appear from hybridization between the varieties in which probably the parents could be interspecific hybrids. *Spathoglottis lobbii*, which was bought from a nursery, was without any flower. Taxonomists reported it as a native in Burma and describe the flower as yellow in colour; it is allied with *S. affinis* as they share most common traits. However, the AFLP analysis clustered the species as a stand alone. This finding may indicate that *S. lobbii* is relatively and genetically isolated from other species.

In the current study, the molecular analysis (AMOVA) has proven that there is a variation among and within the *Spathoglottis* species and hybrids. Among others, a significant difference of 91.14% variation exposed among the *Spathoglottis* species. In other words, the species may be distinct from each other genetically. Table 6.4 and Table 6.5 show results with high values of distance among the species. Based on our investigation, the level of diversity of the *Spathoglottis* species is crucial as it shows a high genetic variation. Although the population sample sizes of each species were small, but there was still a variation within the population of the species. However, the genetic variation within the species is low with 8.86%. Therefore, one genotype was generally dominant in most populations of the species. STRUCTURE analysis shows an optimal overall subdivision of the *Spathoglottis* accessions in six major clusters. Even though the *Spathoglotttis* species have almost the same colour, the STRUCTURE analysis indicates that the accession of the species is in one cluster and some are in different clusters and sharing clusters. This could be a different species or subspecies of *Spathoglottis*. *S. aurea*, *S. affinis*, S. *gracilis* and *S. microchilina* which are clustered as one, with *S. kimballiana* and *S. vanoverberghii* in one cluster, while *S. plicata*, *S. unguiculata* and the hybrid in another cluster. *S. lobbii* and *S.plicata alba* are assigned to one STRUCTURE cluster each.

This certainly proves that the AFLP markers can genetically differentiate the genus *Dendrobium* and *Spathoglottis*. *Dendrobium affine* is proven as a true outgroup and this supports the analysis to be completed. A major morphological characteristic to differentiate the *Spathoglottis* species or cultivar is the colour of their flowers. Information about the genetic relationship among the species estimated the genetic similarity provided based on the DNA marker. The AFLP markers prove that their genetic relationships, as well as their genetic distance, are distinguishable. This work is the first attempt done to study and generate the genetic relationships between *Spathoglottis* species and determine the species that are closely related based on the AFLP markers. Species that are more closely related might be much easier to cross and chances to form new hybrids.

In general, the present results give a clear picture of a higher genetic differentiation among the species and a lower genetic differentiation within the populations. Poor sampling on the population could be the boundary in explaining the correlation between the population genetics. Genetic structure could be more important if the population's history show differences growth processing at different boundaries of a species (Bullock et al., 2000).

6.5.2 *Spathoglottis plicata* population

6.5.2.1 Genetic Diversity among and within *S. plicata*

AFLP data from eleven primers combination revealed significant difference in genetic variation among and within the six populations and individual of *S. plicata* population which most variation described among the population. AMOVA results on the population differentiation value of *S. plicata* was relatively high (Fst= 0.785) which is higher than the other terrestrial species of *Pseudorchis albida* in Ireland ($\phi pt = 0.306$) (Duffy et al., 2011) and *Spiranthes romanzoffiana* in Europe (overall mean *G*ST =

0.187) (Forrest et al., 2004). It was believe that there could be a gene flow among the individuals giving rise to higher genetic variation and gene flow could be predominantly limited between geographical ranges. Higher genetic variation shown among the *S. plicata* populations could imply that this species is predominantly outcrossing. Hamrick and Godt (1990) believed that plants with high genetic diversity typically reflect their wide geographical range of outcrossing. *Spathoglottis plicata* and the other species are believed by Forbes (1884); Ridley (1930) and Lord (1981) to be both outcrossing and self-pollinating species. However, a degree of outcrossing is important in facing an unstable habitat condition and some *S. plicata* population reproduce through various forms of self-pollination due to the absent of pollinator. The populations are expected to become genetically uniform and their survival may be dependent on the availability of suitable habitat and mass seed production. AMOVA and STRUCTURE indicates genetic isolation of populations with distance evident with variation among populations is higher compared to within population.

STRUCTURE is a simple approach that allows handling of dominant markers such as AFLPs (Falush et al., 2007) to cluster individuals in the populations. *Spathoglottis plicata* is reported to be the most widespread species and have many cultivated varieties as described by Ridley (1907); Holttum (1953); Teo (1985); Comber (1990, 2001) and Yong (1993) along the Asia Pacific countries. It is widely distributed throughout the Peninsular Malaysia from North to South and Eastern to Western regions. This is probably a rare long distance seed dispersal event facilitated by wind or human factor. This species has been found to be different in size, colour and some detail of the flower shape which reflect independent variation in the colour of petals and sepals, side lobes and callus of the lip. The gene flow constraints could also influence the species. Hamrick and Godt (1989) proposed that reproductive biology is a key factor in determining the genetic structure of plant population. Breeding systems could also be

major factor influencing the level of genetic diversity within the species. *S. plicata* is known to be self-pollinated in the bud stage (cleistogamy) and also capable of open pollination (chasmogamous) on the same species (Howcroft, 1992).

The STRUCTURE analysis provide a strong support to identified the six populations of *S. plicata* with consistent improvements in the probability function for run with *K*=4 value. Where, only one genotype was dominant in *Spathoglottis plicata* populations in both State of Johor and Negeri Sembilan (Southern region). Whereas, the population of Kedah (Northern region) represent another dominant genotypes as well as Selangor (Central region) having different genotype. Another dominant genotype was found occurred in Pahang (Eastern region). However, Terengganu populations having shared the dominant genotypes that present from Southern and Northern regions. Geographically, the Northern, Eastern and Southern Peninsular Malaysia have different distribution in term of range, altitude, climate and habitat including the rainfall and soil taxonomy. The topography of Peninsular Malaysia is characterized by the central mountain ranges running from north to south. As a matter of fact, the *S. plicata* literature described as a lowland plant but their habitat could also found to be growing in the mountain. It was shown on this statistical analysis result similar to their biological location where Johor (SPJ) and Negeri Sembilan (SPN9) population was sampling from the lowland situation and Kedah (SPK) was on the mountain. The Selangor (SPS) populations are a vegetative propagated bought from a nursery. The Terengganu (SPT) sampling in the open space but found to be a mixture of genetic from the lowland and highlands population. Pahang (SPP) populations were sampled in different latitude.

The factor which could contribute the differentiation of genotype is their soil taxonomy classification parameter which differs among the region. The soils of Malaysia can be divided broadly into 2 groups: (a) the sedentary soils formed in the

interior on a wide range of rock types, and (b) the soils of the coastal alluvial plains (Nieuwolt et al., 1982). Northern region has categories as sedentary soil and southern region as bris soil. Terengganu and Pahang is alluvium and granite soil as there is a high and low elevation. The sedentary soils are developed on igneous, sedimentary and metamorphic rocks, and are strongly weathered with mostly kaolinitic clay minerals. The sedentary soils were categories as the predominantly fine-textured clay and clay loam soils; peat and organic soils; the acid sulphate soils scattered along the west coast plains; and the sandy soils (bris soils) spread along the east coast of the Peninsular Malaysia.

