



**Faculty of Resource Science and Technology**

**DNA PROFILING OF *CANARIUM ODONTOPHYLLUM* MIQ. ACCESSIONS USING  
RAPD MARKERS**

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## LIST OF ABBREVIATIONS

bp	basepair
CIA	Chloroform: Isoamylalcohol
CTAB	Cetyl Trimethylammonium Bromide
DNA	Deoxyribonucleic Acid
dNTPs	Deoxynucleotide-triophosphates
g	gram
kb	kilo basepair
ml	mililiter
mM	milimolar
ng	nanogram
PCR	Polymerase Chain Reaction
pmol	picomole
RAPD	Random Amplified Polymorphic DNA
RAPD-PCR	Random Amplified Polymorphic DNA-Polymerase Chain Reaction
rpm	revolutions per minute
SDS	Sodium Dodecyl Sulphate
TAE	Tris-Acetate-EDTA
UV	ultraviolet
w/v	weight/volume
$\mu$ l	microliter
$\mu$ M	micromolar
$\beta$	beta

# DNA Profiling of *Canarium Odontophyllum* Miq. Accessions using RAPD Markers

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## ABSTRACT

DNA profiling of *Canarium odontophyllum* Miq. accessions was carried out using Randomly Amplified Polymorphic DNA (RAPD) markers. Five RAPD primers that generate reproducible, informative and scorable DNA profile were selected to evaluate the genetic relatedness among *C. odontophyllum* accessions from two different locations namely Sarikei and Sibü. 81 loci were generated. Cluster analysis was carried out using unweighted pair-group method with arithmetic averages (UPGMA) had grouped 10 *C. odontophyllum* samples into two main clusters. The results revealed that samples D1, SBY2, SBW1, SBW2, SBW3, D4, and D2 were in Cluster I, while samples D3, SBY3, and SBY1 were in Cluster II. Cluster I contains mixture of samples from Sarikei and Sibü, while Cluster II contains samples from Sarikei only. This may be due to those samples share common alleles. This situation may be resulted from the movement of seeds across localities. It can be concluded that these samples may be originated from the same mother trees but cultivated at different locations.

Key words: *Canarium odontophyllum* Miq., DNA profiling, Randomly Amplified Polymorphic DNA (RAPD) markers, genetic relatedness, unweighted pair-group method with arithmetic averages (UPGMA).

## ABSTRAK

Pemprofilan DNA untuk akses *Canarium odontophyllum* Miq. telah dijalankan menggunakan penanda Randomly Amplified Polymorphic DNA (RAPD). Lima penanda RAPD yang menghasilkan profil DNA yang boleh diulang, informatif dan boleh diskor telah dipilih untuk menilai hubungan genetik antara akses *C. odontophyllum* daripada dua lokasi berlainan iaitu Sarikei dan Sibü. 81 lokus telah dihasilkan. Analisa kluster yang dijalankan menggunakan unweighted pair-group method with arithmetic averages (UPGMA) telah mengumpulkan 10 *C. odontophyllum* sampel kepada dua kluster utama. Keputusan menunjukkan sampel D1, SBY2, SBW1, SBW2, SBW3, D4, dan D2 berada dalam Kluster I, manakala sampel D3, SBY3, dan SBY1 berada dalam Kluster II. Kluster I mengandungi campuran sampel dari Sarikei dan Sibü, manakala Kluster II mengandungi sampel hanya dari Sarikei. Ini mungkin disebabkan sampel-sampel tersebut berkongsi alel yang sama. Situasi ini mungkin terjadi akibat pergerakan biji benih merentasi kawasan. Ini boleh dikonklusikan dengan menyatakan bahawa sampel-sampel tersebut berasal dari pokok induk yang sama tetapi telah dibiakkan di lokasi yang berbeza.

Kata kunci: *Canarium odontophyllum* Miq., pemprofilan DNA, penanda Randomly Amplified Polymorphic DNA (RAPD), hubungan genetik, unweighted pair-group method with arithmetic averages (UPGMA).

## CHAPTER I

### INTRODUCTION

Malaysia situated in tropical climate regions is blessed with rich natural resources. Sarawak which is the largest state in Malaysia has the richest biodiversity. Therefore, Sarawak still has large forest reserves that provide habitat for animals and unique plants such as *dabai* and *engkalak*. Nowadays, local fruits such as *dabai* are given high commercial value. This is due to their unique taste different from other common fruits. In Sarawak, the Department of Agriculture has identified *dabai* as a potential wild fruits for commercial trade.

