

CONVERSION OF RAPD MARKERS TO CO-DOMINANT BASED  
SEQUENCE CHARACTERIZED AMPLIFIED REGION (SCAR)  
MARKERS IN THREE *Paphiopedilum* SPECIES

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THIS THESIS IS SUBMITTED TO FULFILL THE REQUIREMENT TO OBTAIN A  
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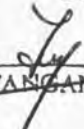
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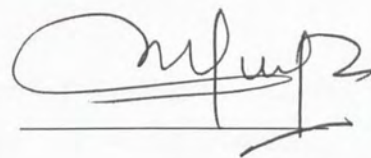
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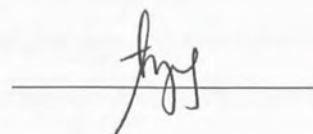
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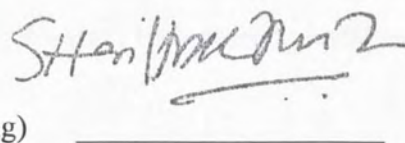
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## ABSTRACT

The genus *Paphiopedilum* is endangered species protected under Convention on International Trade in Endangered Species (CITES). However, the discrimination between the species is difficult due to their similar morphological characters. Therefore, RAPD assay were applied to detect polymorphisms between the three species: *P. rothschildianum*, *P. dayanum* and *P. lowii*. Diagnostic fragments identified for discrimination for each species were converted into SCAR markers by cloning and sequencing the diagnostic fragments and designing SCAR primer pairs to amplify the diagnostic fragments. The reproducibility of these markers was analysed by performing diagnostic PCRs. In this study, PCR amplification with RAPD primers was successful in detecting polymorphisms between species. Three diagnostic fragments were converted into SCAR markers and all primer pairs amplified single distinct bands whose sizes were the same as expected fragments. One of the SCAR primers amplified the expected product size in *P. dayanum* and in one of the *P. rothschildianum* samples. When digested with restriction enzyme *DpnII* found polymorphisms in fragment length between the two samples. In the other two cases, the products of the expected fragments were amplified in *P. lowii* and all samples of *P. rothschildianum* and subsequent RE digestion failed to detect polymorphisms between the species. The overall findings had shown discovery of two SCAR markers to identify *P. rothschildianum* and *P. lowii* apart from *P. dayanum* and one SCAR marker to identify *P. dayanum* and its possible hybrids. Hence, more studies and screenings need to be done in order to develop species-specific markers for the *Paphiopedilum* species.



## ABSTRAK

PENGUBAHAN PENANDA RAPD KEPADA KO-DOMINAN PENANDA  
*SEQUENCE CHARACTERIZED AMPLIFIED REGION* (SCAR)  
DALAM TIGA SPESIS *Paphiopedilum*

Genera *Paphiopedilum* merupakan spesies terancam yang dilindungi di bawah Convention on International Trade in Endangered Species (CITES). Walau bagaimanapun, pengecaman antara spesies adalah sukar disebabkan persamaan ciri-ciri morfologi. Oleh itu, teknik RAPD digunakan untuk mengesan polimorfisma antara tiga spesies: *P. dayanum*, *P. lowii* dan *P. rothschildianum*. Fragmen diagnostik yang dikesan untuk pengecaman setiap spesies akan diubah menjadi penanda SCAR melalui cara pengklonan dan *sequencing* fragmen diagnostik serta mencipta pasangan primer SCAR untuk mengamplifikasi fragmen-fragmen diagnostik tersebut. Kebolehan penanda yang dicipta menghasilkan fragmen diagnostik diuji melalui PCR diagnostik. Dalam kajian ini, amplifikasi PCR menggunakan primer-primer RAPD telah berjaya mengesan polimorfisma antara spesies. Tiga fragmen diagnostik telah diubah menjadi penanda SCAR dan semua pasangan primer mengamplifikasi satu fragmen spesifik di mana saiz fragmen adalah sama dengan fragmen yang dijangkakan. Satu daripada pasangan primer telah mengamplifikasi saiz fragmen yang dijangkakan dalam spesies *P. dayanum* dan salah satu sampel *P. rothschildianum*. Apabila dipotong dengan enzim pembatasan *DpnII*, didapati terdapat polimorfisma antara saiz fragmen antara kedua-dua sampel. Dalam dua kes yang lain, produk untuk saiz yang dijangkakan telah diamplifikasikan dalam *P. lowii* dan semua sampel *P. rothschildianum* serta diikuti pembatasan telah gagal untuk mengesan polimorfisma antara spesies. Keputusan keseluruhan mendapati penemuan dua penanda SCAR untuk membezakan *P. rothschildianum* dan *P. lowii* daripada *P. dayanum* dan satu penanda SCAR untuk mengesan *P. dayanum* dan hibridnya. Maka, lebih kajian dan pengesanan perlu dijalankan untuk menghasilkan penanda yang spesifik untuk mengesan spesies-spesies *Paphiopedilum*.