Genetic diversity within populations and between populations may be influenced by habitat variability with a process of differential gene flow and other factors. Population could also not been separated sufficiently long to accumulate detectable genetic differences. However, gene flow could have increased genetic diversity within the population of Terengganu and Pahang by gene input from external sources such as influence of habitat structure. The genetic diversity of the *Spathoglottis* population could be due to the factor of rainfall distribution (Nieuwolt, l982) in the three related region. It is observed that Kedah rainfall distribution is low compare to the two other regions which has a significant rainfall distribution. The population in Terengganu and Pahang were present with two and three genotypes; this was probably due to the possibility of various humid and moisture percentage in this region. The seasonal climate in the Northern region is reported to have longer dry period. The location of the northern population is in Gunung Jerai at 1000-1500 meter above sea level. The population may have been geographically isolated for long period and isolation sites could be one of the major influencing genetic differentiations at limiting the amount of gene flow. The southern region population may be topologically identical where it clusters together and the population structure for colour is in a genetically similar group.

The UPGMA clusters the Pahang and Terengganu populations into two groups but STRUCTURE analysis obtained that Pahang population have three clusters. This could also account for the overlapping of individuals from different populations and lack of correlation between genetic distances and geographic distances. Gene differentiation and gene flow are important indices to estimate the population genetic structure of a species. The gene flow estimate among populations and the level of migration could prevent continued divergence among populations (Wright, 1951). The breeding system of the *Spathoglottis* species by cross-pollination and the fact that its seeds can be dispersed very long-distance by wind could be the main factor. The possibility showing that the Northern and Southern region were a geographical isolation population and a sign of gene flow hybridization within the Pahang and Terengganu population.

Genetic diversity within and between population was a function of historical events and recent evolutionary processes. Due to limitation knowledge in the evolutionary history and ecology of *Spathoglottis* species, explanations on the levels and patterns of genetic diversity within and among populations rely primarily on the inference from molecular data. However, the information obtained could be further studied to understand all the possibility that could cause to the genetic make up of *Spathoglottis plicata* at their population.

6.5.2.2 Implications for conservation

Spathoglottis plicata is an endangered orchid species. It is classified as Appendix II by CITES because of its indiscriminate extraction. Information on the genetic variation has been increased to guide the conservation of endangered species management world-wide effort (Avise & Hamrick, 1996; Holzapfel et al., 2002). Changing of the environment could be a critical factor in the genetic variation (Frankel & Soule, 1981). In order to preserve the species in future, with the ability to adapt the environmental

changes, there should be a sustained and structured programme for genetic conservation which focuses on maintaining the overall genetic diversity of the species (Fisher & Matthies 1998; Petit et al., 1998 and Fischer et al,. 2000). However, a better understanding is needed on the genetic variation distribution in the gene pool (Qamaruz-zaman et al., 1998). The author suggested that AFLP provided an efficient method for the first assessment on genetic diversity and variation in *S. plicata*. Qamaruz-zaman et al. (1998) used AFLP to decipher the genetic structure studies on endangered orchid species of *Orchissimia* in UK with effective conservation management that led to high effective population size. Pfeifer and Jetschke (2006) also used AFLP for genetic diversity and genetic differentiation within *Himantoglossum hircinum*.

6.6 CONCLUSION

Spathoglottis species are wild terrestrial orchids with little known on its distribution, adaptation and genetics in Malaysia that defeats the orchid conservationist in planning the conservation management plan. This study field was the first attempt made to document and understand the intra and intergenetic variabilities of five native and additional *Spathoglottis* interspecies and one unknown hybrid using markers generated by AFLP. In general, AFLP was potential to elucidate the genetic differences between 5 native and five selected *Spathoglottis* species according to its morphological identification. The colour of flower morphology is in agreement with the genetic data and could facilitate further understanding of the various *Spathoglotis* species distributed not only in Peninsular Malaysia but also in other countries of the Asia Pacific region. The AFLP analysis results suggests that the marker could be used across a wide range of sampling methodologies and is also faster method in resolving the identity of either the species or the hybrids.

The data from this study enable rise to further research on the conservation planning for the endangered species as these populations showed significant variation between populations indicating that there is lack of gene flow in between the populations. There is also an indication of isolation of distance within these populations in accordance to geographical range. It can also be tested to implement the traceability of the species which is necessary for the worldwide management guide and stimulate research of the species. The knowledge of the existence of three subclusters within Peninsular Malaysia will allow for further effort in conservation management decisions to be taken by forest authority in order to protect this species. This is vital as industrialization will continue to affect the forest area coverage and these type of data are pivotal for ensuring the survival of this species in the future.

Therefore, due to lack of studies on *Spathoglottis* for species and population, the AFLP molecular data information obtained here could be further studies throughout the combination of species and it population in future. In order to observe the possibility of genetic differentiations among species that could be due to their geographical isolation with equal sample size of the species and population. Author understands that sampling design methods is important to avoid bias in the results. However, due to the rareness of the species and limitation of sample location, the non-randomly sampling method based on local knowledge of sample (Elzinga et al., 2001) and geographical location were selected with five accessions per species and 25-30 accessions for population. The sample sizes were sufficient enough to gain a primary molecular data information for research and conservation planning.

CHAPTER 7: EARLY INVESTIGATION ON THE EFFECTS OF PVS2 DURATION FOR CRYOPRESERVATION OF *SPATHOGLOTTIS PLICATA* ORCHID PROTOCORM

7.1 INTRODUCTION

The population of wild *Spathoglottis* species grown in this country has been subjected to risks such as erosion, urbanisation and adverse environmental conditions. Therefore, conservation of the *Spathoglottis* germplasm is required to ensure that the species will become extinct. Besides field conservation, cryopreservation is a promising technique which ensures safe, cost-efficient and long-term storage of different germplasm (Sakai & Engelmann, 2007).

In this study, the encapsulation-vitrification method developed is already applicable for many plant types. In this study, explants were embedded in alginate gel followed by dehydration with vitrification solution. This method has been successfully applied to protocorms of *Dendrobium cariniferum* (Pornchuti & Thammasiri, 2008), protocorm like-bodies (PLBs) of *Dendrobium* Bobby Messina (Antony et al., 2011) and *Dendrobium* sonia-28 (Hwa et al., 2009; Ching et al., 2012).

However, there are limited studies on the cryopreservation of *Spathoglottis* using the encapsulation-vitrification method. In this method, the duration of exposure of explants to vitrification solution (PVS2) is crucial (Sakai & Engelmann, 2007). Therefore, optimization of exposure time to PVS2 needs to be considered. The present study aimed to evaluate the effects of PVS2 at different exposure times (5, 10, 15, 20, 25 and 30 min) on encapsulated and non-encapsulated protocorm of *S. plicata*. The survival rate and the regrowth development of explants were also determined based on morphological and histological observations.

7.2 LITERATURE REVIEW

Common plant conservation practices involve field conservation mainly in botanical gardens and in their natural habitats (*in situ* conservation). However, these plants are subjected to natural calamities, diseases and urbanization. Alternatively, plants are kept in laboratories as *in vitro* cultures for short- and medium-term conservation. For long-term conservation, cryopreservation is the standard practice for many plant species.

7.2.1 Cryopreservation

Cryopreservation has been successfully applied to a wide range of orchid species using different explants and techniques (Vendrame et al., 2014). Several techniques of cryopreservation such as vitrification, encapsulation-dehydration and encapsulation-vitrification for conservation of endangered orchid species have been developed by Sakai et al. (2008).

7.2.2 Vitrification

Vitrification is a physical process where a highly concentrated cryoprotective solution solidifies into metastable glass at sufficiently low temperatures without undergoing crystallisation (Fahy et al., 1984). This is a simple, fast and effective cryopreservation method. In this method, plant vitrification solution, PVS2 is used as a cryoprotectant solution before freezing (Engelmann, 2000).

