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DETECTION OF GENETIC VARIATION IN SELECTED SABAH PARK'S GERMPLASM COLLECTION OF SLIPPER ORCHIDS *(Paphiopedilum rothschildianum)* USING RAPD MARKERS

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DECLARATION

I am declaring that this dissertation is the result of my own independent work, except where otherwise stated.

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ABSTRACT

Random Amplified Polymorphic DNA (RAPD) markers were used to detect genetic variation in selected Taman Sabah's germplasm collection of *Paphiopedianum rothschildianum*, an endangered slipper orchid in Sabah. Four populations of the species, which obtained from the locations including Penataran, Telupid, Kota Belud and Ampuan, were selected in the studied. A total of 46 amplified bands were scored from five RAPD markers and a mean of 9.2 per primer and the percentage of polymorphic bands was 93.48%. All populations of the species were found to share one or more RAPD markers with five primers. The cluster analysis of distance values was conducted to construct a dendrogram. The study revealed a moderate level of genetic polymorphism and variability among populations in *P. rothschildianum* and no direct relatonship between geographical distance and genetic variation between the four populations of *P. rothschildianum*.

ABSTRAK

Penanda DNA Polimorfik Amplifikasi Rawak (RAPD) telah digunakan untuk mengesan variasi genetik terhadap himpunan *Paphiopedilum rothschildianum* yang terpilih dari Taman Sabah, iaitu sejenis anggerik yang menghadapi bahaya kepupusan. Empat populasi spesies ini yang berasal dari Penataran, Telupid, Kota Belud dan Ampuan telah dipilih untuk kajian ini. Sejumlah 46 jalur amplifikasi daripada lima tanda RAPD telah diskor. Didapati nilai min mengikut primer adalah 9.2 dan peratusan jalur polimorfik adalah 93.48%. Semua populasi spesies ini didapati berkongsi lebih daripada satu penanda RAPD dengan lima primer yang digunakan. Analisis rangkaian untuk nilai-nilai jarak genetik telah dijalankan bagi membentuk satu dendrogram. Kajian ini telah menunjukkan bahawa terdapat paras polimorfik genetik dan variasi yang sederhana di antara populasi *P. rothschildianum* dan tiada hubungan yang wujud secara langsung bagi jarak geografik dan variasi genetik di antara empat populasi *P. rothschildianum*.

CONTENTS

CHAPTER 1

INTRODUCTION

Paphiopedilum rothschildianum is a rare, distinct slipper orchid and strictly distributed to a number of locations in Sabah only. The species is now classified as an endangered plant according to the The International Union for Convservation of Nature (IUCN) Red Book system. Therefore, research on the *P. rothschildianum*, especially on its genetic variation among populations from known locations is worthy to be carried out as an effort to understand its pattern of genetic variation and may constribute some ideas for other researchers who work on the conservation of the species.

Traditionally, genetic variation was inferred by morphology or growth response of organisms. However, morphological characters are often influenced by the environment. Since 1960's, enzyme electrophoresis was used as a method of estimating genetic variation based on enzyme variation of individuals. Allozymes have been used in the study of genetic variation at the protein level. The advantages of allozymes studies are low cost of chemical and labor and its nature of codominant. However, its limitations are a new allele would only be detected as a polymorphism if a nucleotide substitution has resulted in an amino acid substitution.

In the past decade, the measurement of genetic variation is carried out by modern molecular methods using markers such as Restriction Fragment Length Polymorphism (RFLPs), microsatellites and Random Amplified Polymorphic DNA (RAPDs) at the DNA level. The scope of the research is to detect the genetic variation among populations in selected Taman Sabah germplasm collection of *P. rothschildianum* using RAPD markers. The significant of studying the genetic diversity of *P. rothschildianum* is that the genetic knowledge can be applied to the design of conservation strategies for rare orchids, especially for the purpose of preservation of Sabah's natural heritage for future generation (Liew and Nais, 1997).

