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Development of RAPD technique for plant DNA fingerprinting in Malaysia

Dissertation submitted in partial fulfillment for the Degree of Bachelor of Science in Forensic Science

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CERTIFICATE

This is to certify that the dissertation entitled

Development of RAPD technique for plant DNA fingerprinting in Malaysia

is the bonafide record of research work done by

Muhammad Yusran Bin Abdul Aziz

during the period of 17th December 2006 to 2nd May 2007

under my supervision.

Date: 30 April 2007

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1.0 ABSTRACT

Random amplified polymorphic DNA (RAPD) molecular markers have been widely used in analysis of plant genetic diversity, particularly at species and infra-specific levels. In this research, RAPD was used to study the genetic markers of plants. Three plants selected for this study are *Mitragyna speciosa, Nicotiana spp., L* and *Centella asiatica.* RAPD analysis was carried using seven arbitrary primers of 10 base pair each. Polymerase chain reaction was optimized by varying the DNA concentration, primer concentration and annealing temperature. Each species is represented by the number of scorable band produced. Further optimization is required in order to obtain better quality and quantity of PCR products.

2.0 INTRODUCTION

Within the last decade, technological advancement has increasingly supported the use of genetics in determining population diversity. Many molecular techniques are now available which allow ecologists and evolutionary biologists to determine the genetic architecture of a wide variety of closely related individuals. DNA markers that are known to be genetically linked to a trait of interest can be used for gene cloning, medical diagnostics, and for trait introgression in plant and animal breeding programs. Several different methods for documenting genetic information are used. These methods include isozyme analysis, restriction fragment length polymorphisms (RFLP) and random amplified polymorphic DNA (RAPD) (Mulcahy *et al.*, 1993). Although isozyme analysis and RFLPs are a source of readily obtainable genetic information which is easily reproduced, they often do not show polymorphisms which are necessary to determine variation within a group of genetically similar individuals.

The RAPD technique employs 10 base pair random primers to locate random segments of genomic DNA to reveal polymorphisms. These primers adhere to a specific nucleotide segment of the genomic DNA. The DNA is cut into many segments of a specific length which can be measured using gel electrophoresis. For a mutation to change the RAPD pattern, it must occur in the priming region or must change the length of the DNA between priming regions. In this way the RAPD analysis can provide a simple and reliable method for measuring genomic variation. Because it is a relatively straight-forward technique to apply, and the number of loci that can be examined is unlimited,

RAPD analysis is viewed as having a number of advantages over RFLP's and other techniques (Lynch and Milligan 1994). The advantages of RAPD analysis are its simplicity and rapidity, the DNA sample requirement is only in small quantity and the ability to generate numerous polymorphisms (Cheng *et al.*, 1997). Therefore, it becomes a good technique for genetic analysis (Williams *et al.*, 1990; Chapco *et al.*, 1992; Kiss *et al.*, 1993; Landry *et al.*, 1993 and Wight *et al.*, 1993).

The RAPD technique has further advantages over other systems of genetic documentation because it has a universal set of primers, no preliminary work such as probe isolation, filter preparation, or nucleotide sequencing is necessary (Williams *et al.*, 1990). The ease and simplicity of the RAPD technique make it ideal for genetic mapping, plant and animal breeding programs, and DNA fingerprinting, with particular utility in the field of population genetics. In many instance, only a small number of primers are necessary to identify polymorphism within species (Williams *et al.*, 1990). Indeed, a single primer may often be sufficient to distinguish all of the sampled varieties (Mulcahy *et al.*, 1995). The ease of the RAPD technique could lead to the automation of genetic mapping and to the extension of genetic analysis to cover organisms which lack an ample number of phenotypic markers to completely describe their genome (Williams *et al.*, 1990).

The RAPD technique was first employed by Williams *et al.*, (1990) to examine human DNA samples. Arbitrary amplification of polymorphic DNA sequences has increasingly been reported as a method for the genetic characterization of microorganisms and there

are many variations of this technique. RAPD analysis on eukaryotic DNA is more consistently reproducible, possibly due to the larger genome size, which offers more potential binding sites and perhaps greater stability (Caetano-Anolle's *et al.*, 1991, Munthali *et al.*, 1992, Williams *et al.*, 1990). The majority of reproducibility studies have been concentrated on plant DNA. However, the sensitivity of experimental variables was acknowledged and consistently reproducible results were only possible with rigorously optimized reaction conditions (Devos *et al.*, 1992).

