SEX IDENTIFICATION IN *Canarium odontophyllum* Miq. (DABAI) USING RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD) MARKER

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ABSTRACT

Canarium odontophyllum Miq. is a perennial plant native to Malaysia, Indonesia and Philippines. The pistillate plants bear fruits twice a year and are more preferable than staminate plants. Thus, sex determination in *Canarium odontophyllum* at seedling stage is very important for crop improvement processes because it accelerates the identification of fruitful pistillate plants. In this study, Random Amplified Polymorphic DNA (RAPD) technique was used to amplify DNA segments, with the objective of finding molecular markers associated to sex determination locus in this species. DNA was isolated from one male and three female Dabai trees and subjected to RAPD analysis. Of 52 RAPD primers, one primer (OPL – 03) produced an approximately 1,000 bp amplification band detectable in all pistillate plants, while absent in the staminate plants. The exclusiveness of this female-specific diagnostic band could be used in a breeding program to screen the gender of Dabai trees long before they reach reproductive maturity, resulting in considerable savings of time and economic resources.

Key words: *Canarium odontophyllum* Miq., sex determination, Random Amplified Polymorphic DNA (RAPD), molecular markers, diagnostic band.

ABSTRAK

Canarium odontophyllum Miq. *adalah sejenis tumbuhan yang hanya terdapat di Malaysia, Indonesia dan Filipina. Pokok betina akan menghasilkan buah dua kali setahun dan lebih digemari daripada pokok jantan. Oleh itu, penentuan seks Canarium odontophyllum pada tahap anak benih adalah amat penting untuk proses pemajuan tanaman kerana ia mempercepatkan identifikasi pokok jantan yang berbuah. Dalam kajian ini, teknik Random Amplified Polymorphic DNA (RAPD) telah digunakan untuk mengamplifikasikan segmen DNA dengan objektif untuk mencari marker molekular berkaitan dengan lokus penentuan seks dalam spesies ini. DNA telah dipencilkan daripada satu pokok jantan dan tiga pokok betina Dabai dan digunakan dalam analisis RAPD. Daripada 52 pencetus RAPD, satu pencetus (OPL – 03) telah menghasilkan band amplifikasi bersaiz lebih kurang 1,000 bp yang boleh dikesan dalam semua pokok betina tetapi tidak dalam pokok jantan. Band diagnostik pokok betina yang unik ini boleh digunakan dalam program pembiakan untuk mengesan jantina pokok Dabai sebelum pokok tersebut mencapai tahap matang. Ini akan menjimatkan masa dan sumber ekonomi.*

Kata kunci: Canarium odontophyllum Miq., penentuan seks, Random Amplified Polymorphic DNA (RAPD), marker molekular, band diagnostik.

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LIST OF ABBREVIATIONS

CIA	Chloroform: Isoamylalcohol
CTAB	Cetyl Trimethylammonium Bromide
DNA	Deoxyribonucleic Acid
dNTPs	Deoxynucleotide-triphosphates
OD	Optical density
PCR	Polymerase Chain Reaction
PVP	Polyvinylpyrrolidone
RAPD	Random Amplified Polymorphic DNA
RNA	Ribonucleic Acid
SCAR	Sequence Characterized Amplified Region
TBE	Tris-Borate-EDTA
UV	ultraviolet
bp	base pair
g	gram
kb	kilo base pair
ml	milliliter
mM	milimolar
ng	nanogram
nm	nanometer
rpm	revolutions per minute
µl	microliter
β	beta

CHAPTER I

INTRODUCTION

In Malaysia, *Canarium odontophyllum* or better known as 'Sibu Olive' is a minor tropical fruit which serves as a food source for the local population in Sarawak (Morico *et al.*, 1998). The common names for this wild fruit species are dabai, danau majang, or kedondong. In Brunei, this species is known as kembayau among the Bruneian. *C. odontophyllum* is native to Sarawak, Brunei, Philippines and Indonesia (Whitmore, 1972). In Sarawak, this fruit species is only found in upper Rejang River (Tan, 2006).