New methods that have been developed for vitrification are encapsulation-vitrification, droplet-vitrification and vitrification using aluminium plate's methods (Matsumoto & Niino, 2014). Encapsulation-vitrification method is the earliest combined procedures that incorporate vitrification and encapsulation-dehydration methods. This was first used by Matsumoto et al. (1995) on wasabi shoot tips

encapsulated in alginate beads. This method was easy to manage and eliminated dehydration step.

Encapsulation-vitrification procedures was further developed and applied to protocorms of *Dendrobium cariniferum* (Pornnchuti & Thammasiri, 2008; Thammasiri, 2008), *Dendrobium candidum* (Yin & Hong, 2009), *Dendrobium nobile* (Mohanty et al., 2012) and *Dendrobium sonia*-28 (Ching et al., 2012).

7.3 MATERIALS AND METHODS

7.3.1 Plant materials

The mature *Spathoglottis plicata* seed pods contain minute seeds (Figure 7.1). The seed from the seed pods were placed into a small bottle and sterilized using 5% (v/v) domestic bleach Clorox (containing 0.025% active ingredient sodium hypochlorite) and a drop of detergent, Tween® 20. The sterilized seeds were cultured in half strength modified Murashige and Skoog (MS) medium (1962) supplemented with 150 ml/l coconut water, 20g/l sucrose and 1.8 g/l phytagel at 24^{o}C under 12 hours photoperiod using vit-lite fluorescent (56 µmol S^{-1} m^{-2}) for 5 to 6 weeks until protocorms were formed. Subsequently, *in vitro* cultured protocorms (about 1-2 mm) of *S. plicata* were used as explants in this study (Figure 7.2).

Figure 7.1: Mature seed pods of *Spathoglottis plicata*

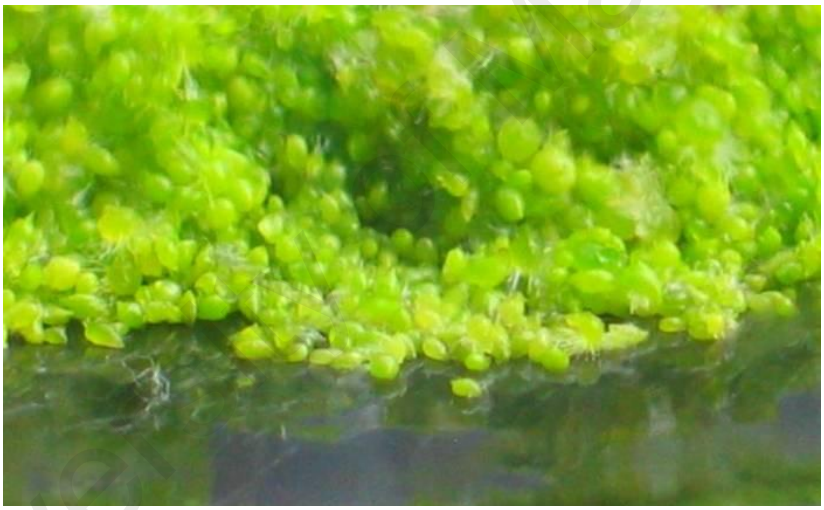


Figure 7.2: Protocorms of *S. plicata* cultured on ½ MS media

7.3.2 Preparation of MS stock solution

Murashige and Skoog (MS) medium (1962), slightly modified by using the 150 ml/l coconut water as growth regulator was used as basal medium (Appendix F). Stock solutions of Macro Element Stock (10X), Micro Element Stock (1000X), Iron Stock (10X) and Vitamin Stock (1000X) were prepared separately. All stock solutions were prepared in distilled water.

7.3.3 Preparation of culture and recovery medium

One litre of half strength MS basal medium was prepared using 50 ml of macroelement Stock, 0.5 ml microelement Stock, 5 ml iron stock and 0.5 ml vitamin stock. Coconut water of 150 ml/l and 20g/l sucrose were added to prepare one liter of MS culture medium. The pH of the medium was adjusted to 5.7 using 1.0 or 0.1 N NaOH before adding the Phytagel at 1.8 g/l. The MS medium was then autoclaved at 121^{o}C and 15 p.s.i. for 30 minutes. After autoclaving, about 10-12 ml of the sterilized medium was dispensed into each sterilized vial in the laminar airflow cabinet and covered with heavy duty aluminium foil. The vials were stored in a cool clean room and used within one or two weeks after preparation. Similar MS culture medium was prepared for recovery medium to be used to stabilize the protocorm after vitrification. The sterilized MS medium was dispensed into 9 cm sterile plastic petri dishes.

7.3.4 Preparation of preculture, loading, vitrification and unloading solutions

All solutions used for cryopreservation procedures in this experiment were dissolved in half strength MS basal medium. The pH of all solutions was also adjusted to 5.7 prior to sterilization and stored in screw-cap bottles in refrigerator before use. Each solution contained the following additional components used in this study as given in Table 7.1.

Table 7.1: Type of solutions and its components

Type of Solutions	Components
Preculture	0.3M Sucrose
Loading	2M glycerol 0.4M sucrose
Plant Vitrification Solution 2 (PVS2)	30% (v/v) glycerol 15% (v/v) ethylene glycol 15% (v/v) demethylsulfoxide (DMSO) 0.4M sucrose
Unloading	1.2 M sucrose

7.3.5 Preparation on encapsulation media

For encapsulation of protocorm, two types of solutions were prepared. These are sodium alginate [2% (w/v)] with 0.3M sucrose and another solution with 100mM calcium chloride ($CaCl_2$). Both solutions were prepared in half strength MS basal media. These medium was placed in a sterilized bottle and autoclaved.

7.3.6 Encapsulation-Vitrification procedure

The procedure involves the following steps:

a) Preculture

Fresh protocorms (1-2mm) were placed in 100ml conical flasks (Pyrex, USA) containing 25ml preculture solution. The flasks were placed on a rotary shaker (Protect Orbital Shaker Model 722) with 100 rpm speed for 24 hours.

b) Encapsulation

The encapsulation step used was based on a modified method described by Matsumoto and Sakai (1995). The precultured protocorms were

immersed in 25 ml calcium-free half strength liquid MS medium containing 2% (w/v) sodium alginate with 0.3M sucrose placed in 100 ml beaker. Each single protocorm was drawn up into a pipette fitted with modified tip (cut tips to a width of about 4 mm) and dropped into 100mM $CaCl_2$ solution to form alginate beads. The beads in the beaker were left to harden in a laminar flow for 20 min with occasional agitation. The alginate beads were then washed with MS liquid medium and transferred to empty 9 cm petri dishes with milipore filter paper (7 cm) to remove any excessive solutions.

c) Loading treatment

Both encapsulated and non-encapsulated protocorms were transferred aseptically into a 2ml sterilized cryovial (brand). One ml of loading solution was added and incubated for 20 min.

d) PVS2 dehydration

The loading solution was pipetted out and replaced by 1 ml of the PVS2 solution. The encapsulated and non-encapsulated protocorms were dehydrated with PVS2 solution for different durations of 5, 10, 15, 20, 25 and 30 min. The PVS2 was replaced with fresh solution after 5 min and dehydrated further for the targeted exposure time.

e) Storage in liquid nitrogen

After PVS2 treatment, both encapsulated and non-encapsulated protocorms were suspended in 0.5 ml of fresh vitrification solution and

the cryovial was put in the cryo holder and plunged into liquid nitrogen for at least 1 hour.

f) Thawing

Cryovials with frozen encapsulated and non-encapsulated protocorms were removed from liquid nitrogen and warmed in 40 ℃ water bath until the PVS2 became liquid again. After thawing, the PVS2 solution was removed.

g) Unloading

The non-frozen encapsulated and non-encapsulated protocorm were treated with unloading solution for 20 min. One ml of fresh unloading solution was added into the cryovial and shaken for 10 min. The unloading solution was replaced and held for another 10 min.

h) Recovery

Finally, after unloading the encapsulated and non-encapsulated protocorms were removed and placed into sterilized filter paper over the recovery medium for overnight stabilization. The encapsulated and non-encapsulated protocorms were then cultured on semisolid half strength MS culture medium and place under 12 hours photoperiod using vit-lite fluorescent light (56 µmol S^{-1} m^{-2}).

