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JUDUL: DNA FINGERPRINTING OF PINEAPPLE (Ananas comosus var. comosus) USING RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD) MARKERS

Ijazah: SARJANA MUDA SAINS DENGAN KEPUJIAN (BIOTEKNOLOGI)

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DNA FINGERPRINTING OF PINEAPPLE (*Ananas comosus* var. *comosus*) USING RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD) MARKERS

MUHAMMAD AZFAR BIN ABDULLAH

THIS DISSERTATION WAS PREPARED AS ONE OF
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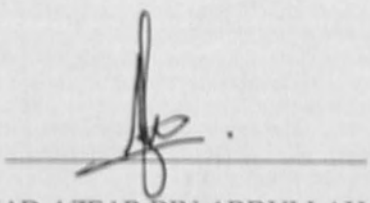
DECLARATION

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

31 March 2005



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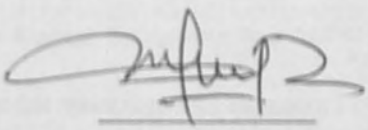
(DR. VIJAY KUMAR) 

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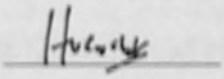
(PROF. HO COY CHOKE) 

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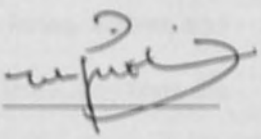
(MRS TEOH PEIK LIN) 

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ABSTRACT

RAPD (random amplified polymorphic DNA) markers generated by arbitrary primers have been successfully employed to detect genetic polymorphisms between different varieties of pineapple (*Ananas comosus* var. *comosus*), namely Paun, Madu and Sarawak variety. The samples were taken from Babagon, Tamparuli and Sipitang respectively. Their genetic relatedness was also determined. DNA was extracted from the pineapple samples using a modified protocol of the salting out procedure (Miller, 1988). PCR was performed for two samples per variety using RAPD primers OPA4, OPA8 and OPA10. PCR products were resolved in 2 % agarose gel. A total of 32 scorable DNA fragments ranging in size from 100-1300 bp were obtained from the PCR amplification of which 68.7 % were polymorphic. The genetic distances (Nei & Li, 1979) between and within the varieties range from 0.1330 – 0.3191. Putative variety-specific amplification products were also obtained (OPA4.3 and OPA10.11 for Paun variety; OPA4.7 for Madu variety). These products could be used as a diagnostic marker in the identification of a particular variety. The genetic distance values were used to construct a Neighbour-Joining dendrogram. Cluster analysis revealed two major groups on which the first group consists of the Paun and Madu variety, while the second group consists solely of Sarawak variety. This information is important to make informed decision in order to improve the development of the crop.

ABSTRAK

Penanda RAPD (random amplified polymorphic DNA) yang dihasilkan oleh primer yang rawak telah berjaya digunakan untuk mengesan polimorfisma genetik di antara nenas (*Ananas comosus* var. *comosus*) yang berlainan variasi, iaitu Paun, Madu dan Sarawak yang diambil dari Babagon, Tamparuli dan Sipitang. Perhubungan genetik di antara variasi-variasi juga telah ditentukan. DNA diekstrak menggunakan protokol *salting out* (Miller, 1988) yang telah diubah suai. PCR dijalankan dengan menggunakan dua sampel bagi setiap variasi menggunakan primer RAPD OPA4, OPA8, dan OPA10. Produk PCR dipisahkan di dalam 2 % gel agarose. Sejumlah 32 fragmen DNA dengan saiz julat antara 100-1300 bp telah dihasilkan daripada amplifikasi PCR, di mana 68.7 % fragmen DNA adalah polimorfik. Jarak genetik yang ditentukan (Nei & Li, 1979) di antara dan sesama variasi menunjukkan nilai antara 0.1330 – 0.3191. Produk amplifikasi putatif yang variasi-spesifik juga telah diperoleh (OPA4.3 dan OPA10.11 untuk variasi Paun; OPA4.7 untuk variasi Madu). Dinyatakan di sini amplifikasi ini boleh digunakan sebagai penanda diagnostik bagi pengecaman sesuatu variasi. Nilai jarak genetik yang diperoleh telah digunakan untuk menghasilkan satu *Neighbour-Joining* dendrogram. Analisis kelompok ini menunjukkan terdapat dua kumpulan utama, di mana kumpulan pertama terdiri daripada variasi Paun dan Madu, manakala kumpulan kedua terdiri hanya daripada variasi Sarawak. Maklumat ini adalah penting bagi membuat keputusan yang baik bagi kemajuan tanaman ini.