C. odontophyllum is a seasonal fruit which brings crops twice a year. The fruiting season usually falls in the end of year following Durian season. This fruit belongs to Burseraceae family and is a species of *Canarium* genus. *C. odontophyllum* grows in altitudes up to 700 metres and it is a medium-sized, upright growing tree with large, pinnate leaves. The fruits of *C. odontophyllum* are oblong in shape and have a dark purple, edible skin. The fresh inside has oily yellowish appearance and covers a single, large three angled seed (Whitmore, 1972).

The genus *Canarium*, in the family *Burseraceae*, contains at least 75 tropical tree species. Being one of the species of *Canarium* genus, *C. odontophyllum* is a nutritious indigenous fruits with high content of energy, protein and pottasium. The fruits which are pesticide residue free have high potential to be promoted for wider use and commercial purposes. It has been previously introduced from Borneo to Queensland, Australia (Whitmore,

1972). Other species of *Canarium* are rich in oil and successfully applied to biodiesel generation in USA, Germany and Europe country (Voon and Kueh, 1999).

The life cycle of *C. odontophyllum* is characterized by alternation of generations, during which the diploid plant body (sporophyte) produces haploid spores (microspores and megaspores) that develop into multicellular haploid gametophytes producing haploid gametes (pollen and egg). Therefore, the terms "male" and "female" refer to the "stamen" and "pistil" on the reproductive structure (i.e. flower) of the plant. The pistil shelters ovule while the stamen produces pollen grains. *C. odontophyllum* is a typical dioecious plant in which pistil and stamen are developed on separate individuals, which are distinguished as "pistillate plants" and "staminate plants" (Wen *et al*., 2004).

The fruiting of *C. odontophyllum* requires both staminate and pistillate trees. The species grow easily from seeds with both male and female trees in a population. Male trees will not bear fruit but to produce pollen for the female flowers. Since the presence of existing male trees is sufficient to produce pollen, newly grown male trees are normally removed. *C. odontophyllum* are slow growing plants and have no distinguishing features to identify the sex until flowering. The plants commence flowering only after 4 years of maturity. Therefore, it is a general practice for farmers to plant Dabai seedling in one area , allowing them to grow for 4 years until the sex types are identified, and then to eliminate the undesired male trees to develop the orchards with only female trees (Chaves-Bedoya *et al.*, 2007). On account of the dioecious nature and long juvenile period, the farmers have encountered a number of losses due to undesired waste of time, space and production cost (Wen *et al.*, 2004). An ideal solution to overcome this constraint is by determining the gender of *C. odontophyllum* at early

stage, for instance at the seedling stage. This would help farmers while selecting the seedlings and facilitate breeding, with a saving of time and economic resources such as soil, fertilizers and water (George *et al.*, 2007).

Sex determination of *C. odontophyllum* at early stage can be done by utilization of PCR-based DNA marker to diagnose and select a genotype, long before the phenotype is apparent. This is particularly important in *C. odontophyllum*, which has a long juvenile period. In the present study, Random Amplified Polymorphic DNA (RAPD) molecular marker was employed for screening the gender of *C. odontophyllum* before they reach reproductive maturity (i.e, at seedling stage). RAPD assay described by Williams *et al.* (1990) is a simple tool for identification of polymorphism and has been used widely in genetic mapping, detection of phenotypic variation and evolutionary studies. It is also very useful in studies of sexual differentiation and identification of dioecious plants (Sakamoto *et al.*, 2005). Singh *et al.* (2002) emphasized that sex-linked RAPD genetic marker may not only be useful in breeding programmes, but would also allow the understanding of the genetic and molecular basis of dioecious plants.

Polymerase chain reaction (PCR) is an *in vitro* enzymatic DNA amplification strategy of producing millions copies of DNA fragments in a short period of time (Karp, 2002). PCR is carried out using a thermal cycling machine to create repeated cycle of more than 20 times, with each cycle comprising denaturation, annealing and extension steps. PCR had been proven to have great importance in all kinds of DNA studies, such as evolutionary studies, ecological studies, and Human Genome Project. In the present study, 52 RAPD primers (Operon Technologies, Inc) were utilized in the PCR analysis to generate detectable RAPD

bands. RAPD-PCR analysis can then be conducted to identify the diagnostic bands associated to sex determination locus.