The choice of primers for use in RAPD analysis is one of the most critical factors, and several primers must first be screened. It appears that some arbitrary primers may work better than others and may provide results that are more reproducible (Penner *et al.*, 1993). It has also been indicated that the use of a combination of oligonucleotide primers in a single RAPD reaction can give more detailed and reproducible patterns (Kaemmer *et al.*, 1992, Klein-Lankhorst *et al.*, 1991, McClelland *et al.*, 1994, Micheli *et al.*, 1994, Welsh *et al.*, 1991). The method need to be optimized for each arbitrary primer and DNA template used in order to increase the efficiency of the RAPD analysis (Kangfu *et al.*, 1992).

Kratom or the scientific name *Mitragyna speciosa* a medical leaf harvested from a large tree native to Southeast Asia was first documented by Dutch colonial botanist Korthals. It is botanically related to the Corynanthe, Cinchona and Uncaria genii and shares some

similar biochemistry. It is in the same family as coffee, and the psychoactive plant *Psychotria viridis*. Other species in the Mitragyna genus are used medicinally in Africa.

Kratom is a well established psychoactive drug in its native region and elsewhere in the world. In Southeast Asia, the fresh leaves are usually chewed, often continuously by workers or manual laborers seeking a numbing, stimulating effect. Elsewhere, the leaves are often made into a tea or extracted into water and then evaporated into a tar that can be swallowed. Kratom is not often smoked, although this method does provide some effect.

The leaf of *Mitragyna speciosa* has been used in Thailand for its opium-like effect (Burkill, 1935) and its coca-like stimulant ability to combat fatigue and enhances tolerance to hard work under a scoring sun (Grewal, 1932; Suwanlert, 1975). There are descriptions of its use as a cure for fever, as a tonic, treatment for diarrhea and substitute for morphine in treating addicts (Suwanlert, 1975; Jansen and Prast, 1988). From the leaves of *Mitragyna speciosa*, mitragynine was obtained as the major constituent (66.2% base on the crude base extract) together with its analogues, paynantheine (8.6%), speciogynine (6.6%), 7-hydroxymitragynine (2.0%) and speciociliatine (0.8%) (Takayama, 2004). Studies on the pharmacological effects of mitragynine on guinea-pig ileum, radioligand binding assay and the tail-flick test in mice, and found that mitragynine acts on opioid receptors and possesses analgesic effects (Watanabe *et al.*, 1997; Yamamoto et al., 1999; Takayama *et al.*, 2002). Mitragynine-related compounds also express interesting opioid activities; mitragynine pseudoindoxyl and 7-hydroxymitragynine, especially, were found to exhibit potent antinociceptive activity in

mice (Takayama *et al.*, 2002; Takayama, 2004; Matsumoto *et al.*, 2004). In addition, some pharmacological studies have revealed that mitragynine has an antinociceptive action through the supraspinal opioid receptors (Matsumoto *et al.*, 1996a; Thongpradichote *et al.*, 1998) and descending noradrenergic and serotonergic systems (Matsumoto *et al.*, 1996b).

Tobacco (*Nicotiana spp., L*) refers to a genus of short-leafed plants of the nightshade (Solanaceae) family indigenous to North and South America. Tobacco leaves are often smoked in the form of a cigar or cigarette, or in a smoking pipe. Tobacco can also be chewed, "dipped" (placed between the cheek and gum), or sniffed into the nose as finely powdered snuff. Many tobacco smokers and some other users become addicted and use every day.

Tobacco contains nicotine, a powerful neurotoxin that is particularly harmful to insects. All means of consuming tobacco result in the absorption of nicotine in varying amounts into the user's bloodstream, and over time, develop tolerance and dependence. Absorption quantity, frequency and speed seem to have a direct relationship with how strong a dependence and tolerance. Long term tobacco use carries significant risks of developing various cancers as well as strokes, and severe cardiovascular and respiratory diseases. It has been shown that tobacco may cause lasting brain changes just like morphine or cocaine effect. The substantially increased risk of developing cancer as a result of tobacco usage seems to be due to the plethora of nitrosamines and other carcinogenic compounds found in tobacco and its residue as a result of anaerobic heating, either due to

smoking or to flue-curing or fire-curing. The use of flue-cured or fire-cured smokeless tobacco in lieu of smoked tobacco reduces the risk of respiratory cancers but still carries significant risk of oral cancer. In contrast, use of steam-cured chewing tobacco (snus), avoids the carcinogenicity by not generating nitrosamines, but the negative effects of the nicotine on the cardiovascular system and pancreas are not ameliorated.