According to Aman (1999), *dabai* or *Canarium odontophyllum* is a wild fruits species that belongs to family Burseraceae and genus *Canarium*. This genus has about 75 species. It is native to tropical Africa, from southern Nigeria east to Madagascar, Mauritius, India, southern China, Indonesia and Philippines. There are widely distributed in the lowland forest. In Sarawak, *C. odontophyllum* can be found in the upper Rejang River such as Sarikei, Sibul, Kapit and Limbang (Voon, 1998).

*C. odontophyllum* is an indigeneous fruit of Sarawak (Voon, 1998). This fruit is also known as ‘Sibu Olive’. Other names for this fruit are *kembayau* in Brunei, *kedondong*, *dabang*, *dawai*, *kurihang* and *danau majang*. In Sarawak, *C. odontophyllum* trees are grown to obtain its fruits. It is grown in orchards or can be found naturally along the riverbanks. Other species of *Canarium* are rich in oil and successfully applied to biodiesel generation in USA, Germany and Europe country (Voon & Kueh, 1999).

DNA-based markers are effective tool for the assessment and identification of the genetic relatedness among germplasm in many plant species (Weising *et al.*, 1995). One example of the DNA-based marker is RAPD or Randomly Amplified Polymorphic DNA. RAPD also can be categorized as PCR-based marker due to the use of PCR and this arbitrary primer to amplify DNA from total genomic DNA. According to Dassanayake and Samaranayake (2003), RAPD is a PCR-based technique that uses amplification-based scanning technique driven by arbitrary priming oligonucleotides to generate amplification products for a given strand of DNA. One advantage of using RAPD marker is prior knowledge of the DNA sequences is not needed. Thus, this marker is suitable for use in this study as the DNA sequence of *C. odontophyllum* is unknown. This technique is also preferable because it is fast and cheap. RAPD primers are commercially available and the method for its detection is easy using gel electrophoresis.

PCR or Polymerase Chain Reaction is an *in vitro* technique to amplify DNA quickly by incubation with special primers, DNA polymerase molecules, and other PCR ingredients (Campbell & Reece, 2002). This technique has become very important in DNA studies because it enables specific part in DNA to be selected and amplified exponentially (Elliot & Elliot, 2005). PCR procedure consists of three-step cycles which are denaturation, annealing and extension that bring about a chain reaction to produce DNA molecules exponentially. PCR has been successfully used in Human Genome Project and the use of this technique has been increasingly important in human health, evolutionary studies, modern systematics and ecological studies. In this study, RAPD markers were used as primers to bind specific sequences in *C. odontophyllum* DNA and the PCR amplified the sequences to detectable

amount. RAPD-PCR analysis then was used to establish the DNA profiles of *C. odontophyllum*.

DNA profiling or DNA fingerprinting or DNA typing is a technique that is used to establish a DNA profile unique to particular individual or organism. The DNA profiles provide genetic information between species and individual that can contribute to the evolutionary and ecological studies (Weising *et al.*, 1995). DNA profiling also can be used to determine genetic relatedness and to evaluate the genetic diversity among plant species. Before the use of PCR-based technique in DNA profiling, RFLP was used widely for this analysis. Nowadays, DNA markers such as RAPDs are used in DNA profiling to produce specific DNA bands that can distinguish a species from others. Various satellite DNA are increasingly used as markers for DNA fingerprinting such as ISSRs (Campbell & Reece, 2002).

With degradation of our rainforest now, there is an urgent need for us to conserve the tropical plants in every way as soon as possible before the unique plant species become extinct. According to Wickneswari and Ho (2003), the advancement in breeding programme need complete understanding in the genetic information in order to produce genetically improved planting materials, for germplasm conservation and management of natural resources. In case of *C. odontophyllum*, further and deeper researches are needed to improve the quality and yield of this fruit and subsequently to commercialize it for international market. Thus far, not much effort has been done in determining the genetic relatedness in *C. odontophyllum* accessions using molecular markers. Thus, it is worth to use DNA-based

markers and DNA profiling analysis to identify and determine the genetic relatedness of *C. odontophyllum* accessions collected from different locations.

The goal of this study is to determine the genetic relatedness of *C. odontophyllum* Miq. accessions using RAPD markers.