7.3.7 Survival assessments

After 8 weeks of recovery, the viability and the survival of cryopreserved and non-cryopreserved, encapsulated and non-encapsulated protocorms were assessed based on

growth observations. Survival is defined as the ability for protocorm regrowth, and explants to regenerate *in vitro* on a half MS medium in culture room at 24^{o}C under 12 hours photoperiod using vit-lite fluorescent light (56 µmol S^{-1} m^{-2}). The regrowth was classified into 8 categories (Figure 7.3) for survival assessments. Categories A to G were considered surviving explants and category H was presumed to be dead.

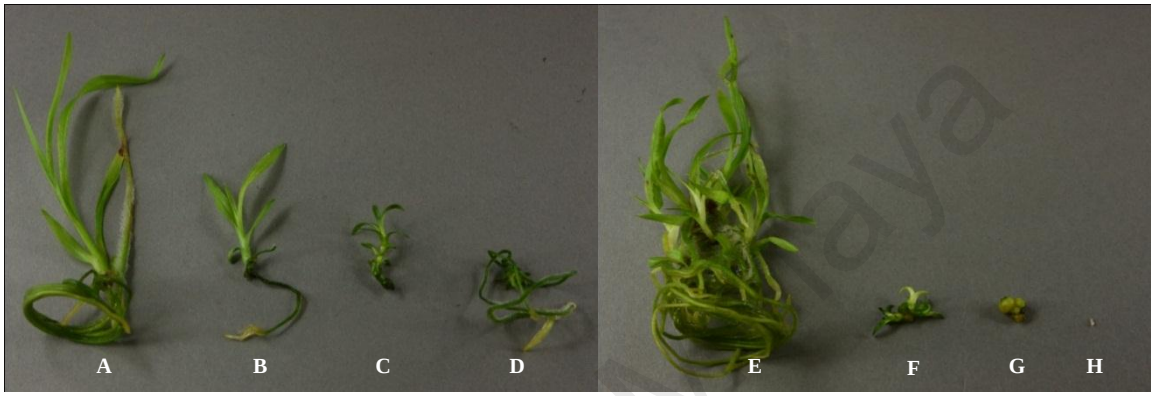


Figure 7.3: Morphological characterisation of *S. plicata* plantlet. **(A)** Normal plantlet with root and shoot > 1 cm; **(B)** Normal plantlet with root and shoot < 1 cm; **(C)** Shoot formation only; **(D)** Root formation only; **(E)** Plantlet with multiple shoot and root; **(F)** Abnormal plantlet; **(G)** Callus formation; **(H)** Dead protocorm.

7.3.8 Statistical data analysis

The experiments were conducted in a randomized complete block design (RCBD) and consisted of three replicates containing 10 explants for each parameter tested. The SAS software was used for analysis of variance (ANOVA). Means were compared using the Duncan' Multiple Range Test (DMRT).

7.3.9 Histology analysis

Histological analysis was performed on PVS2 dehydration (encapsulated and non-encapsulated) for cryopreserved and non-cryopreserved *S. plicata* protocorms. This was done after 8 weeks of growth recovery. Histology observations were conducted on fresh, precultured, loading treatment, PVS2 solution exposure to encapsulated and non-

encapsulated protocorm without and after cryopreservation treatment to observe occurring of cryo injuries. The histology techniques used in this study used resin developed by ORSTOM – CIRAD team LRGAPT France (Maril et al., 1995). The protocorms were sliced, fixed in Glutaraldehyde-Paraformaldehyde-caffeine (GPC) fixative mixture and then dehydrated in ascending ethanol percentage. The filtration protocorm tissues were prepared with basic resin (Leica Historesin Embedding Kit) 24 to 48 hours at 4℃ under slight vacuum. The specimens were embedded, the resin was fully polymerized and 3 micrometer sections were then sliced using a microtome. Specimens were stained with 1% periodic acid for five minutes, rinsed with distilled water at pH 4.5 and submerged in Schiff's reagent for 20 minutes in the dark. Finally, Napthol Blue Black (1g Naphthol Blue Black in 100ml 7% acetic acid) was used at 60℃ for 5 minutes for counter staining and slides were observed viewing under light microscope.

7.4 RESULTS

7.4.1 Effects of PVS2 exposure time

The encapsulated and non-encapsulated *S. plicata* protocorms were unable to grow after thawed from liquid nitrogen. In the early stage, the protocorm was greenish in colour but turned to brown after a few weeks in culture tubes. There was no significant difference on the survival of non-cryopreserved encapsulated and non-encapsulated *S. plicata* protocorm at different exposure times to PVS2 as shown in Table 7.2. However, the untreated (FC) protocorms of 5-6 weeks in culture had 100% survival rate. After preculture with 0.3M sucrose for 24 hours (C1), the non-encapsulated and encapsulated protocorms survived 73.3% and 86.67% respectively. Non-treated encapsulated protocorms (C2) exhibited 78.33% survival. However, after being treated with loading

solution (0 min) the survival rate decreased to 60% for encapsulated protocorm and 10.83% for non-encapsulated protocorm.

The encapsulated and non-encapsulated protocorms were further treated at different durations (5, 10, 15, 20, 25 and 30 min) in order to determine the best exposure period to PVS2 solution. The encapsulated protocorm showed 36% to 52% survival rate with increasing rates of PVS2 exposure times. Exposure time at 5 min to PVS2 gave the highest survival rate at 52%. Whereas, the survival rate at 10 and 20 min was 47% and 36% at 15 and 25 min. However, there was no significant difference on the PVS2 exposure time to the survival of encapsulated protocorms. The non-encapsulated protocorms produced lower than 10% survival rate after 5-15 min exposure time to PVS2 and no survival after 20 min. Longer exposure to the PVS2 solution was potentially injurious to *S. plicata* protocorms. The present study suggested that encapsulation of the *S. plicata* protocorm with 2% sodium alginate before dehydration with PVS2 solution prevented the protocorm from injury.