The genetics studies of rare plants can provide valuable insights into the patterns and extent of genetic diversity and provide clues about biology and evolutionary history of a species (Hogbin and Peakall, 1999). According to Koontz *et al.*, 2001, genetic markers provide a direct window into the variation present in a species and this have revealed differences among populations that are often masked by morphological similarity. Because of this, determining the levels and patterns of genetic diversity in rare species is even more important for developing well-informed management strategies. It suggests that the population of species is threatened by extinction, as the loss of genetic variability elements is irreversible. The entire population can hardly have a chance to recover in a short span from the sudden diminishment in genetic diversity cause by drift. Therefore, the species face with a critical need for conservation.

1.1 Objective of Research

The goal of the research is to detect the level of genetic variability in *P. rothschildianum* by RAPD markers. Therefore, the objectives are:

1. To extract total genome of *P. rothschildianum* using CTAB method.
2. To amplify DNA of *P. rothschildianum* obtained from four populations in Sabah, namely, Penataran, Ampuan, Telupid and Kota Belud using RAPD-PCR.
3. To detect genetic variation of *P. rothschildianum* among populations using RAPDistance program (version 1.04).
4. To construct a dendrogram and carry out cluster analysis.

CHAPTER 2

LITERATURE REVIEWS

2.1 Taxonomy of *Paphiopedilum* Family

The Orchidaceae is considered the largest family of plants in the world (20,000 and 30,000 species) while Sabah houses about 1500-2000 species of orchid in 143 genera (Lamb, 1996). *Paphiopedilum* is derived from the Greek 'Paphian' meaning an epithet for Aphrodite and 'pedilon' meaning slipper (Yong, 1990). There are seven subgenera in *Paphiopedilum*, including *Barbata, Pardalopetalum, Coryopedilum, Paphiopedilum, Cochlopetalum, Concoloria and Parvisepalum* (Cribb, 1997). The evolutionary relationship from ITS sequence in the genus *Paphiopedilum* is given in Appendix 2. *P. rothschildianum* is the *Paphiopedilum* subgenus of subgenera *Corypedilum*. All *Paphiopedilum* produce large flowers on small plants. Moreover, it is a genus of tropical Asiatic origin (Cribb, 1997).

Slipper ochids are characterized by their curious flowers and gain their common name from their slipper-shaped lower petal. The plants have very short stems bearing a number of opposite and alternate arranged leaves. All of the Bornean slipper ochids are rare and their habitat is strictly limited, their colonies usually being small and widely separated (Cribb, 1998).

2.2 Distribution Pattern of *Paphiopedilum rothschildianum*

P. rothschildianum, which has been called "aristocrat of all slipper orchids", was first discovered by Baron Ferdinand de Rothschild. The founder of this plant, Baron Ferdinand de Rothschild is an eminant Victorian orchid grower and has the distinction of having the most spectacular, has the distinction of having the most spectacular orchid in the genus named after him. Another important contribution came from J. Waterstradt, who was an orchid collector for Rothschild, has discovered the presence of *P. rothschildianum* in Mt.Kinabalu in 1894. In addition, Rolfe of Kew Herbarium has done later review of the species (Cribb, 1997).

Geographically, *P. rothschildianum* has a narrow distribution pattern (Appendix I). It is mainly found in New Guinea and in the east Malaysian mountian of Mount Kinabalu. In Sabah, it is narrowly endemic to a mountain range of 600-1200 m altitude. Considering of spatial patterns of species richness, there is a relationship between species richness and an increase in latitude or altitude (Lott and Winiger, 1996).

In fact, *P. rothschildianum* is classified as an endangered plant species and has only been found in three localities, one of which was destroyed by fire few years ago (Cribb, 1997). Therefore, it needs to be protected from becoming extinct. According to the The International Union for Convservation of Nature (IUCN) Red Book system, a species can be classified as safe, vulnerable, endangered and critical depending upon its perceived degree of risk. This can be done by noting the probability of its declining by a specific percentage in the next fifty years. For an endangered species, its observed decline is 50% in 10 years or 3 generations, its geographical range is under 500 m^2 or 5 locations, its total population (N) less than 2500 and extinction probability exceed 20 % in 20 years or 5 generations (Dobson, 1996).