Centella asiatica is a small herbaceous annual plant of the family Apiaceae, native to Australia, Pacific Islands, New Guinea, Melanesia, and Asia. Common names include Gotu Kola, Asiatic Pennywort, Antanan, Pegaga, and Brahmi. It is used as a medicinal herb in Ayurvedic medicine and traditional Chinese medicine. Botanical synonyms include *Hydrocotyl asiatica* L.

Centella asiatica or 'pegaga' is one of the local herbs that is claimed to possess various physiological effects. Reports from different places have revealed that *C. asiatica* has been used for wound healing, memory improvement, treating mental fatigue (Goh *et al.*, 1995), bronchitis, asthma, dysentery, leucorrhoea, kidney trouble, urethritis (Jaganath *et al.*, 1999), antiallergic and anticancer purposes, curing leukorrhea and toxic fever (Kan *et al.*, 1986). It is also commonly used as porridge for feeding pre-school children in Sri Lanka in combating nutritional deficiencies (Cox *et al.*, 1993). Even though this precious herb is surrounded with various claims, the underlying mechanisms involved in its physiological effects are lacking. More scientific data are required before recommendation for increase in its consumption/utilization can be given with confidence.

Table 1: Scientific classification of *Mitragyna speciosa.*

Kingdom	Plantae
Division	Magnoliophyta
Class	Magnoliopsida
Order	Gentianales
Family	Rubiaceae
Genus	*Mitragyna*
Species	*speciosa*

Binomial name

Mitragyna speciosa

Table 2: Scientific classification *Centella asiatica.*

Kingdom	Plantae
Division	Magnoliophyta
Class	Magnoliopsida
Order	Apiales
Family	Apiaceae
Genus	*Centella*
Species	*asiatica*

Binomial name

Centella asiatica

Table 3: Scientific classification of *Nicotiana spp., L.*

Kingdom	Plantae
Class	Magnoliospida
Order	Solanales
Family	Solanaceae
Genus	*Nicotiana*
Species	*acuminata, alata, attenuata, benthamiana, clevelandii, excelsior, forgetiana, glauca, glutinosa, langsdorffii, longiflora, obtusifolia, paniculata, quadrivalvis, repanda, rustica, suaveolens, sylvestris, tomentosa*

3.0 LITERATURE RIVIEW

Random amplified polymorphic DNA (RAPD) molecular markers have been widely used in analysis of plant genetic diversity, particularly at species and infra-specific levels. RAPD markers provide a fast and easy approach to the problem of cultivar identification. Many horticultural crops have been fingerprinted using RAPD markers. In situations where sequencing of genes has provided little resolution, they offer relatively higher polymorphism, as well as simplicity of procedures and relatively low cost (Ye Sun *et al.*, 2005).

RAPD requires very small quantities of DNA, no cloning, sequencing or hybridization is necessary (Jorge Fraga *et al.*, 2001). For these reasons, it has a specific advantage over other molecular techniques generally used for genomic characterization. The ability to detect highly variable regions of DNA has tremendous potential application in biomedical investigations, detection of several types of damaged and mutated DNA, characterization of isolates, construction of genetic maps, study of populations, strain identification, taxonomic and epidemiological studies and analysis of simple and complex phenotypic traits (Ellsworth *et al.*, 1993; Carlton *et al.*, 1995; Howard *et al.*, 1996).