## CONTENT

	Pages
DECLARATION	i
CERTIFIED BY	ii
ACKNOWLEDGEMENT	iii
ABSTRACT	iv
ABSTRAK	v
CONTENT	vi
LIST OF TABLES	viii
LIST OF FIGURES	ix
LIST OF ABBREVIATIONS & SYMBOLS	x
<b>CHAPTER 1 INTRODUCTION</b>	<b>1</b>
1.1 Introduction	1
1.2 Objectives	3
<b>CHAPTER 2 LITERATURE REVIEW</b>	<b>5</b>
2.1 Genus <i>Paphiopedilum</i>	5
2.1.1 Background	5
2.1.2 Distribution and Ecology	6
2.1.3 Vegetative Morphology	7
2.1.4 Floral Morphology	8
2.2 <i>Paphiopedilum</i> Species	9
2.2.1 <i>Paphiopedilum rothschildianum</i>	9
2.2.2 <i>Paphiopedilum lowii</i>	10
2.2.3 <i>Paphiopedilum dayanum</i>	12
2.3 <i>Paphiopedilum</i> on CITES	13
2.4 Distinguishing between Wild and Artificially Propagated <i>Paphiopedilum</i>	15
2.5 Random Amplified Polymorphic DNA (RAPD)	16
2.5.1 Application of RAPD Analysis	18
2.6 Sequence Characterized Amplified Region (SCAR)	22
<b>CHAPTER 3 METHODOLOGY</b>	<b>25</b>
3.1 Plant Materials	25





3.2	Methods	26
3.2.1	DNA Extraction	26
3.2.2	Polymerase Chain Reaction (PCR)	27
3.2.3	Gel Extraction	28
3.2.4	DNA Cloning	29
3.2.5	Plasmid Mini-prep	30
3.2.6	Sequence Analysis & Data Analysis	31
3.2.7	SCAR Primers Designing	31
3.2.8	SCAR Primers Testing	32
<b>CHAPTER 4</b>	<b>RESULTS AND DATA ANALYSIS</b>	<b>34</b>
4.1	DNA Extraction	34
4.2	PCR Amplification	36
4.3	Gel Extraction	43
4.4	DNA Cloning	45
4.5	Plasmid Mini-prep	46
4.6	Sequence Analysis	49
4.7	Data Analysis	52
4.8	SCAR Primers Design	54
4.9	SCAR Primers Testing	55
<b>CHAPTER 5</b>	<b>DISCUSSION</b>	<b>60</b>
5.1	DNA Extraction	60
5.2	PCR Amplification	61
5.3	Gel Extraction	62
5.4	DNA Cloning for Diagnostic Fragments	63
5.5	Plasmid Mini-prep	64
5.6	Sequence Analysis	64
5.7	Data Analysis	65
5.8	SCAR Primers Design	66
5.9	SCAR Primers Testing	67
<b>CHAPTER 6</b>	<b>CONCLUSION</b>	<b>71</b>
	REFERENCES	74
	APPENDIXES	79



## LIST OF TABLES

Table No.		Page
2.1	Differences between wild and artificial <i>Paphiopedilum</i>	16
3.1	The incubation period and temperature of restriction enzyme <i>DpnII</i> used with its recognition sites	33
4.1	Band coring and reproducibility rate for RAPD-PCR of OPA2	38
4.2	Band coring and reproducibility rate for RAPD-PCR of OPA12	40
4.3	Band coring and reproducibility rate for RAPD-PCR of OPA18	42
4.4	The count of colonies in blue-white screening and efficiency rate	45
4.5	DNA sequence of diagnostic fragment of <i>P. rothschildianum</i>	49
4.6	DNA sequence of diagnostic fragment of <i>P. dayanum</i>	50
4.7	DNA sequence of diagnostic fragment of <i>P. lowii</i>	51
4.8	Sequence definition and their respective accession number in GenBank	52
4.9	Putitive alignment of nucleotide sequence encoded by PRO2-1, PDO12-20 and PLO18-1 using Blastn algorithm	53
4.10	Primer sequences for the SCARs and their annealing temperature	55