2.3 Biology of *Paphiopedilum rothschildianum*

Flowering occurs at the end of the rains in most Bornean slipper orchids. February to April is the best season to see slipper orchids in flower but flowering can also occur in November. Flowers normally can last for between 4-8 weeks. The plant can live for many years. Overall, its peak flowering time in north temperate countries is 4-6 months.

P. rothschildianum has a chromosome complement of 2n = 26. It is a distinct species thus easilly to be identified. The leaves of *P. rothschildianum* are distinctive and its upper surface is uniformly green. Leaves can grow more than a meter. It has

long petals, spreading widely, and horizontally giving flower a spread of 18-32 cm. Its staminode is linear and often bifid at apex (Cribb, 1998).

The floral fragrance of *P. rothschildianum* emanates from the centre of flower. The flower will emit a peppery fragrance to attract its pollinator. Pollinator of this species was found to be the syrphid fly, *Dideopsis aegrota.* A number of hybrids have been found including Kimballinum (*P. dayanum x P. rothschildianum)* and *P.Transvaal (P. rothschildianum x P. chamberlainianum* (Cribb, 1998).

2.4 Genomic DNA of Orchid

Plant cells contain separate genome in the nucleic, chloroplast and mitochondria. Thus, the genetic information is contained within three different organelles. As in plant, total genomes contain chloroplast DNA (cpDNA) and mitochondrial DNA (mtDNA). The plant mtDNA may be homogenous or heterogenous and it could be made up of several different large circular DNA molecules. There is a number of techniques to isolate genomic DNA such as boiling, alkaline extraction, chelating resin, protein digestion and so on (Hoelzel, 1998).

2.5 Measurement of Genetic Diversity

According to the definition of IUCN, genetic diversity is defined as the range of genetic material found in the world's organisms while species diversity is the variation between species of one region (Virchm, 1999).

The genetic diversity can occur among populations of a species with different ecological and life history features. If one population is undergone a large decrease in size, the population is expected to lose genetic variation, consequently, individual fitness, resistance and parasites, and its ability to respond to the environmental will be declined (Belliger *et al.*, 2003). In addition, the main determinants of this genetic reduction are human induce activity, natural catastrophes, mutation and adaptation.

Genetic difference occurs among individual within population of a plant. Individuals within a population share some percentage of alleles. The shared portion of the gene pool can be divided into two basic classes of genes, namely monomorphic gene that is common and essential to all individual. In plant, the monomorphic proportion of the total genome is 50 %. On the other hand, polymorphic genes are only specific to an individual.

Therefore, each species distributes its genetic diversity in a pattern reflecting its biology and can be valued by measuring its total alleles at all loci. Even if

variation within a population is low but variability among population could be in the contrary.

Variety among population could be quatified by statistic including Wright's inbreeding coefficiet (FST) and Nei's coefficient of gene variation (GST). These indices can show how heterozygosity is partitioned among populations based on differences in allele frequencies.

2.6 Research of Orchid using RAPD markers

There is a number of researchs on plant species using RAPD markers on orchid (Chen *et al.*, 1995). For example, an endangered orchid, *P. miracrathum* has been studied by using RAPD markers. In addition, RAPD technique also has been used in genotypic identification of breeding lines, hybrids and clones in many plants (Filippis *et al.*, 1996).

A recent study on *Paphiopedilum sp.* using RAPD markers to analyse the genetic variabilities has been carried out (Aung and Tan, 1997). In the study, the researchers have selected seventeen Asian slipper orchids (*Paphiopedilum* sp.) for the population studies. They found that all species shared one or more RAPD markers with 17 primers out of 30 primers used and amplified DNA ranged from 0.2 to 2.8 kbp.

The RAPD act as an effective genetic marker and the amplified RAPD product always segregate in a Mendelian fashion. In the research, Filippis *et al.*, (1996) found that RAPD-PCR has some limitations such as the concentratin of $MgCl_2$ must be optimised for each combination of primer-template tested in order to maximise the generation of DNA products consistently. RAPD also can be applied to delineate genetic difference between individuals, but without providing information about the region amplified. It works by detecting the presence of absence of a DNA sequence and generate polymorphism (Li *et al.*, 2001).