Table 7.2: Percentage of survival of encapsulated and non-encapsulated *S. plicata* protocorm after different exposure times to PVS2

Exposure Times to PVS2	**Survival (%)**	
	Encapsulated (En)	**Non-encapsulated (N-En)**
FC	100.00^{a}	100.00^{a}
C1	86.67^{ab}	73.33^{b}
C2	78.33^{abc}	-
0 min	60.00^{bcd}	10.83^{c}
5 min	51.67^{cd}	3.33^{c}
10 min	46.67^{d}	7.41^{c}
15 min	35.83^{d}	3.33^{c}
20 min	46.67^{d}	0.00^{c}
25 min	35.83^{d}	0.00^{c}
30 min	43.71^{d}	0.00^{c}

Means with the same letter (s) within a column are not significantly different at 0<0.05 based on DMRT. Note: FC: Fresh Control (Untreated Protocorm), C1: Preculture Protocorm, C2: Encapsulated Protocorm, 0 min: Loading solution treatment

7.4.2 Morphological characterization of regrowth plantlets

The development and growth of surviving encapsulated and non-encapsulated protocorms of *S. plicata* (5-30 min exposure times to PVS2) was recorded and shown in Table 7.3. There were differences on the plantlets growth, their morphological characterization and categories as shown in Figure 7.3. Most of the encapsulated protocorms that survived had developed into normal plantlets (A) with 6.67% to 36.30% after 5-30 min exposure to PVS2 solution. PVS2 dehydration at 30 min produced normal plantlet with shoots and root formation of about 36.30%. Subsequently, the non-encapsulated protocorm showed about 10% abnormal plantlets (F) after 5 min of dehydration with PVS2 solution. At 10 min of dehydration, 11% developed into normal plantlets and 11% formed only shoots. At 15 min, 10% of the protocorm produced callus (G) but did not develop into plants. However, no regrowth was observed in the protocorm after exposing it to 20, 25 and 30 min with PVS2 dehydrating solution. Although, both encapsulated and non-encapsulate protocorms did not survive in liquid nitrogen, the result still showed the potential of the encapsulation-vitrification technique where the encapsulated protocorms had developed high percentage of normal plantlets compared to non-encapsulated protocorms.

Table 7.3: Morphological characterisation of surviving *S. plicata* protocorm after different exposure times to PVS2

Treatment	Morphological categorization (%)															
	A		B		C		D		E		F		G		H	
	En	N-En	En	N-En	En	N-En	En	N-En	En	N-En	En	N-En	En	N-En	En	N-En
FC	10.37	43.33	-	8.33	25.93	28.33	-	-	53.33	3.33	-	6.67	10.37	10.00	-	-
C1	65.00	48.33	-	6.67	3.33	14.17	-	-	8.33	4.17	-	-	10.00	-	13.33	26.67
C2	37.50		-		10.83		6.67		4.17		-		19.17		21.67	
0 min	36.67	10.00	-	-	3.33	-	-	-	6.67	-	-	-	13.33	6.25	40.00	83.75
5 min	27.50	-	-	-	4.17	-	-	-	-	-	-	10.00	20.00	-	48.33	90.00
10 min	26.67	11.11	-	-	3.33	11.11	-	-	10.00	-	-	-	6.67	-	53.33	77.78
15 min	6.67	-	-	-	7.50	-	-	-	-	-	-	-	21.67	10.00	64.17	90.00
20 min	17.78	-	-	-	6.67	-	-	-	3.33	-	-	-	18.89	-	53.33	100
25 min	13.33	-	-	-	14.17	-	-	-	-	-	-	-	8.33	-	64.17	100
30 min	36.30	-	-	-	7.41	-	-	-	-	-	-	-	-	-	56.30	100

Note: FC: Fresh Control (Untreated Protocorm), C1: Preculture Protocorm, C2: Encapsulated Protocorm, 0 min: Loading solution treatment; Category (A) Normal plantlet with root and shoot > 1 cm; (B) Normal plantlet with root and shoot < 1 cm; (C) Shoot formation only; (D) Root formation only; (E) Plantlet with multiple shoot and root; (F) Abnormal plantlet; (G) Callus formation; (H) Dead protocorm.

7.4.3 Histological observation

Histological observation of *S. plicata* protocorm indicated that majority of the cells were injured either during the dehydration step with PVS2 (encapsulated and non-encapsulated) or during freezing or thawing. Cross section of the fresh protocorm (Figure 7.4a) and precultured protocorm (Figure 7.4b) showed the presence of complete outer layer of cell wall without damage and was polyhedral in shape. Whereas, protocorm after treatment with loading solution (Figure 7.4c) and osmotic dehydration with PVS2 solution (Figure 7.4d), showed the presence of breakage and shrinkage on the outer layer of the cell wall with rupture in cell and cell membrane. However, most encapsulated *S. plicata* protocorm after being treated with the loading solution and dehydrated with PVS2, grew as normal plantlets. Histological sections of the non-exposed encapsulated protocorm (Figure 7.4e) and exposed to loading solution (Figure 7.4f) showed the presence of complete outer layer of cell wall and presence of dense cytoplasm.

All the encapsulated or non-encapsulated *S. plicata* protocorms were neither viable nor survived into plantlets after freezing in liquid nitrogen. Cross sections showed the breakage of the cell walls and with a possibility of plasmolysis (Figure 7.5a-f). Most cells displayed detachment of the plasma membrane, breakage of the cell wall and loss of cytoplasm. The cellular nuclei in cryopreserved protocorms appeared damaged and flocculated as well.