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LIST OF ABBREVIATION

%	percentage
°C	degrees Celsius
µl	micro liter
bp	base pair
cm	centimeter
dH_2O	distilled water
DNA	Deoxyribonucleic Acid
dNTPs	Deoxynucleside triphosphates
kbp	kilo base pair
KCl	Potassium chloride
kg	kilogram
m	meter
mg	milligram
$MgCl_2$	Magnesium chloride
ml	milliliter
mM	millimolar
NaCl	Sodium chloride
ng	nanogram
PCR	Polymerase Chain Reaction
pmol	picomolar
RAPD	Random Amplified Polymorphic DNA
RNA	Ribonucleic Acid
rpm	revolutions per minute
SDS	Sodium dodecyl sulphate
Tris-HCl	Tris-hydrochloride
UV	ultra violet
V	volt
v/v	volume over volume

CHAPTER 1

INTRODUCTION

Pineapples are the third most exported tropical fruit in the world, the first and second being bananas and mangoes respectively. Although a lot of work has been done on pineapples, most of them are focused on methods of cultivation, pineapple nutritional contents, pineapple processing, and secondary metabolites of pineapples. Not much work has been done at the genetic level.

Research on pineapples regarding the relatedness between the wide arrays of varieties is lacking. The lack of scientific information has hampered in the development of the crop. With many different techniques of DNA markers are being developed, it is possible to obtain information based on the genetic fingerprints. This research will hopefully provide information and knowledge of the relatedness of the various pineapple varieties, and uncover unique polymorphic bands that can be used as diagnostic marker.

In this study, the relatedness of the pineapples from the different plantations will be analyzed and determined using the Random Amplified Polymorphic DNA (RAPD) markers, originally described by Williams *et al.* (1990), coupled to the Polymerase Chain Reaction (PCR) method. PCR has become an invaluable tool for molecular study. DNA sequences can be amplified *in vitro* from pictogram quantities of DNA or even single copies of a gene within a matter of hours.

The use of RAPD has been a major success in the research of polymorphic markers for plant genome fingerprinting. This method have been proven as a powerful analysis tool in the study of genetic relationship inter and intra organisms. Information obtained can also be used in genome mapping projects or to find differentially expressed genes from different tissues or stage of development.

1.1 Objectives

It is assumed that different pineapple varieties would give different banding patterns, subsequently producing a unique DNA fingerprinting profile. By comparing the banding pattern, the genetic similarity and the genetic distances can be calculated and determined.

In this research, three main objectives have been identified. The objectives are as follows:

1. To determine the genetic similarities and distances of the various pineapples from Babagon, Tamparuli, and Sipitang.
2. To identify levels of polymorphism in each of the pineapple variety.
3. To identify unique DNA fragments that could be used as a diagnostic marker for each of the varieties.

CHAPTER 2

LITERATURE REVIEW

2.1 Pineapples

2.1.1 Pineapple Origins

Pineapple (*Ananas comosus* var. *comosus*) comes from the flowering plant family of *Bromeliaceae*. It is believed to have originated from Southern Brazil and Paraguay. Among the common names of pineapples include "ananas" (Dutch and French), "nanas" (Southern Asia and South Indies), and "pina" (Spanish). Pineapples were first cultivated and domesticated by the Indians, who later brought it up through Central and South America to Mexico (California Rare Fruit Growers, 1996).

Caribbean Indians placed pineapples or pineapple crowns outside the entrances to their dwellings as symbols of friendship and hospitality. Europeans adopted the motif and the fruit was represented in carvings over doorways in Spain, England, and later in New

England for many years. The plant has become naturalized in Costa Rica, Guatemala Honduras and Trinidad but the fruits of wild plants are hardly edible (Maxwell and Maxwell, 1984).

In the regions of Asia, the pineapples were first introduced in the Philippines by Spanish explorers. India received its first pineapple seeds in 1548 through Portuguese traders, who brought it from the Moluccas, and pineapple was growing in China by 1594. Pineapples were first canned in Malaya by a retired sailor in 1888 and export to Singapore soon followed. In recent years, pineapples can be found cultivated all over the world and are usually consumed fresh, as pineapple juice, fruit pulp, or canned (Correia *et al.*, 2004).