## CHAPTER II

### LITERATURE REVIEW

#### 2.1 *Canarium odontophyllum* Miq.

*Canarium odontophyllum* Miq. is a wild fruit species that belongs to Burseraceae family and genus *Canarium* (Aman, 1999). Burseraceae is a family of trees or shrubs that can produce flowers, fruits and seeds. *Canarium* genus has about 75 species that is native to tropical Africa, from southern Nigeria east to Madagascar, Mauritius, India, southern China, Indonesia and Philippines. It is widely distributed in lowland forest. The taxonomical classification of *C. odontophyllum* is shown as below:-

Kingdom	:	Plantae
Subkingdom	:	Tracheobionta
Superdivision	:	Spermatophyta
Division	:	Magnoliophyta
Class	:	Magnoliopsida
Order	:	Sapindales
Family	:	Burseraceae
Genus	:	<i>Canarium</i>
Species	:	<i>odontophyllum</i>
Scientific name	:	<i>Canarium odontophyllum</i> (Mique)

*C. odontophyllum* is an indigenous fruit to Sarawak. The vernacular names for *C. odontophyllum* are *dabai* in Sarawak, *kembayau* in Brunei, *kedondong*, *dabang*, and *dawai*. In Indonesia, it is known as *kurihang* and *danau majang* (Whitmore, 1972). In Sarawak, this fruit can be found in upper Rejang River such as in Sarikei, Sibul, Kapit and Limbang divisions. In Sarawak, the Department of Agriculture has identified *dabai* as a potential wild fruits for commercial trade due to their unique taste that differ from other commercial fruits.

These plants are large evergreen trees up to 40 – 50 meter tall with alternate pinnate leaves. *C. odontophyllum* tree can grow up to 20 meter tall. The bark usually pale grey or pale brown and the surfaces have resin patches in black, brown or white colour. Canopy of *C. odontophyllum* tree is compact and round shaped. The branches are usually on the top of the tree. The leaves are pinnately compound and the leaflets are arranged in 3 to 8 pairs. According to Kueh (2003), the leaflets shape is oblong to lanceolate with the size of 9.5 – 28 cm x 4 – 11 cm. The schematic diagram of *C. odontophyllum* leaves, young shoot, flowers and infructescence is shown in Appendix B.



**Figure 2.1:** The leaflet of *C. odontophyllum* from Sibul (SBW2).



**Figure 2.2:** *C. odontophyllum* female tree from Sarikei (D2).

*C. odontophyllum* produces two sex-types flowers which are male and female flowers. It takes 4 years for its seedling to mature sexually and produce flowers. The female flowers are fertilized by cross pollination and then produced fruits. From the seedlings, there is only a 50 percent chances to get female trees. The male trees do not produce fruits. Thus, it is undesirable and usually being removed. The presence of wild male trees is sufficient to produce pollen for fertilization with female flowers.

*C. odontophyllum* is a seasonal fruit. This plant is also a heavy bearer that bears fruits two times annually. The planting of this plant is done in small scale or the fruits can be obtained from naturally grown trees along the riverbank. No specific variety of this plant is recommended for planting until recently the Department of Agriculture, Sarawak had produced two new clones of *C. odontophyllum* namely Laja and Lulong. These two clones

would be the signature clones for *C. odontophyllum* (Anon., 2007). It is also stated that research also had been successfully done in prolonging *C. odontophyllum* shelf life up to eight months to meet the market demand of this fruit.

*C. odontophyllum* is a very nutritious fruit species with high value of energy, protein and potassium (Voon & Kueh, 1999). The nutritional content of *C. odontophyllum* is shown in Appendix C. The fruits are dark purple in colour. Immature fruits are white in colour and turn to pink before mature. The fruits have smooth skin with oily yellowish flesh. The edible portion of this fruit is its flesh or also known as mesocarp. This mesocarp surrounds the hard three angular seed about 6 to 8 mm thick (Whitmore, 1972). Before the fruits can be eaten, it must be soaked in hot water for 5 to 10 minutes to soften the mesocarp. This fruit must be eaten with sugar and it taste similar to avocado.



**Figure 2.3:** *C. odontophyllum* fruits.

## 2.2 RAPD markers

RAPD or Random Amplified Polymorphic DNA is a type of PCR reaction that amplifies random segments of DNA. The RAPD markers are DNA fragments obtained from PCR amplifications of random segments of genomic DNA with single primer arbitrary nucleotide sequence. According to Sall *et al.* (2000), RAPD is a multiplex marker system that conventionally uses single-primer PCR to amplify random DNA fragments. This technique was developed by William *et al.* (1990), Welsh and McClelland (1990). William *et al.* (1993) stated that RAPD marker system has found widespread applications in plant molecular biology. It has been used widely for studying genetic variation, the construction of linkage maps and in bulked segregant analysis (BSA) for identifying markers linked to genes of interest.