## LIST OF FIGURES

Figure No.	Page
4.1 Electrophoresis analysis of DNA extracted from three <i>Paphiopedilum</i> species	35
4.2 Electrophoresis analysis of banding profiles of RAPD-PCR of OPA2	39
4.3 Electrophoresis analysis of banding profiles of RAPD-PCR of OPA12	41
4.4 Electrophoresis analysis of banding profiles of RAPD-PCR of OPA18	41
4.5 Electrophoresis analysis of gel purification of extracted PCR products	44
4.6 Electrophoresis analysis of plasmid mini-prep for <i>P. rothschildianum</i> diagnostic band	47
4.7 Electrophoresis analysis of plasmid digestion with restriction enzyme <i>EcoRI</i>	47
4.8 Electrophoresis analysis of plasmid mini-prep for <i>P. dayanum</i> diagnostic band	48
4.9 Electrophoresis analysis of plasmid mini-prep for <i>P. lowii</i> diagnostic band	48
4.10 Electrophoresis analysis of SCAR-PCR amplification of SPRO2-1	56
4.11 Electrophoresis analysis of RFLP with <i>DpnII</i> for SPRO2-1 amplification products	56
4.12 Electrophoresis analysis of SCAR-PCR amplification of SPDO12-20	58
4.13 Electrophoresis analysis of RFLP with <i>DpnII</i> for SPDO12-20 amplification products	58
4.14 Electrophoresis analysis of SCAR-PCR amplification of SPLO18-1	59
4.15 Electrophoresis analysis of RFLP with <i>DpnII</i> for SPLO18-1 amplification products	59



## LIST OF ABBREVIATIONS & SYMBOLS

bp	base pair
g	gram
kb	kilo base
M	molar
ng	nanogram
mM	millimolar
μl	microlitre
rpm	revolution per minute
pmol	picomole
%	percent
°C	degree Celcius
dH <sub>2</sub> O	de-ionized distilled water
DNA	Deoxyribonucleic
dNTP	Deoxynucleoside triphosphate
EDTA	Ethylenediamine tetraacetic acid
MgCl <sub>2</sub>	Magnesium chloride
min	minutes
NaCl	Natrium chloride
PCR	Polymerase Chain Reaction
RAPD	Random Amplified Polymorphic DNA
RE	Restriction Enzymes
RFLP	Restriction Fragment Length Polymorphism
RNase	Ribonuclease
s	seconds
SCAR	Sequence Characterized Amplified Region
SDS	Sodium dodecyl sulphate
<i>Taq</i>	<i>Thermus aquaticus</i>
TBE	Tris Boric EDTA
TE	Tris-HCl EDTA
Tris	Tris (hydroxymethyl) aminomethane





Tris HCl	Tris (hydroxymethyl) aminomethane hydrochloride
U	unit
V	volt



## CHAPTER 1

### INTRODUCTION

#### 1.1 Introduction

The genus *Paphiopedilum* belongs to the family Orchidaceae and subfamily Cyripedioideae. *Paphiopedilum* is known as the slipper orchids because their lip resembles a lady's slipper. The range of *Paphiopedilum* species are found from India eastward across southern China to the Philippines and throughout south-east Asia and the Malay Archipelago to New Guinea and the Solomon Islands (Cribb, 1998). In Malaysia, the *Paphiopedilum* or slipper genus, one of the rarest orchids, is found only in a remote part of Mount Kinabalu.

*Paphiopedilum* orchids are listed on Appendix I of the Convention on International Trade in Endangered Species (CITES), under which trade is banned or regulated with special permits. This increases their desirability of the *Paphiopedilum* species and causes it to be hunted by orchid collectors. These collectors are obsessed with the *Paphiopedilum* species to the extend of committing crime by smuggling. For instance, in the year 2004, the head of research and development of Medpharm Ltd.,



a pharmaceutical company, was sentenced to 50 months in jail by the British court for smuggling 126 rare and protected slipper orchids, including the rare Sabah species, into Britain (John & Kaur, 2006)

The *Paphiopedilum* genus is protected under local legislation as well. In Sarawak, all orchids are protected species under the Wildlife Protection Ordinance established at year 1998. Meanwhile, in Sabah, the *Paphiopedilum* species are totally protected under the Wildlife Conservation Enactment established at year 1997. The penalty of those in possession of the species is a maximum fine of RM50,000, five years' jail or both. However, local communities continued to collect and sell the *Paphiopedilum* species for as low as RM5 to the tourists which are illegal and violate the value of the *Paphiopedilum* species.