RAPD markers have been demonstrated to be an useful genetic markers for a variety of eukaryotic organisms, including humans, fungi and plants. Besides, RAPD has been applied in analysing individual protoplasts and thus provide a screening method for identification, selection and possible suitability for culture of fused protoplast (Monna *et al.*, 1998). However, RAPD markers are dominant markers, and dominant markers are less informative. Dominant markers have lower information content than co-dominant ones (Wang and Porter, 2001). The method has been utilized for setting of the genetic markers for segregation analysis in yellow birch, tomato, conifers, wheat, lettuce and rice. Besides, RAPD of *Pratylenchus* populations from coffee, banana and citrus have been desmostrated by Renata and Mario in their papers (Renata and Mario, 1996).

2.7 Sample Preservation and Preparation

For sample collection, there is a number of important considerations need to done in order to get a good source of DNA. It is because plant material varies in structure and chemical composition and these compounds, including polysaccarides and secodary metabolites that are the main causes of problems in DNA extration, vary among organs, tissue types, and tissue age in the plant.

The preservation method plays an important role in preparing a good quality of DNA extraction. Specimens must be collected from the environment, and any exogenous contamination has to be removed. The yield and quality of DNA is affected by the condition of the original tissue. By the way, it is best to harvest the freshest material possible.

Freshly harvested materials are to be kept cool and moist in an ice chest, freezing does subsequent preservation. Once frozen tissue should remain frozen until thawed in an extraction buffer. It is important to protect DNA from degradation. Once the leaves are pluck away from original plant, it starts the process of degradation. This is because the leaves will start degrades by cellular enzyme once is harvested.

Once the leaf samples are collected, silica gel technique is immediately applied to preserve the leaves. Silica gel is a blue crystal with a high water-absorbing

capacity. It is an expensive dessicant, but can be used indefinitely. However, it must be used in an airtight container to be effective. If the silica gel becomes saturated with moisture from the air, it will not have the capacity to dry plant materials. As silica gel absorbs moisture, it turns pale blue-gray or even pinkish gray. Because the silica gel will give the effect of rapid drying, thefore, silica gel technique can be used to preserve orchid leaf before return to the laboratory for further treatment of freezing at –80°C.

2.8 Genomic DNA Extraction

According to Michels (2002), the very first step in the preparation of DNA extracts is to rupture the cells and release the contents. Extraction of DNA for a particular species is difficult when the species is high in polysaccharides or secondary metabolites. In the case of plant, the optimal technique is achieved by detergent isolation techniques including SDS isolation technique and CTAB (cetyl trimethyl ammonium bromide) method (Tachi *et al.*, 2003). In the case of *Phalaenosis* Orchid, its DNA has been extracted using a modified method of Gawel and Jarret (1991) (Chen *et al.*, 1995).

The CTAB buffer contains detergents that lyse the cell membranes. CTAB is a cationic detergent, it is used to solubized the plants membranea and form a complex with DNA.This method is chosen for the study because the extensive preparation of the plants tissues is not required and is adaptable to numerous types of tissue

including leaves, roots, seeds, embryos, endosperm, pollen and suspension cultures (Hoelzel, 1998).

In addition, detergent is a substance, which is effective in solubilization of membranes without the loss of biological activity, and it acts as a protein denaturant. The PVP of the buffer will bind up polysacharides found in the plant tissue that may co-precipitate with the DNA and PVP can decrease the effect of polyphenols, quinines and tannins (Hoelzel, 1998).

In the DNA extraction, major problems which encountered are that intact DNA must be isolated from a complex and unknown chemical mixture, DNA degradation due to the presence of native DNase, secondary plant compounds and polysaccharide contaminants. The function of essential components of DNA isolation buffer used for plant material is to protect the DNA from degradatation by native enzyme or secondary compound released due to the disruption of the cell (Milligan, 1998).

The extracted DNA of all samples must be undergone qualitative and quantitative analysis in order to ensure that the concentration is high enough and the DNA is in high purity derived of any contaminants such as proteins, RNAs and polysaccharides. This is imperative for subsequent analysis such as PCR-RAPD. Generally, this can be done by examining UV absorbance with a spectrophotometer, by fluorimeter, or by comparison with DNA standards on agarose gels (Milligan, 1998).

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