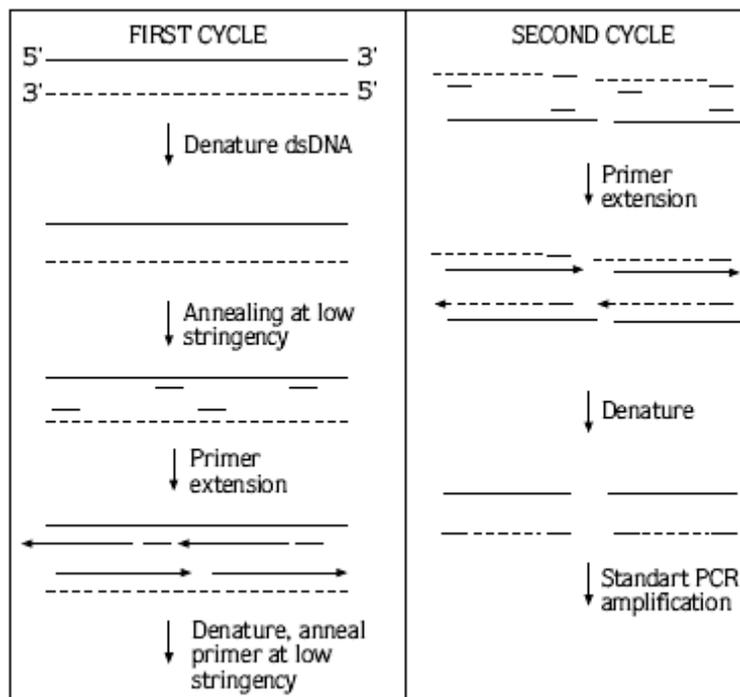
RAPD can be categorized as a molecular marker. Molecular marker is a DNA sequence that can be detected to monitor its inheritance in a particular species. RAPD is also known as DNA-based marker and PCR-based marker. This is due to the use of PCR and arbitrary primer to amplify DNA from total genomic DNA. Other than RAPDs, AFLPs (Amplified Fragment Length Polymorphisms), SCARs (Sequence Characterized Amplified Regions), and SSRs (Short Sequence Repeats) are also grouped in the PCR-based marker. SCARs are markers that had been developed by sequencing specific fragments generated from RAPD. Thus, SCARs have advantages of co-dominant markers and more reproducible.

DNA polymorphism was used in the development of the RAPD markers. The polymorphisms of amplified fragments are caused by several factors. The factors are base substitutions or deletions in priming sites, insertions that caused priming sites to be far to allow amplification, and insertions or deletions that change the size of the amplified fragments. Other than RAPD, DNA polymorphism can be detected by using several related techniques such as arbitrarily primed PCR (AP-PCR) analysis and DNA amplification fingerprinting (DAF) analysis.

Specific knowledge of DNA sequence of target organism is not needed for RAPD analysis. Arbitrary, short primers for 8 – 12 nucleotides are created as RAPD markers. The analysis will proceed with PCR that uses a large template of genomic DNA and hope that the primers will anneal to specific site and amplify the DNA fragments. The amplification depends on the positions that are complementary to the primers' sequence. Several conditions that do not produce fragments such as primers annealed too far apart or 3' ends of the primers are not facing each other, and if a mutation has occurred in the template DNA complementary to the primer. Thus, this reaction results in formation of different patterns of amplified DNA fragments that can be visualized on the gel. According to Dassanayake and Samaranayake (2003), any organism can be characterized using this difference.

One advantage of the RAPD analysis is the knowledge of the DNA sequence for the target gene is not require as the primers will bind randomly in the sequence. Other than that, RAPD is fast, easy and cheap to assay due to commercially sets primers are available. However, there are several limitations of RAPD. First limitation is that nearly all RAPD markers are dominants which means it is not possible to differentiate whether a DNA segment

is amplified from heterozygous (1 copy) locus or homozygous (2 copies) locus. Second limitation is the influence of the PCR reaction. PCR is an enzymatic reaction. Thus, the quality and concentration of template DNA, concentrations of PCR components and the thermal cycling conditions can influence the result. The mismatches between the primer and the template DNA may result in the absence of the product or produce small amount of PCR product. Similar size fragments also can be produced but these fragments may not be homologous. This can lead to the wrong interpretation of the results. To overcome this problem, a standardized PCR and laboratory protocol is needed. Replication of experiments such as bands visualizations of PCR products using gel electrophoresis also must be done to ensure the accuracy of the results.