In RAPD analysis, sources of DNA polymorphisms may include base changes within the priming site sequence, deletions in the priming site, insertions that render priming sites too distant to support amplification, and deletions or insertions that change the size of a

DNA fragment without preventing its amplification (Williams *et al.*, 1990). Limitations of RAPD markers include questionable reproducibility of some bands, requirement for stringent standardization of reaction conditions, comigration of different amplification products and dominant inheritance (Bachmann, 1994). Despite these caveats RAPD method offers the highest potential for generating large numbers of markers with the greatest ease. Schnell *et al.*, (1995) demonstrated that almost all south Florida mango cultivars could be distinguished using just two RAPD primers. The technique has been used successfully with other vegetatively propagated crops including apple (Koller *et al.*, 1993), grapes (Qu *et al.*, 1996), annonas (Ronning *et al.*, 1995) and avocado (Fiedler and Bufler, 1995).

Random amplified polymorphic DNA (RAPD) was used to investigate relationships between species. Based on a study conducted by Sun *et al.*, (1998), RAPD molecular markers were used to investigate relationships between a sample of *Bambusa* species from South Eastern China that have been placed in *Bambusa* or in several segregate genera, *Dendrocalamopsis*, *Leleba*, *Lingnania*, *Neosinocalamus* and *Sinocalamus*.

Random amplified polymorphic DNA (RAPD) can also be used in the study of genetic diversity. Rachel (2002) demonstrated genetic diversity of three populations of the seagrass *Halodule wrightii* from Christmas Bay, a Flour Bluff cooling pond in Corpus Christi, and Florida Bay. In this study, to obtain reproducible data for RAPDs, PCR conditions were optimized. $MgCl_2$ concentrations were tested at 1.0–6.0 mM. A concentration of 1.5 mM produced the most reliable bands. DNA amounts of 10, 1, and

0.1 ng exhibited successful amplifications that were indistinguishable, while 100 ng failed to amplify.

Sudheesh *et al.*, (2002) also demonstrated that RAPD can be used for genetic diversity, the genomic diversity within *Vibrio parahaemolyticus* and *V. alginolyticus* was assessed by using random amplified polymorphic DNA (RAPD)-PCR. Fingerprinting of genomic DNA was carried out on 25 bacterial strains. PCR reaction conditions have been optimized for important parameters such as primer annealing temperature and concentrations of $MgCl_2$, template DNA, *taq* DNA polymerase, dNTPs and primer.

Tsai *et al.*, (2002) used RAPD markers for phenetic relationship and identification of subtribe Oncidiinae genotypes. Molecular markers among 24 accessions of subtribe Oncidiinae genotypes were investigated based on random amplified polymorphic DNA (RAPD) analysis. Phenetic similarity based on the percentage of band sharing was estimated, ranging from 0.25 to 0.71 among the 24 accessions.

Random amplified polymorphic DNA (RAPD) technique was also used in the study of genetic similarities. Study was conducted by Schnell *et al.*, (1999) to examine genetic similarities among cocoyam cultivars based on randomly amplified polymorphic DNA (RAPD) analysis. Eighteen cultivars of cocoyam (*Xanthosoma* spp.) and two cultivars of taro (*Colocasia esculenta* (L.) Schott) from the USDA/ARS germplasm collection were evaluated for genetic relatedness using RAPD data. PCR amplifications were carried out using RAPD 10 mer primers. Among cocoyam cultivars the genetic similarity ranged

from 0.86 to 0.97 with a mean of 0.91. Cluster analysis identified two main clusters with some unexpected groupings. These data indicate that very little genetic variation exists within the accessions used in this study and that this *Xanthosoma* spp. collection is of limited value as a genetic resource.

Míriam O. Rocha *et al.*, (2003) had demonstrated the use of Isoenzymes and RAPD (random amplified polymorphic DNA) analysis to characterize three Brazilian human isolates of *Giardia duodenalis* and its clones. The dendogram constructed with the RAPD data, using seven primers, revealed a great heterogeneity between Brazilian isolates and the Portland-1 strain. There was no relationship to the clinical characteristics of the isolates. Although a lot of similarity has been observed among Brazilian isolates and its clones, individual polymorphism was detected, which could be releated to the clonal reproduction of this protozoan.