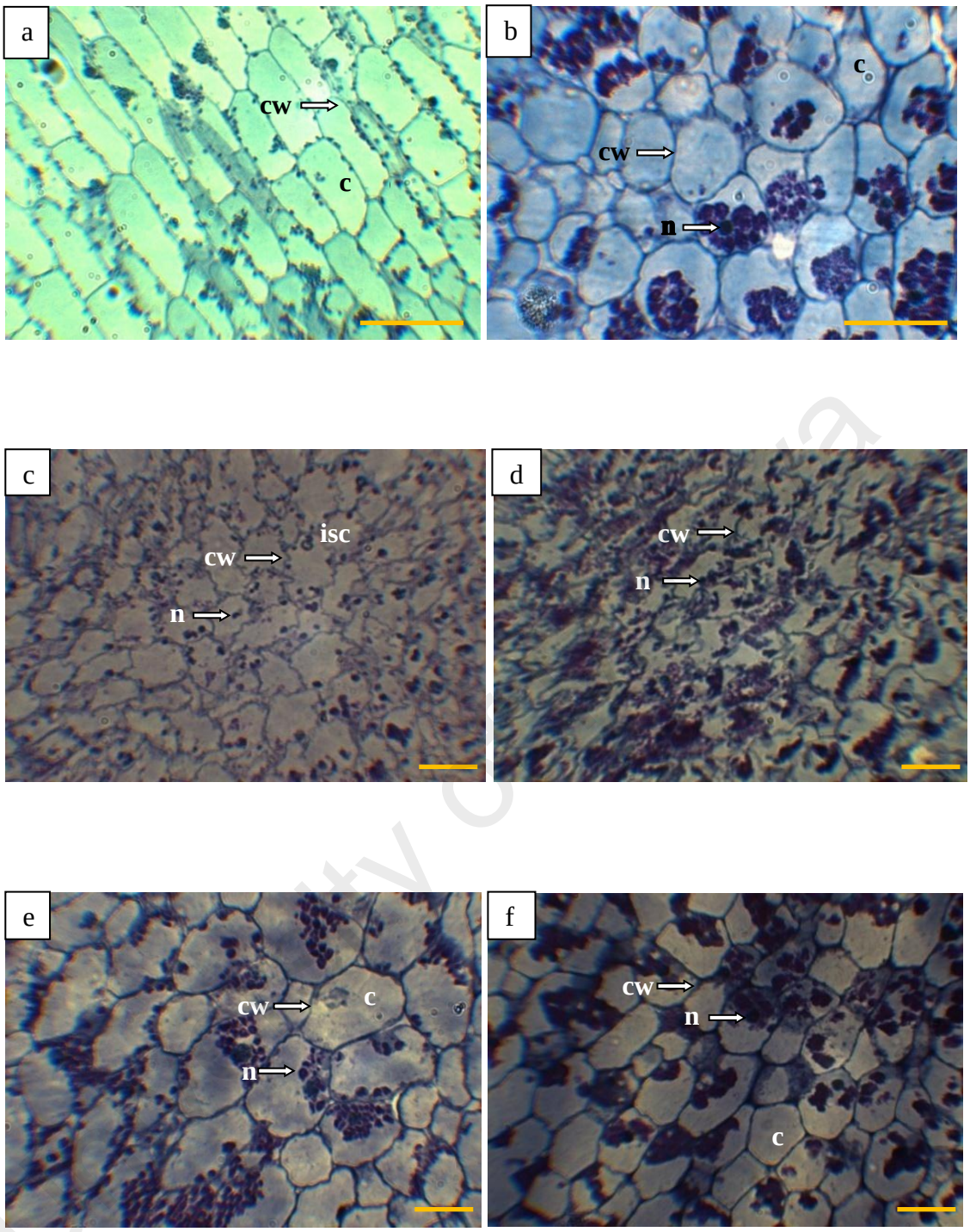


Figure 7.4: Histology cross section of non-cryopreserved *S. plicata* protocorm X400. Scale: Bar = 50 µm. (a) Untreated protocorm (Fresh Control); (b) preculture protocorm; (c) protocorm expose to loading solution; (d) protocorm dehydrated with PVS2; (e) encapsulated protocorm (f) encapsulated protocorm expose to loading solution and dehydrated with PVS2; cw; cell wall, n; nucleus, c; cytoplasm, isc: irregular shaped cells

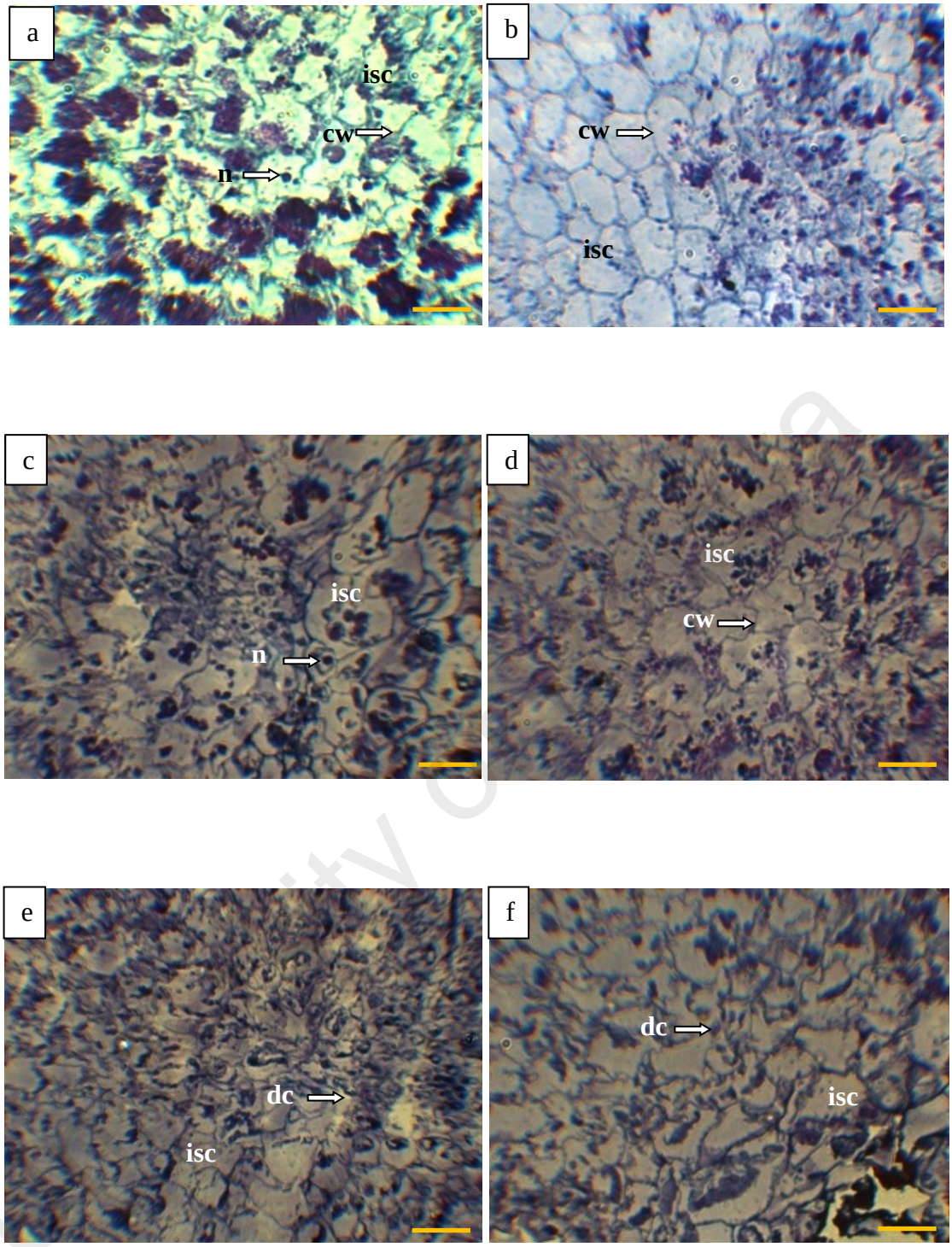


Figure 7.5: Histology cross section of cryopreserved protocorm of *S. plicata*. X400. Scale: Bar = 50 µm. (a) Untreated protocorm (Fresh Control); (b) preculture protocorm (expose to 0.3 M sucrose); (c) protocorm expose to loading solution (d) protocorm dehydrated with PVS2; (e) encapsulated protocorm expose to loading solution; (f) encapsulated protocorm dehydrated with PVS2; cw; cell wall, n; nucleus, dc; dense cytoplasm, isc: irregular shaped cells.

7.5 DISCUSSION

7.5.1 Effects of PVS2 solution exposure time on encapsulated and non-encapsulated protocorms

Optimising the time of exposure during dehydration to PVS2 is important in producing a high level of shoot formation after vitrification. Vendrame et al. (2007) reported that the key to a successful cryopreservation by vitrification technique is the control of dehydration and prevention of injury by chemical toxicity. Five to thirty minutes of exposure durations to PVS2 were optimized on encapsulated and non-encapsulated *S. plicata* protocorm in order to attain a high percentage survival using vitrification technique. Safrinah et al. (2009) optimized exposure time from 0 to 30 min on precultured *Mokara* orchid shoot before plunging into liquid nitrogen. They also determined 10 min as the optimal exposure time to PVS2. Antony et al. (2011) dehydrated the *Dendrobium* Bobby Mesinna for 0-140 min and 60 min was considered as the best PVS2 treatment. Ching et al. (2012) obtained high survival rate of encapsulated *Dendrobium* sonia-28 after dehydrated in PVS2 for 150 min.

The non-encapsulated *S. plicata* protocorm survived after 5-15 min dehydration in PVS2 but produced lower than 10% survival rate. Observation showed that only 11% developed into normal plantlets after dehydration with PVS2 solution at 10 min, while the others formed either abnormal plantlets or only shoots or callus. No regrowth was observed in protocorm after exposing to 20, 25 and 30 min dehydrating solution, PVS2. Longer exposure to the PVS2 solution affected the *S. plicata* protocorms and was potentially injurious. The tissue might be exposed to chemical toxicity and osmotic stress if over exposure to PVS2 solution (Suranthan et al., 2012). It could also cause phytotoxic effects of either individual component or combination of osmotic effect on cell viability (Sakai et al., 2000) and caused disruption to the plasma membrane. Histological observation conducted on non-encapsulated protocorm showed the

presence of breakage on the outer layer of the cell wall after dehydration to PVS2 at different exposure times.