**Figure 2.4:** Schematic diagram of RAPD reaction for 2 loci (Welsh & McClelland, 1990).

### **2.3 Polymerase Chain Reaction (PCR)**

PCR or Polymerase Chain Reaction is an *in vitro* technique to amplify DNA quickly by incubation with special primers, DNA polymerase molecules, and other PCR ingredients (Campbell & Reece, 2002). According to Karp (2002), PCR is a molecular biology technique which enzymatically amplified DNA fragments. Elliot and Elliot (2005) stated that this technique is important in DNA studies as it allow specific DNA fragment to be quickly amplified exponentially.

The PCR is also called as molecular photocopying. PCR is a fast and inexpensive technique that can be used to amplify small segments of DNA. Studies of isolated pieces of DNA are nearly impossible without PCR method because only significant amount of DNA and specific sequence are necessary for molecular and genetic analysis. PCR has been successfully used in Human Genome Project and the use of this technique has been increasingly important in human health, evolutionary studies, modern systematics, and ecological studies.

The PCR method was discovered by Kary Mullis in the mid-1980s. At that time, he devised a method of replicating genes called PCR. In 1993, he was awarded a Nobel Prize for Chemistry for Polymerase Chain Reaction. Then, the first generation of PCR machine called thermocycler was built by PerkinElmer Company. Alternative to this machine are water baths with set temperature can be used for PCR method but it is time consuming because the reaction need to be transferred from one water bath to another in order to complete one cycle.

First generation thermocycler requires the use of mineral oils to avoid condensation of the reaction mixture. Fortunately, second generation thermocycler was designed to overcome several problems of first generation PCR machine. Heated lid is used to overcome the mineral oil problem. Ramping time which is the time interval to change the temperature has made faster such as 1°C per second.

There are three major steps in PCR, which are repeated for 20 to 40 cycles. These steps are done in an automated cycler or thermocycler. This machine can heat and cool the tubes with the reaction mixture in a very short time. The entire cycling process of PCR only takes few hours to complete depending on the thermal cycling profile required by the primer used.

First step is denaturation. The temperature required for this step is between 92 °C to 99 °C. During this process, heat is used to induce the disruption of hydrogen bonding between complementary bases. Double-stranded DNA is split into single-stranded molecules. The importance of this step is that the DNA template is made accessible for binding of primers. Second step is annealing. The required temperature for this step is depending on the length and nucleotide sequence of the primers. There are formulas whereby the annealing temperature can be calculated. In this step, the synthetic oligonucleotide primers recognize and bind to the complementary sequences on DNA template. Both upstream (forward) and downstream (reverse) primers bind to the template. The third step is extension at 72 °C. This temperature is the suitable working temperature for DNA polymerase. The dNTPs or nucleotides are sequentially added from primer based.

Optimization of PCR is important to increase efficiency of the PCR reactions. To produce specific and unique product (strong single band), the condition that is required for the reaction need to be optimized empirically. The annealing temperature is important to determine the specificity of the reaction. At low temperature, primers may anneal to similar non-specific sequences in the DNA template and result in production of multiple products. High annealing temperature gives more stringent conditions for primer annealing and more specific product. The optimization can be done by testing at 3 to 5 °C increments until maximum specificity is reached. Nowadays, the optimization is made easier by using gradient PCR machine because this machine can have different annealing temperatures for each tube. Specificity also can be increased by reducing the concentration of primers, DNA or *Taq* polymerase, minimizing the times allowed for annealing and extension steps or by reducing the free magnesium ions concentration.

## **2.4 DNA profiling**

DNA profiling or DNA fingerprinting or DNA typing refers to the techniques to reveal the specific DNA profile for a particular organism. A DNA profile is unique and independent of environment. It is consistent for different parts and developmental stages of the organism. The DNA fingerprinting can distinguish plants from different families, genera, species, cultivars and even siblings' plants with the aids of markers (Hong, 2007). Other than that, genotypic alteration in plant species can be identified by DNA profiling. In germplasm conservation, DNA profiling is used to select DNA for storage. In addition, the DNA fingerprinting is also used for forensic analysis and paternity or maternity genetic verification in human.