To date, the propagation of *C. odontophyllum* depends mainly on traditional seedling cultivation. Four years are required for this tree to reach its flowering stage and there are only 50:50 probabilities to obtain female trees from the total seedlings. Much effort has been made over recent years to obtain a method for early sex determination at seedling stage but no methodology has yet been developed that is efficient for distinguishing female from male Dabai seedlings prior to flowering (Lemos *et al.*, 2002). Therefore, it is worthwhile to develop a rapid RAPD technique for early sex identification of *C. odontophyllum*. The objectives of this study are :-

(1) To optimise RAPD-PCR protocol for *C. odontophyllum*, and

(2) To identify RAPD markers that associated to sex determination locus in *C. odontophyllum*

CHAPTER II

LITERATURE REVIEW

2.1 *Canarium odontophyllum* Miq.

C. odontophyllum has various vernacular or common names. It is locally known as dabai in Sarawak, Malaysia and kembayau in Brunei Darussalam. *C. odontophyllum* is also called 'Sibu Olive' by local population in Sibu, Sarawak. There are different common names for *C. odontophyllum* in different countries. In Indonesia, its local names are kurihang and danau majang (Whitmore, 1972). The taxanomic classification of *C. odontophyllum* is as below:-

Kingdom	:	Plantae
Subkingdom	:	Tracheobionta
Superdivision	:	Spermatophyta
Division	:	Magnoliophyta
Class	:	Magnoliopsida
Order	:	Sapindales
Family	:	Burseraceae
Genus	:	*Canarium*
Species	:	o*dontophyllum*
Scientific name	:	*Canarium odontophyllum* (Mique)

2.1.1 Distribution

C. odontophyllum is native to Malaysia (Sarawak and Sabah), Indonesia (Kalimantan and Sumatera) and Philippines (Palawan). It has been domesticated in Sarawak and widely cultivated in Sibu, Sarikei, Kapit and Limbang (Voon, 1998). The species was also introduced into Queensland, Australia for commercial purpose and food source. *C. odontophyllum* grows up to 700 metres above sea level in its natural habitat.

2.1.2 General Morphology

The tree of *C. odontophyllum* is medium in size with maximun height of 21 metres. It is an upright growing tree with straight trunk and big stems. The bark is light grey or brown, and rough with small resin patches. They are very heavy bearers and may yields up to 800 kilograms per tree (Voon, 1998).

Figure 2.1: 20 years old female Dabai trees that achieve up to 20 metres in height

C. odontophyllum tree has round shaped, compact canopy with large, pinnate leaves. Leaves are thin and furry and the twigs are dull golden in colour. New foliage appears in green or red velvet. Leaflets are oblong, lanceolate, 9.5 to 28 cm long and 4 to 11cm wide (Kueh, 2003).

C. odontophyllum fruits are olive-like and held above the dark green foliage. The fruits are white in colour when immature, turning blue-black when ripe. They are oblong in shape, 35 to 40 mm long, 20 to 25 mm wide, and have a thin, edible skin. The whitish or yellow, edible mesocarp inside is 6 to 8 mm thick and covers a single, large three angled seed (Whitmore, 1972).