Encapsulation-vitirification is a modification method of vitrification procedures where the encapsulated protocorm were easier to manipulate during vitrification because of its relatively large size with alginate beads. The concentrations of sodium alginate play a crucial role in obtaining beads with optimum hardness and rigidity. The PLBs of *Dendrobium* Bobby Mesina orchids and *Brassidium* shooting orchid hybrids were encapsulated using 2.5% (Zainuddin et al., 2011) and 3.5% (Yin et al., 2011) alginate respectively. In this study, the *S. plicata* protocorms were encapsulated with 2% sodium alginate before exposure to PVS2 solution. Most of the encapsulated protocorms that had survived developed into normal plantlets (A) [as described in para 7.3.6 and Figure 7.3] in between 6.67% to 36.30% (Table 7.3) upon exposure to PVS2 solution. Protocorms in PVS2 dehydration solution for 5 min showed 52% survival rate where 28% developed into normal plantlets with shoot and root formation whereas at 30 min exposure normal plantlets were formed at 36.30%

Encapsulated protocorms had developed better percentage of normal plantlet formation compared to non-encapsulated protocorms. Thus, suggesting encapsulation of the *S. plicata* protocorm with 2% sodium alginate before dehydration with PVS2 solution prevented the protocorm from toxicity injury. Cross sections of *S. plicata* encapsulated protocorm indicated the presence of a complete outer layer of the cell wall. Sodium alginate was shown to protect the explants from mechanical injury (Saiprasad, 2001). Khor et al. (1998) reported that the protocorms and seeds of *S. plicata* could tolerate the encapsulation treatments with complex co-acervation of alginate-chitosan and alginate-gelatin, giving high percentage of viability after desiccation. The survival rate of the encapsulated *Dendrobium sonia*-28 PLBs increased with increasing use of PVS2 dehydration duration from 0-150 min (Ching et al., 2012). It is suggested that

encapsulation using alginate solution on *S. plicata* could enhance further the protection to protocorm and also reduce osmotic stress to PVS solution (Ginibun et al., 2014).

7.5.2 Histology

Histology is an important tool in accessing the extent of cellular damages caused by cryopreservation. Cross sections of non-cryopreserved *S. plicata* encapsulated or non-encapsulated protocorm indicated the presence of a complete outer layer of the cell wall while cross sections of cryopreserved protocorm showed breakage at the outer layer of the cell wall or the breakage of crystals that damage the cells during cryopreservation. All the cryopreserved protocorm showed damaged cell walls and symptoms of plasmolysis, rupture inside cell and cell membranes. This could be due to crystallization during freezing which is a complex process that comprises a number of critical steps: nucleation, growth of crystals and recrystallization which were main elements affecting the survival of cells subjected to cryopreservation.

Similar histological observations obtained on cryopreserved *Vanda Kaseem's Delight* PLBs (Poobathy et al., 2012) that were subjected to recovery for three weeks, and untreated PLBs that were obtained from the stock culture. The structure and morphology of the nucleus, cell wall and cytoplasm could be clearly observed in the transverse sections of both groups of PLBs. The cell wall structure and integrity were maintained in non-cryopreserved PLBs. Sections of non-cryopreserved PLBs also displayed the presence of a single nucleus in each cell, surrounded by the cellular cytoplasm. However, ruptures and damages in cell wall structure could be observed in cryopreserved PLBs, most likely as a result of ice crystallization occurring in the freeze-thaw cycle. The cellular nuclei in cryopreserved PLBs appeared damaged and flocculated as well. Cryopreservation procedures may result in cell distortion, cell and nuclear shrinkage, nuclear envelope rupture and plasmolysis (Wen et al., 2010). As reviewed by Koichi et al. (2012), the primary site of freezing injury is reflected to be the

plasma membrane and such cryoprotective effects might vary with the composition of the plasma membrane. Similar results were obtained in this study. The cell wall and the plasma membrane were the sites of cellular injury during cryopreservation. Following the freeze-thaw cycles, the membrane permeability, corresponding to cellular viability, has frequently decreased (Fujikawa, 1995). The damages and ultrastructural changes in the cells could be attributed to intracellular or extracellular ice formation. The major factor of cryopreservation failure was linked to lethal intracellular ice crystal formation (Mazur 1984; Wen et al., 2010).

7.6 CONCLUSION

The dehydration periods with PVS2 affected the survival rate and plantlet growth of encapsulated and non-encapsulated protocorm of *S. plicata*. The protocorm without cryopreservation that was encapsulated with 2% sodium alginate gave the highest survival rate of 52% at 5 min exposure to PVS2. Although, none of the protocorms survived after freezing in liquid nitrogen, the result suggested that encapsulation-vitrification technique could be a potential technique for cryopreservation of *S. plicata* protocorms. However, further investigations on other important parameters using vitrification technique in the future are necessary. The alternative strategies for future studies on *Spathoglottis* species are by attempted a cryo-storing seeds.

CHAPTER 8: CONCLUSION

The importance of studying the *Spathoglottis* group of terrestrial orchids has been accelerated due to its increased popularity for landscaping and as ornamental potted plants. This genus comprises of fast-growing terrestrial orchids that are easy to maintain and tend to bloom all year round. The wild species and its populations which grow in this country are constantly exposed to threats such as erosion, construction and adverse environmental conditions with human interference that involves horticulture and developmental activities.

This study was initiated to gain insights and information on the morphological and molecular characteristics as well as the genetic structure and relationships of the five *Spathoglottis* species in Malaysia, namely, *S. plicata, S. aurea, S. microchilina, S. affinis* and *S. gracilis*. Overall, using the different approaches and methodologies, the study has fulfilled its objectives. The study was able to develop a structured description of the morphological characteristics of the Malaysian native terrestrial orchid of *Spathoglottis* species. It was also able to carry out genetic analysis on the samples obtained and generate information on their genetic relationships and identity.

Visual observations of the five *Spathoglottis* species were able to identify 21 qualitative characteristic to distinguish between the different species. The colour of the flower and the shape of the lips are the important morphological characteristics for identifying the differences between the *Spathoglottis* species. The characterisation indicates that *S. plicata* are distinguishable from *S. aurea, S. microchilina, S. affinis* and *S. gracilis* with its distinct purple colour flower. The other four species had yellow flower colour but were distinguishable from each other by their lip shape (Figure 3.1). The bract, flower, dorsal and lateral sepal, petal, lip, lip side lobes, lip mid-lobe claw,

lip mid-lobe and lip callus will be recommended as useful grouping characteristics for *Spathoglottis* species or varieties identification in the future.

In the molecular marker study, all the three techniques of IRAP, chloroplast DNA (cpDNA) barcoding and AFLP could potentially be used to analyse the genetic diversity of *Spathoglottis* species (Table 8.1). The IRAP studies had identified twelve single and combined IRAP primers: LTR 6150, 3'LTR, Sukkula, LTR 6149+Sukkula, LTR 6149+Nikita, LTR 6149+3'LTR, LTR 6150+Nikita, LTR 6150+3'LTR, 5'LTR1+3'LTR, 5'LTR2+3'LTR, 5'LTR2+Nikita and 3'LTR+Nikita which could be utilised for genetic analysis of the five Malaysian *Spathoglottis* species. All the 12 markers confirmed the utility of employing Barley (*Hordeum vulgare*) and Banana *(Musa acuminata*) RTNs based markers for the *Spathoglottis* species analysis. The IRAP dendrogram results could clearly differentiate the species and also classify them into their basic flower colour groups.