Leaf shape in orchids is relatively uniform for many genera where most orchids have simple, linear, lanceolate, oblanceolate, ovate, or elliptic leaves. The *Paphiopedilum* species also have regular leaf morphology similar to the ordinary orchid genus when the plant is not flowering. Therefore, it is hard to determine the *Paphiopedilum* species morphologically. Hence, it is difficult to stem smuggling.

There have been a few studies using Random Amplified Polymorphic DNA (RAPD) analysis to determine the genetic diversity and relationship between *Paphiopedilum* and *Phragmipedium* species and cultivars (Chung *et al.*, 2006) and a preliminary analysis of the level and apportionment of genetic diversity in *Paphiopedilum micranthum* using RAPD analysis (Li *et al.*, 2002). Thus far, there is no study has been made for species identification for *Paphiopedilum* species.





Therefore, in this study, RAPD markers are used to create species-specific SCAR markers for differentiation of each of the *Paphiopedilum* species.

RAPD markers work by amplifying random sequences from a DNA template with a single arbitrary 10-mer primer. RAPD markers are dominant because polymorphisms are detected as the presence or absence of bands. Thus, it could not differentiate between homologous and heterozygous loci. Therefore, RAPD markers are transformed into a codominant marker called Sequence Characterized Amplified Region (SCAR) which allows for a reproducible amplification of a single RAPD fragment. SCAR markers are PCR-based markers that represent single, genetically defined loci that are identified by PCR amplification of genomic DNA with pairs of specific oligonucleotide primers. The sequence of the SCAR marker is designed from the amplified RAPD products that were cloned and sequenced.

In this study, species specific codominant RAPD-SCAR markers will be designed for three *Paphiopedilum* species: *P. rothschildianum*, *P. lowii* and *P. dayanum* for identification of each of these *Paphiopedilum* species.

## 1.2 Objectives

- a. To determine specific Random Amplified Polymorphic DNA (RAPD) polymorphic markers among *Paphiopedilum lowii*, *P. dayanum* and *P. rothschildianum*.





- b. To convert the diagnostic RAPD marker for *P. lowii*, *P. dayanum* and *P. rothschildianum* into species-specific Sequence Characterized Amplified Region (SCAR) markers by performing cloning, sequencing and primer design.
  
- c. To test for reproducibility of the species-specific SCAR markers on *P. lowii*, *P. dayanum* and *P. rothschildianum* by PCR amplification.



## CHAPTER 2

### LITERATURE REVIEW

#### 2.1 Genus *Paphiopedilum*

##### 2.1.1 Background

*Paphiopedilum* derives from the Greek 'Paphio', an epithet for Aphrodite (the goddess known as Venus to the Romans), and 'pedilon' meaning slipper. Thus, the genus *Paphiopedilum* is also known as the slipper orchids, so called for the shape of the deeply saccate lip of their flower, represent a small but remarkable offshoot of the main line of orchid evolution (Cribb, 1998).

*Paphiopedilum* is the largest genus of slipper orchids with some 80 species occurring in the Asian tropics from southern India to New Guinea and the Philippines. They can be found growing on the ground, on rock and cliff surfaces and attached to trees and other vegetation. Most are ground-growing, growing in leaf litter or in cracks in rocks containing organic matter. They occur in a wide range of habitats from

branches of large trees in the rainforests of Thailand to the harsh serpentine soils on Mount Kinabalu (McGough *et al.*, 2006).

### 2.1.2 Distribution and Ecology

The range of *Paphiopedilum* extends from India eastward across southern China to the Philippines and throughout south-east Asia and the Malay Archipelago to New Guinea and the Solomon Islands. Of the seven Indian species, six are confined to the north-east, along the foothills of the Himalaya from eastern Nepal to the Naga Hills, and also in the Khasia Hills. Meanwhile, there are eighteen species have been reported from China (Pridgeon *et al.*, 1999).

The genus is well represented in the south-east Asia, notably in Thailand and Vietnam which can boast 12 species but with few in adjacent countries. In Borneo, there are twelve species of which seven are endemic. Meanwhile, Sumatra has ten species and six endemics, whereas Java has only three species and one endemic (Cribb, 1998).