Intra-specific diversity of *Aureobasidium pullulans* strains was conducted by Urzì *et al.*, (1999) using random amplified polymorphic DNA (RAPD). In this study, *Aureobasidium pullulans* strains isolated from environmental sources and from stones was studied by assessment of morphological, biochemical and physiological characters as well as random amplified polymorphic DNA (RAPD) using microsatellite or minisatellite DNA primers $(GTG)_5$, $(GACA)_4$, M13. The arbitrary primers $(GTG)_5$, $(GACA)_4$ and M13 were synthesised by Pharmacia. The microsatellite DNA sequence $(GTG)_5$, $(GACA)_4$ and the minisatellite phage M13 core sequence were used as

oligonucleotide primers. The results showed that both classical and molecular techniques evidenced a phenotypic and genetic diversity of analysed *A. pullulans* strains.

Genetic variation between Pakistani wheat (*Triticum aestivum* L.) genotypes has been conducted by Bhutta *et al.*, (2005) by Random Amplified Polymorphic DNA (RAPD) markers. In this study, the genetic variation and relationships among 277 individual plants from 10 wheat genotypes were evaluated using RAPD markers. A total of 190 DNA fragments were generated by 25 random primers, with an average of 7.6 easily detectable fragments per primer. Of these, 84 fragments (44.64%) were polymorphic among the 10 genotypes. Several RAPD marker bands showed unique patterns of mean frequency that differed among the wheat germplasm groups.

RAPD were also used to discriminate among 25 *Feijoa sellowiana* cultivars and accessions (Maria Teresa Dettori and Maria Antonietta Palombi, 2000). Fifty ten-mer oligonucleotide primers were tested on five accessions. Twenty-two primers, showing clear polymorphic patterns, were chosen to amplify the DNAs of all 25 genotypes; each amplification was repeated three times separately. Only 11 primers were finally analysed, yielding a total of 23 polymorphic RAPD markers.

4.0 OBJECTIVES

The objectives of this project are:

1. To develop singleplex RAPD technique for plant DNA fingerprinting.
2. To study genetic markers of *Mitragyna speciosa, Nicotiana spp., L* and *Centella asiatica.*

5.0 MATERIALS AND METHODS

5.1 MATERIALS

5.1.1 Chemical

5.1.1.1 10X TBE Buffer

An amount of 53.9 gram Tris base and 3.72 gram EDTA is dissolved in 400 ml distilled water. A total of 23 g boric acid was then added to the solution. The pH of the solution was adjusted to pH 8 with the remaining 4.5 g boric acid and top up to 500 ml using deionize water and autoclaved.

5.1.1.2 1X TBE Buffer

An amount of 100 ml of 10X TBE buffer was diluted using 900 ml of deionize water.

5.1.1.2 0.5X TBE Buffer

An amount of 50 ml of 10X TBE buffer was diluted using 950 ml of deionize water.

5.1.1.3 Ethidium bromide

EtBr is intercalating agent that is commonly use in agarose gel in molecular biology laboratories. EtBr will fluorescent under UV light. Precaution is needed when handling EtBr because it is carcinogenic. A working solution was prepare by adding

5.1.2 ReddyMix PCR Master Mix

ReddyMix PCR Master Mix was purchased from AB gene. This ready-to-used master mix was used in all RAPD-PCR reaction. It contained all components required for PCR reaction, including the dye and precipitant to facilitate gel loading.

5.1.3 DNeasy® Plant Mini Kit.

DNeasy® Plant Mini Kit was purchased from QIAGEN and used for DNA extraction for all the samples. The kit is consists of: DNeasy Spin Columns, QIAshredder™ Mini Spin Columns, Collection Tubes (2 ml), Buffer AP1, Buffer AP2, Buffer AP3/E, Buffer AW, Buffer AE and RNase A.

5.1.4 Primer

All arbitrary primer used were purchased from Repfon Glamor Sdn. Bhd.

Table 4: The arbitrary primers used and the sequence

Primer	Sequence (5' to 3')
OP-1	CAATCGCCGT
OP-2	GTTGCGATCC
OP-3	TTCGAGCCAG
OP-4	GTGAGGCGTC
OP-5	CTCACCGTCC
OP-6	GTAACCAGCC
OP-7	TTGGCACGGG
OP-8	GAAACAGCGG
OP-9	GGAGCCCAC
OP-10	GCCGTCTACG
OP-11	GGCATCGGCC
OP-12	GTGAGCGTC
OP-13	ACAACTGGG
OP-14	TGCCGGCTTG
OP-15	CACAGACACC