The chloroplast DNA barcoding study successfully amplified the four coding regions of *matK, rbcL-a, rpoB* and *rpoC1* regions from chloroplast DNA in the *Spathoglottis* species. The data analysis had proven that the chloroplast DNA markers either individually or in combination have the potential in differentiating the *Spathoglottis* species by arranging and representing the data in haplotypes. The *rbcL-a*, *rpoB* and *rpoC1* regions were combined and produced high resolution, which revealed eight haplotypes from 28 variable sites for identification and classification among the species in the phylogenetic analysis. The phylogenetic analysis clearly revealed the relationships of the five Malaysian native species, five additional species, a hybrid of *Spathoglottis* and the out group of *Dendrobium affine*. The Malaysian species were clustered in a group where *S. plicata* was separated from the *S. microchilina, S. affinis, S. aurea* and *S. gracilis*. It also showed that the *Spathoglottis* species could be

differentiated based on their flower using, the coding sequences of chloroplast DNA as the genetic marker for phylogenetic analysis at the species level of *Spathoglottis*.

In the AFLP studies, eight *Eco*RI + 3 bases/*Mse*I + 3 base primer sets (Figure 6.2a) were successfully amplified for the *Spathoglottis* species whereas eleven *Eco*RI + 3 bases/*Mse*I + 3 bases primer sets were amplified for the *Spathoglottis plicata* population work. In general, the AFLP markers results were able to display the genetic differences among the five *Spathoglottis* species native to Peninsular Malaysia, five additional species, a hybrid of *Spathoglottis;* and the out group of *Dendrobium affine* according to their morphological characters. The morphological trait of flower colour was in agreement with the genetic data. The AFLP analysis results suggests that the markers can be used across a wide range of sampling methodologies and are also a quick way of resolving the identity of either the species or the hybrids.

The AFLP markers were also able to reveal the genetic variation among and between the six *Spathoglottis plicata* populations of Selangor, Kedah, Terengganu, Pahang, Negeri Sembilan and Johor. There was a unique AMOVA result from the populations of Terengganu and Pahang with 39.75% variation among the two populations and high variation of 60.25% within the populations. Thus, there is need to understand the detailed genetic relationship between the individuals of these two populations in terms of future conservation planning.

The result shown in Table 8.1 for the three molecular markers studied represents a useful first step towards elucidating the genetic structure within the *Spathoglottis* species with each marker having its own advantages and limitations. Some aspects may need continued improvement for example it will be recommended to optimise single and combination IRAP primers additionally due to the differences between LTR sequences of barley and genus *Spathoglottis*; cloning and sequencing of *Spathoglottis* RTNs for future RTN-marker based genome analysis will potentially add even more

information for the characterization of this genus. This study can also help to establish the use of chloroplast DNA markers as a molecular tool to help in species identification for taxonomy at the molecular level for applications in conservation, breeding and varietal protection. AFLP markers on the other hand are highly informative markers that will allow for the documentation of genetic variation among and within the *Spathoglottis* species and thus aid in their conservation. In general the results show the utility of each of the systems and all three markers can be used on their own or in combination depending on the capacity or needs of the institutions. However, authors recommended that the AFLP was the best molecular marker to studies the genetic for *Spathoglottis* species in Peninsular Malaysia.

The results from this study suggests that conservation strategies either *ex situ* or *in situ* have to be developed for this species taking into account the diversity and variation that has been demonstrated. In the meantime conservation technologies also need to be developed to ensure that no species becomes extinct from the wild, while strategic for protection are being put into place. As an example an in situ technology that could be used immediately is demonstrated in this study through the development of a cryopreservation technique to preserve *Spathoglottis* plant materials.

To develop a robust, internationally accepted strategy using the approaches developed in this study it is recommended that future extended work is conducted on the morphology and molecular genetics of *Spathoglottis* with larger numbers of species; extended number of samples of each species and more individuals of *Spathoglottis* species or their cultivars as well as wider geographical coverage. It is necessary to have networking with researchers from Malaysia with other countries where *Spathoglottis* is native especially in the Asia Pacific region. Through the examination of larger numbers of sample per species and population, the *Spathoglottis* morphological characterizations could be further validated and a detailed international descriptor could be developed. It

could be further used in establishing information on genetic diversity and ultimately in tandem with other approaches such as molecular markers described in this study, for introducing varietal protection and conservation strategies and legislation. Ultimately these studies could also overcome the taxonomic challenge of the *Spathoglottis* genus.

Table 8.1: Marker systems developed for *Spathoglottis* species and their application

Marker system used	**Summary information**	**Advantage/Limitation**	**Application**
Inter-Retrotransposon Amplified Polymorphim (IRAP)	Amplified and identified twelve single and combined IRAP primers: LTR 6150, 3'LTR, Sukkula, LTR 6149+Sukkula, LTR 6149+Nikita, LTR 6149+3'LTR, LTR 6150+Nikita, LTR 6150+3'LTR, 5'LTR1+3'LTR, 5'LTR2+3'LTR, 5'LTR2+Nikita and 3'LTR+Nikita. Indicated 17-65% similarity among the Malaysian native *Spathoglottis* species. *Spathoglottis* species were in Cluster 1 and divided into two groups, where *S. plicata* was in Group 1 flower colour purple. Whereas, Group 2 included all the native Malaysian species of *S. aurea, S. gracilis, S. affinis* and *S. microchilina* flower colour yellow.	Retrotransposon-based markers. Requires neither restriction enzyme digestion nor ligation to generate the marker bands. Retrotransposons are long and produce large genetic change at the point of insertion.	As markers to detect genotypes, measure diversity or reconstruct phylogeny. Can be applied in a similar way for DNA fingerprinting, genetic diversity, genome mapping and gene tagging in plants.
DNA Barcoding/ Chloroplast DNA	Amplified four coding regions of *matK*, *rbcL-a*, *rpoB* and *rpoC1* regions. Combination of *rbcL-a*, *rpoB* and *rpoC1* regions generated eight haplotype with 28 intraspecific variable sites.	Requires minute amount of sample to identify a species accurately using DNA. Determined from the complete DNA sequences for nuclear, mitochondrial and chloroplast genomes.	Method in the identification of new species. Marker for phylogenetic relationships studies between closely related species.

	Spathoglottis species were Clustered and divided into two groups, where *S. plicata* in Group 1 which the flower colour are purple. Whereas, Group 2 included all the native Malaysian species of *S. aurea, S. gracilis, S. affinis* and *S. microchilina* flower colour are yellow.	Examining the sequences from a standard DNA region ("gene") for species identification. May have resolution limitations depending on species and region used.	
Amplified Fragment Length Polymorphism (AFLP)	Eight *Eco*RI + 3 bases/*Mse*I + 3 bases primer sets amplified in *Spathoglottis* species. *Spathoglottis* species were in Cluster 1 and divided into two groups, where *S. plicata* in Group 1 flower colour purple. Whereas, Group 2 included all the native Malaysian species of *S. aurea, S. gracilis, S. affinis* and *S. microchilina* flower colour yellow. Eleven *Eco*RI + 3 bases/*Mse*I + 3 bases primer sets were amplified for the *Spathoglottis plicata* population. The *S. plicata* population were indicated as Negeri Sembilan and Johor population in the same genetic groups; The Kedah and Selangor population represented another genetic group. Whereas, the Terengganu and Pahang population had a mixture of genetic groups.	Capability to detect various polymorphisms in different genomic regions. PCR-based tool used in genetics research and DNA fingerprinting. Uses restriction enzymes *Eco*RI and *Mse*I to digest genomic DNA. Reproducibility and robustness where small amounts of DNA are digested and detection of AFLP fragments does not depend on hybridization.	Identification of genetic variation among and between species and population. Determine the genetic diversity, population structure and phylogenetic inference.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

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