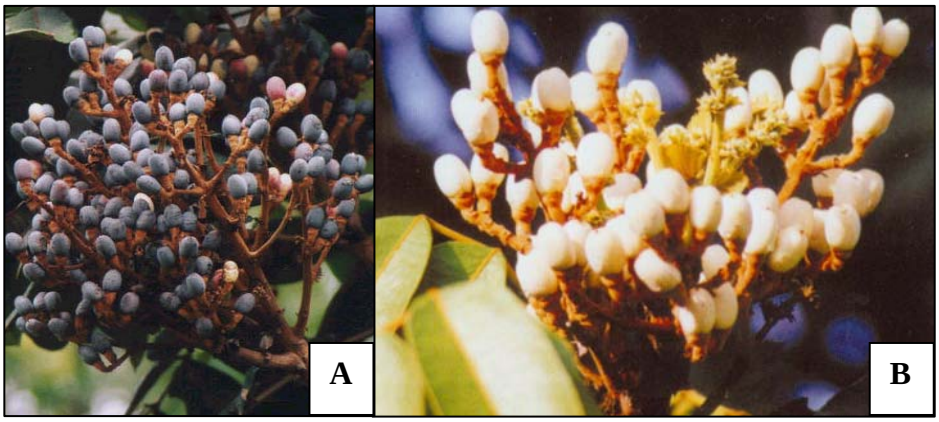

Figure 2.2 : (A) Mature Dabai fruits (B) Immature Dabai fruits

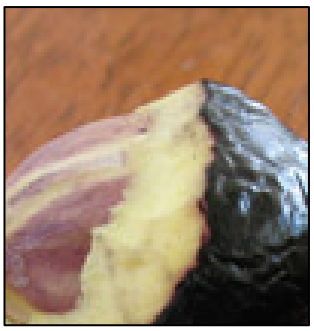

Figure 2.3: Edible part of Dabai fruit

2.1.3 Uses and Economic Importance

Voon and Kueh (1999) reported that *C. odontophyllum* is a very nutritious fruit species with high value of energy, protein and potassium. Its range of nutrients is generally comparable with other commonly cultivated fruit species. This indigenous fruit which is pesticide free is important food sources for rural population. In addition to human food sources, *C. odontophyllum*'s fruit may be eaten by certain lemurs such as Red-bellied Lemur in Madagascar's eastern rainforests.

According to Morico *et al.* (1998), *C. odontophyllum* is one of the indigenous fruit species that have the potential for exploitation. The recent International Conference on Plant Genetic Resources (PGR), stressed the need to establish large-scale cultivation of *C. odontophyllum*, through advanced scientific techniques and financial resources (Morico *et al.*, 1998). It could be exploited as new, multi-purpose tree and as sources of germplasm for improving existing cultivated fruit crops.

2.2 Sex Determination in Plant

Plant sex determination is the expression of femaleness or maleness in plants (Alstrom-Rapaport *et al.*, 1998). It is an identification technique which studies the differential expression of sex-related genes at DNA level (Singh *et al.*, 2002). According to Dellaporta and Calderon-Urrea (1993) and Hardenack *et al.* (1994), all plant species possess necessary sex-determining genes for the development of either staminate (male) or pistillate (female) flowers, leading to the diversity of floral types and sex determination mechanisms among

flowering plants. Example of corresponding genes are homeotic genes-MADS box genes (Coen and Meyerowitz, 1991).

In the plant kingdom, dioecy is a widespread condition in angiosperms or flowering plants, approximately 6% of the 240,000 angiosperm species are dioecious (Negrutiu *et al.*, 2001). Dioecism has arisen independently in different families and genera, resulting in extremely diverse sex determination mechanisms in dioecious plant species (Durand and Durand, 1990). Some dioecious species such as *Silene latifolia* Poiret (white champion) have heteromorphic sex chromosomes, with a well characterized male (XY) and female (XX) chromosome system (Hormaza *et al.*, 1994). More often, the gender of dioecious plant species is controlled by the expression of alleles at one to several loci (Durand and Durand, 1991). Well documented examples include dioecious species of genera *Asparagus*, *Vitis*, *Spinacia* and *Mercurialis* (Irish and Nelson, 1989). Louis (1989) also reported that in *Mercurialis*, cytokinin regulation cause male-to-female conversion, whereas the opposite effect has been observed in *Asparagus officinalis* L. (Bracale *et al*., 1991).

Several attempts have been carried up to distinguish between male and female dioecious plants at molecular level including immunochemistry, isozyme pattern and RNA hybridization (Singh *et al.*, 2002). However, isozymes present some limitations as biochemical marker because they exhibit polymorphisms that are susceptible to post-translation modification, environmental conditions and developmental stages of the seedlings (Yao and Tigerstedt, 1993). On the other hand, DNA-based molecular markers do not encounter these limitations and appear to be the best option for gender screening. Besides its

relevance to the understanding of evolution in dioecious plants, it is also applied in plant breeding and improvement programmes (Alstrom-Rapaport *et al*., 1998).

2.3 Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction (PCR) is a molecular biological technique for amplifying DNA without using a living organism, such as E. coli or yeast. This technique is commonly used in medical and biological research labs for a variety of tasks, such as the detection of hereditary diseases, the identification of genetic fingerprints, the diagnosis of infectious diseases, the cloning of genes, and paternity testing (Karp, 2002).