2.1.2 Morphology of Pineapples

The pineapple plant is a terrestrial herb 0.75-1.5 m in height with a spread of 0.9-1.2 m; a very short, stout stem and a rosette of waxy, strap-like leaves, long-pointed, 50-180 cm in length. The leaves are usually needle tipped and generally bearing sharp, upcurved spines on the margins. The leaves may be all green or variously striped with red, yellow or ivory down the middle or near the margins. At blooming time, the stem elongates and enlarges near the apex and puts forth a head of small purple or red flowers, each accompanied by a single red, yellowish or green bract. The stem continues to grow and acquires at its apex a compact tuft of stiff, short leaves called the "crown" or "top". Occasionally a plant may

bear 2 or 3 heads, or as many as 12 fused together, instead of the normal one (California Rare Fruit Growers, 1996).

As individual fruits develop from the flowers they join together forming a cone shaped, compound, juicy, fleshy fruit to 30 cm or more in height, with the stem serving as a fibrous but fairly succulent core. The tough, waxy skin (rind), made up of hexagonal units, may be dark-green, yellow, orange-yellow or reddish when the fruit is ripe. The flesh ranges from nearly white to yellow. If the flowers are pollinated, small, hard seeds may be present, but generally one finds only traces of undeveloped seeds. Since hummingbirds are the principal pollinators, these birds are prohibited in Hawaii to avoid the development of undesired seeds. Offshoots, called "slips", emerge from the stem around the base of the fruit and shoots grow in the axils of the leaves. Suckers (aerial suckers) are shoots arising from the base of the plant at ground level; those proceeding later from the stolons beneath the soil are called basal suckers or "ratoons" (California Rare Fruit Growers, 1996).

2.2 Pineapple Varieties

In international trade, the numerous pineapple cultivars are grouped in four main classes: "Smooth Cayenne", "Red Spanish", "Queen", and "Abacaxi", despite much variation in the types within each class (Morton, 1987).

2.2.1 Smooth Cayenne

"Smooth Cayenne" or "Cayenne", "Cayena Lisa" in Spanish (often known in India, Sri Lanka, Malaysia and Thailand as "Sarawak" or "Kew") was selected and cultivated by Indians in Venezuela long ago and introduced from Cayenne (French Guyana) in 1820. From there it reached the Royal Botanical Gardens, Kew, England, where it was improved and distributed to Jamaica and Queensland, Australia (Samson, 1986).

The plant is free from spines except for the needle at the leaf tip, with the size ranging from 1.8-4.5 kg, cylindrical form, shallow eyes, orange rind, yellow flesh, low fiber, juiciness and rich mildly acid flavor. It has become of greatest importance worldwide even though it is subject to disease and does not ship well. Mainly, it is prized for canning, having sufficient fiber for firm slices and cubes as well as excellent flavor (Sprang, 2002).

Different strains of "Smooth Cayenne" have been produced. Each different strain differs in the aspect of size, shape, sweetness, and acidity. Some of them include "Hilo", "St. Michael", "Giant Kew", and "Perolera" (Morton, 1987).

2.2.2 Red Spanish

"Red Spanish" is produced and used mainly for canning. The fruit is more or less round, orange-red externally, with deep eyes, and ranges from 1.36-2.7 kg. The flesh is pale-

yellow, fibrous, with a large core, aromatic and flavorful. The fruit is hard when mature, breaks off easily and cleanly at the base in harvesting, and stands handling and transport well. It is highly resistant to fruit rot though subject to gummosis (Sprang, 2002).

"Cabezona" ("Bull Head" or "Pina de ague") is a prominent variant (a natural tetraploid) of 'Red Spanish' long grown in Puerto Rico in the semiarid region of Lajas and also in El Salvador. The plant is large, over 1 m high; the leaves are gray-green. The fruit is conical; averages 1.8-2.75 kg and may reach 8 kg or more. It is orange-yellow at maturity, has few fibers and sweet-acid flesh. The stem is large and extends up into the base of the fruit and if the fruit is broken off when harvested it leaves a cavity. It is marketed as fresh pineapple and is resistant to gummosis. It has been cultivated frequently in the Philippines (Morton, 1987). Among other strains of "Red Spanish" includes "Valera", "Valera Amarilla", "Valera Roja", and "Castilla" (Maxwell and Maxwell, 1984).