The Philippines are another rich centre of diversity of the genus with eight species, seven of which are endemic. To the south and east the number of species drops rapidly. Four species have been recorded from Sulawesi and the Moluccas, one from each being endemic. In New Guinea four species, all endemic, are found with two reported from the Solomons (Pridgeon *et al.*, 1999).





Five species of *Paphiopedilum* have been reported as growing epiphytically; *P. parishii*, *P. lowii* and *P. villosum* are usually found growing on trees while *P. hirsutissimum* and *P. glanduliferum* are facultative epiphytes. The remaining species are either terrestrial or lithophytic (Cribb, 1998).

### 2.1.3 Vegetative Morphology

*Paphiopedilum* leaves are leathery with a prominent middle rib. The leaves are V shaped in cross-section. Leaves may be short and strap-like or oblong to linear. The leaves are usually short, less than 20 centimetres in length. An exception to this rule is the multiflowered group which includes species such as *Paphiopedilum sandermanum*, *P. rothschildianum* and *P. lowii*. Leaf colour ranges from plain or glossy green to mottled purple and can be quite useful in identification (McGough *et al.*, 2006).

The leaves of the *Paphiopedilum* are often distinctive and taxonomically useful at the subgeneric and even in some cases species level. Leaf shape varies from linear-ligulate in sections *Paphiopedilum* and *Coryopedilum* to elliptic-oblong in most of the species of subgenus *Brachypetalum*. The upper surface of the leaves is uniformly green in all the species of sections *Paphiopedilum* and *Coryopedilum*. Paler margins are characteristic of some species. Mottling or tessellation of the upper surface is characteristic of the species of subgenera *Parvisepalum* and *Brachypetalum*. The tessellation of the leaves of section *Barbata* species is distinct from that of *Brachypetalum*. Meanwhile, faint tessellations can be seen on the upper surface of *P. victoria-regina* and *P. victoria-mariae* in section *Cochlopetalum* but not at all in other species. The lower surface of the leaves is also distinctively marked in many species.





In most species of subgenera *Parvisepalum* and *Brachypetalum*, the leaves are densely purple-spotted beneath (Cribb, 1998).

#### 2.1.4 Floral Morphology

The most distinctive features of the *Paphiopedilum* plant are to be found in its flower. The flower has an inferior unilocular ovary with parietal placentation; a conspicuous upper or dorsal sepal and a less obvious synsepal formed by the united lateral sepals; two spreading petals that are usually deflexed or reflexed and often twisted; a deeply saccate ventral pouched lip, usually with more or less obvious side lobes; and a central short column bearing a ventral stalked trilobed stigma, two lateral and ventral anthers and an apical, usually shield-shaped, staminode (Cribb, 1998).

Petal shape, marking and indumentum are all taxonomically useful characters. In subgenera *Parvisepalum* and *Brachypetalum* the petals are more or less elliptic to circular in shape and usually less than twice as long as broad. Petals that taper from base to apex are found in all the species of section. In *Cochlopetalum* the petals are more or less linear, spreading and spirally twisted. The petal margins are also markedly ciliate, the ciliae being multicellular hairs. The remaining species in the genus have oblong to spatulate petals, the latter feature being particularly marked in species such as *P. lowii*, *P. haynaldianum*, *P. bullenianum*, *P. appletonianum* and *P. hookerae*.

The third petal in the flower of *Paphiopedilum* is highly modified to form a slipper-shaped lip. The lip is effectively three-lobed with mid-lobe deeply saccate and



the side-lobes infolded to give the lip an apparent claw. Four types of lips can be distinguished in the genus. An inflated, thin-textured and brightly coloured lip is characteristic of subgenus *Parvisepalum*. In subgenus *Brachypetalum* the lip is smaller, ovoid, and more solid-textured. In both these subgenera the apical margin is involute in the manner of many *Cypripedium* species and of *Phragmipedium besseae* and *P. schlimii*.

In section *Barbata*, *Cochlopetalum*, *Paphiopedilum* and *Pardalopetalum* the margin of the mid-lobe is not involute but the side-lobes are well developed and incurved to form a tube at the base of the lip. Finally, in section *Coryopedilum*, the side-lobes are much reduced and represented only by acute ear-like lobes that point into the saccate mid-lobe of the lip. In both the latter cases the form is paralleled in the genus *Phragmipedium* (Cribb, 1998).