The PCR is a process by which DNA could be artificially multiplied through repeated cycles of duplication driven by an enzyme called DNA polymerase. This enzyme occurs naturally in living organisms, where it functions to duplicate DNA when cells divide. It works in PCR by binding to a single DNA strand and creating the complementary strand. The double-stranded DNA is separated into two single strands by denaturation stage at 96°C. However, DNA polymerase tends to be destroyed at this temperature. Therefore, DNA polymerase is usually taken from thermophilic bacteria that can sustain high temperature of over 110°C. One of the first thermostabe DNA polymerase was obtained from Thermus aquaticus and it was named Taq polymerase. Taq polymerase is widely used in current PCR analysis. A disadvantage of Taq is that it sometimes makes mistakes when copying DNA, leading to mutation in the DNA sequence (Brown, 1990).

The DNA fragment to be amplified is determined by selecting primers. Primers are short, artificial DNA strands with not more than fifty nucleotides (usually 18-25 bp). During the annealing stage, these primers will bind to the complementary sequences on beginning and end of the DNA fragment to be amplified. The choice of the length of the primers and their melting temperature (T_m) depends on a number of considerations. The optimum length of a primer is generally from twenty to forty nucleotides with a melting temperature between 60°C and 75°C. Primers that are too short would anneal at several positions on a long DNA template, which would result in non-specific copies. On the other hand, melting temperatures of over 80°C may result in denaturation of DNA polymerase (Dales and Schantz, 2002).

2.4 Random Amplified Polymorphic DNA (RAPD)

Random ampified polymorphic DNA (RAPD, pronounced "rapid") was originally applied in the genetic mapping technique as described by Williams *et al.* (1990). The basic principle of RAPD employs single primer of arbitrary nucleotide sequence (10 to 15 bases) to amplify genomic DNA using the polymerase chain reaction. Unlike traditional PCR analysis, RAPD does not require any prior knowledge of the DNA sequence of the target organism. A particular short arbitrary primer may or may not amplify a segment of DNA, depending on position that is complementary to the primer's sequence.

RAPD technique requires only the presence of a single, randomly chosen arbitrary primer. The RAPD primers may be synthesized privately or purchased from one reliable commercial source, the Operon Technologies, Inc., which has 500 random primers available. A major criterion to be considered when selecting or synthesizing a RAPD primer is that the

primer should conventionally has a (G+C) content between 60-70% and has no self-complimentary ends.

When conducting a RAPD assay, the primers are screened individually and identified to produce probably male or female-related RAPD bands. Through direct PCR amplification, the primers will hybridise to several hundred sites in either male or female plant DNA depending on their specificity. Male-specific primers hybridise with male plant DNA to generate male-related bands or vice versa. The selected primers will then be used to detect the presence and absence of bands in all male and female entries, leading to confirmation of gender in a particular plant species (Singh *et al*., 2002).

Since RAPD marker is able to produce multiple bands using a single decamer primer, it is considerably a fast technique, easy to perform and comparatively cheap. It also possesses the advantage of being feasible to the analysis of most plant species because universal primers are utilized without any need for prior sequence information.

The RAPD markers in general would be useful for genome mapping, as well as for estimation of genetic relatedness and thereby act as an aid for taxonomic investigations (Persson and Nybom, 1998). It has been widely used for sex identification in dioecious species and phylogenetic studies (Wiessman *et al*., 1998; Belaj *et al*., 2000). The RAPD markers could also prove very useful in the studies of population genetics, evolutionary studies and disease diagnostics (Hormaza *et al*., 1994).

Promising results have already been obtained in various dioecious species using RAPD markers. Mulcahy *et al*. (1992) had successfully identified four RAPD markers linked with gender in *Silene latifolia*, a dioecious species with heteromorphic sex chromosomes. Hormaza *et al*. (1994) also detected a female-linked RAPD marker in *Pistacia vera* L. and Sakamoto *et al*. (1995) reported the finding of six male-specific RAPD markers, MADC (male-associated DNA sequences) 1 to MADC 6 in *Cannabis sativa*.