2.2.3 Queen

"Queen" (also called "Common Rough" in Australia) is the leading cultivar in South Africa, Queensland and the Philippines. The plant is dwarf, compact, more cold-resistant and more disease-resistant then "Smooth Cayenne". It matures its fruit early but suckers freely and needs thinning, and the yield is low. The fruit is conical, deep-yellow, with deep eyes; weighs 0.45-1.13 kg; is less fibrous than "Smooth Cayenne", but more

fragrant; it is juicy, of fine flavor with a small, tender core. It is sold fresh and keeps well. It is mainly used for canning (Maxwell and Maxwell, 1984).

2.2.4 Abacaxi

"Abacaxi" (also called "White Abacaxi of Pernambuco", "Pernambuco", "Eleuthera", and "English") is well known in Brazil, the Bahamas and Florida. The plant is spiny and disease-resistant. Leaves are bluish-green with red-purple tinge in the bud. The numerous suckers need thinning out. The fruit weighs 1-5 kg, is tall and straight-sided; sunburns even when erect. It is very fragrant. The flesh is white or very pale yellowish, of rich, sweet flavor, succulent and juicy with only a narrow vestige of a core. It is rated by many as the most delicious pineapple variety. It is too tender for commercial handling, and the yield is low thus the fruit is marketed fresh (Samson, 1986).

"Sugarloaf" (also called "Pan de Azucar") is closely related to "Abacaxi", and found mainly at Central and South America, Puerto Rico, Cuba and the Philippines. The leaves of the plants and crowns pull out easily and this fact gave rise to the unreliable theory that pineapple ripeness is indicated by the looseness of the leaves. The fruit is more or less conical, sometimes round; not colorful; weighs 0.68-1.36 kg. Flesh is white to yellow, very sweet, juicy. Among several strains of "Sugarloaf" are "Papelon", and "Black Jamaica", and "Montufar" ('Sugar Slice' of Guatemala) (Morton, 1987).

2.2.5 Pineapples in Malaysia

"Mauritius" (also known as "Malacca Queen", "Red Ceylon" and "Red Malacca") is one of the two leading pineapple cultivars in Malaysia; also important in India and Ceylon. The leaves are dark green with broad red central stripe and red spines on the margins. The fruit is small, weighs 1.36-2.25 kg, yellow externally; has a thin core and very sweet flesh. It is sold fresh and utilized for juice (Samson, 1986).

"Singapore Red" (Also called "Red Jamaica", "Singapore Spanish", "Singapore Queen", and "Singapore Common") is second to "Mauritius" in popularity. The leaves are usually all-green but sometimes have a reddish stripe near the margins; they are rarely spiny except at the tips. The fruits, cylindrical, reddish, with deep eyes, are small - 1.6-2.25 kg - with slender core, fibrous, golden-yellow flesh; insipid raw but valued for canning. The plant is disease and pest-resistant (Morton, 1987).

The related "Green Selangor" (also called "Selangor Green", "Green Spanish", and "Selassie") of Malaysia has all-green leaves prickly only at the tips. The flesh is golden-yellow, often with white dots. This cultivar is grown for canning (Samson, 1986).

2.2.6 Genetic Studies of Pineapple

Although there are four major pineapple varieties as described above, there are numerous sub-varieties and various types of cultivars, some of which are closely related. Most

varieties have their own unique morphological characteristic, but differentiating them sometimes could be difficult. Therefore, it is important that analysis to distinguish pineapple varieties at the genetic level be conducted through the use of DNA fingerprinting. The fingerprints can be created using various types of genetic markers that are available, such as Random Amplified Polymorphic DNA (RAPD), Direct Amplified Length Polymorphism (DALP), and Restriction Fragment Length Polymorphism (RFLP).

2.3 Random Amplified Polymorphic DNA (RAPD)

Random Amplified Polymorphic DNA (RAPD) was first introduced by Williams *et al.* (1990). The DNA polymorphism assay is done based on the amplifications of random DNA segments with single primers of arbitrary nucleotide sequence. RAPD employs short primers of arbitrary sequences to amplify random portions of the sample DNA by PCR. Since each primer is short, it will anneal at many sites throughout the target DNA (Franklin *et al.*, 1999).

RAPD is considered to be non-coding and therefore selectively neutral. Such markers have found widespread use in population genetic studies whose characterizations of genetic diversity and divergence within and among populations are based on assumptions of Hardy–Weinberg equilibrium and selective neutrality of the markers employed (Liu *et al.*, 2004).

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