## 2.2 *Paphiopedilum* Species

### 2.2.1 *Paphiopedilum rothschildianum*

The *P. rothschildianum* belongs to the subgenus of *Paphiopedilum* in section *Coryopedilum*. This orchid is endemic to Mount Kinabalu in north-east Borneo (Pridgeon *et al.*, 1999). Of all the species in the genus, *P. rothschildianum* is one of the rarest in nature. It has been located in only three sites on the lower slopes around Mount Kinabalu, in one of which it is certainly now extinct. It usually grows on ledges on steep slopes and cliffs of ultra-basic rock where it seems to thrive in the open as well



as in shaded places. Fortunately, *P. rothschildianum* grows only inside the Kinabalu Park and is afforded some protection.

*P. rothschildianum* is a terrestrial or lithophytic herb often growing in large clumps. The leaves of this specie are several, linear to narrowly oblanceolate, acute, up to 60 cm long, 4 to 5 cm wide, sparsely ciliate at base and green in colour. The inflorescence is 2 to 4-flowered and erect; the peduncle is up to 45 mm long and purplish while the bracts are ovate-elliptic, obtuse, up to 5.5 cm long, ciliate and hairy on mid-vein, pale green or yellow in colour with purple-striped. The flowers are very large within 14 to 30 cm in diameter while the ovary can be up to 7.5 cm long and pale green with sparsely spotted purple. The dorsal sepal is ovate, 5.4 to 6.8 cm long, 3.3 to 4.8 cm wide and in the shade of ivory-white or yellow with maroon veins. The synsepal is similar but smaller compare to dorsal sepal with 4.8 to 6 cm long and 3.2 to 4.4 cm wide. Petals are narrowly tapering to rounded apex, 8 to 14 cm long, 0.7 to 1.5 cm wide, ciliate, papillose towards apex and in the shade of yellow or ivory-white marked with maroon. The lip is subcorrect, grooved on back, 5.3 to 6 cm long, 2.2 to 2.5 cm wide and golden in colour with heavily purple-suffused. The staminode is linear, bifid at apex, geniculate, 14 to 16 mm long, 4 to 5 mm wide, densely glandular-pubescent on margins and at the base and pale yellow-green in colour (Cribb, 1998).

### 2.2.2 *Paphiopedilum lowii*

The *P. lowii* belongs to the subgenus of *Paphiopedilum* in the section of *Pardalopetalum*. This specie is the most widespread of the multiflowered species, found throughout the Malay Peninsular, Sumatra, Java, Borneo and the Celebes (Sulawesi)





(Pridgeon *et al.*, 1999). It is also one of the few epiphytic species in the genus, although it may also occasionally be found growing on rocks. *P. lowii* can be found growing at altitudes between 250 m and 1600 m in riverside, lower mountain and mountain rain-forest either as an epiphyte on the trunks and branches of trees or as a lithophyte in moss- or humus-filled hollows of rocks especially limestone and of boulders.

*P. lowii* is an epiphytic herb that grows on trees. Its leaves are commonly about four to six, linear-ligulate, unequally roundly bilobed at apex 22 to 40 cm long, 2.8 to 6 cm wide and mid-green in colour. The inflorescence is erect-arcuate and 3 to 7-flowered; the peduncle is green, mottled purple, shortly pubescent and up to 70 cm long while the bracts are elliptic, obtuse, 2 to 4.5 cm long, 2.2 cm wide and in the shade of yellow with purple marks. The flowers are 9 to 16.5 cm across while pedicel and ovary is 4.5 to 7 cm long and greenish in shade. The dorsal sepal is elliptic-ovate, obtuse, 3 to 5.5 cm long, 2.5 to 3.5 cm wide, undulate and ciliate on margins, pale green colour and mottled dull purple in basal half with recurved basal margins. The synsepal is elliptic, obtuse, 2.2 to 4.8 cm long, 2 to 2.8 cm wide, 2-keeled on outer surface and pale green in colour. The petals often once-twisted in middle, spatulate, subacute to obtuse, 5 to 9.3 cm long, 1.5 to 3.1 cm wide, ciliate and pale yellow in colour with a purple apical third and maroon-spotted in basal two-thirds. The lip is 3.5 to 6 cm long, 2.1 to 3.1 cm wide and in the shade of dull ochre-brown. The staminode is obovate, apically three-toothed with a long erect hook at the base, 10 mm long, 7 mm wide and pale ochre to brownish green in colour (Cribb, 1998).





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