Moreover, Paran and Michelmore (1993) had reported the conversion of RAPD markers to sequence-characterized amplified regions (SCARs) by developing primers that are much more longer and specific than RAPD sequences. This modified RAPD technique has significantly improved the reproducibility and reliability of PCR assays. The strategy has been successfully used to generate genetic markers for dioecious plants such as *Olea europaea* (Hernandez *et al*., 2001) and *Carica Papaya* L. (Urasaki *et al*. 2002).

According to Urasaki *et al*. (2002), sex determination in *Carica papaya* is controlled by a single gene determining male, female and hermaphrodite plants. Male and hermaphrodite papaya plants have heterozygous sex chromosomes while female papaya plants have homozygous sex chromosomes. After screening 25 arbitrary RAPD primers, a 450 bp RAPD marker fragment named PSDM (Papaya Sex Determination Marker) specific for male and hermaphrodite papaya plants had been successfully detected. It is also claimed that sex determination system in *C. odontophyllum* is of male, female and hermaphrodite state (Sim, personal communication). In the present study, the specificity of RAPD markers allowed development of male, female or hermaphrodite-related diagnostic bands to identify the gender of *C. odontophyllum*.

CHAPTER III

MATERIALS AND METHODS

3.1 Plant Material

The fresh leaf samples of one adult male and three female *C. odontophyllum* trees were collected from Sarikei. The leaf samples were washed using sterile distilled water and wiped with tissue papers until dry. The leaf samples were then wiped with 70% ethanol using tissue. After that, the leaf samples were wiped to dry using dry and clean tissue. The samples were wrapped in tissue papers and packed in clean plastic bags and stored at - 20ºC until use.

3.2 Total Genomic DNA Isolation of *Canarium odontophyllum*

Total genomic DNA was extracted from fresh young leaves of male and female *C. odontophyllum* trees based on a modified CTAB procedures described by Doyle and Doyle (1990). 4 mL of Cetyl Trimethylammonium Bromide (CTAB) extraction buffer and 80 µL of mercaptoethanol were prepared separately and mixed in a Falcon tube. The extraction buffer was preheated at 65ºC in water bath for 30 minutes. Using analytical balance, 0.5 g of leaves were weighted and cut into smaller pieces. Small pieces of leaf tissues were ground under liquid nitrogen by using mortar and pestle. The pulverised leaf tissues were immediately suspended in a 1.5 ml Falcon tube containing preheated, CTAB extraction buffer. The buffer mixture was agitated gently at room temperature for 1 minute and then incubated in water bath at 65ºC for 30 minutes.

After that, 500µl sample and equal volume of Chloroform:Isoamylalcohol 24:1 (CIA) were mixed in a microcentrifuge tube. The mixture was inverted 25 times and then centrifuged at 13000rpm for 10 minutes. The final aqueous phase was transferred into a new microcentrifuge tube. Second volume extraction was done by again adding equal volume of CIA, inverting the tube and centrifuged at 13000rpm for 5 minutes. ⅔ volume of -20ºC isopropanol was then added to precipitate the DNA and the mixture was stored at -20^{o}C for at least 30 minutes.

Then, the supernatant was removed following centrifugation at 13,000 rpm for 15 minutes at 4ºC and 1 ml of wash buffer was added. The DNA pellet was obtained after second centrifugation at 14,000 rpm for 30 minutes at 4ºC. The pellet was air-dried at room temperature and then suspended in 25 µl TE buffer for storage at -20ºC. 5µl of the extracted DNA concentration for each sample was determined using 0.8% agarose gel electrophoresis method.

3.3 DNA Purification

The DNA samples that successfully extracted from young leaves of *C. odontophyllum* trees were purified using a Wizard Genomic DNA Purification Kit (Promega, USA). 15 µl of DNase-free-RNase A was added to the DNA samples and the entire volume was incubated for 15 minutes at 37ºC. The RNA-free DNA samples were cool down to room temperature, followed by protein purification with 100 µl of Protein Precipitation Solution. After centrifugation at 13,000 rpm for 5 minutes, the aqueous phase containing DNA was transferred into a new microcentrifuge tube. DNA precipitation was done by adding equal