Assessment of Gene Polymorphism in Terpene Synthase among Local Variety of Pineapple through Gene Sequence Analysis

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DECLARATION

I hereby declare this work embodies in this report is the result of the original research and has not been submitted or institutional.

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TABLE OF CONTENT

List of Tables

List of Figures

List of Abbreviation

mL	Mililiter
µL	Microliter
cm	Centimeter
M	Molarity
ng	Nanogram
nm	Nanometer
bp	Base pair
µM	Micromolar
mm	Micrometer
µg	Microgram
PCR	Polymerase chain rule
MEGA	Molecular Evolutionary Genetics Analysis
CTAB	Cetyltrimethylammonium bromide
SDS	Sodium dodecyl sulfate
DNA	Deoxyribonucleic acid

Assessment of Gene Variation in Terpene Synthase Gene among Varieties of Local Pineapple

ABSTRAK

Nenas atau Ananas comosus adalah industri hortikultur yang penting di seluruh kawasan tropika dan subtropika. Kajian mengenai sebatian nenas menunjukkan lebih daripada 280 sebatian aroma yang menemui sifat rasa nenas. Nenas digunakan secara meluas sebagai buah segar dan di dalam tin seperti jus yang diproses dan juga digunakan sebagai ramuan dalam makanan kerana rasa manisnya yang unik. Dalam nenas, aroma meruap yang paling banyak yang menyumbang kepada rasa nanasnya adalah terutamanya dari ester dan terpena. Terpena adalah enzim yang bertanggungjawab terhadap sintesis sesquiterpenoid dalam tumbuhan aromatik. Pengenalpastian pelbagai variasi dalam gen dan analisis kesannya boleh membawa kepada pemahaman yang lebih baik mengenai kesannya terhadap fungsi gen. Dalam kajian ini, pengoptimuman kaedah pengekstrakan menggunakan dua kaedah pengekstrakan yang berbeza menghasilkan hasil DNA yang berbeza. Kaedah SDS menghasilkan hasil DNA yang lebih tinggi berbanding dengan kaedah CTAB. Amplifikasi PCR dengan menggunakan primer untuk Exon 2 dan DNA genomik yang diekstrak menggunakan kaedah SDS menghasilkan semua jalur tunggal tertentu seperti yang dijangkakan. Walaupun DNA genom yang diekstrak dengan menggunakan kaedah CTAB gagal amplifikasi PCR untuk primer yang sama untuk dua jenis nanas, walaupun terdapat jalur yang jelas dalam elektroforesis gel. Sejumlah 15 jalur diperkuat menggunakan primer Exon 2, Exon 3 dan Exon 6 untuk semua lima jenis nenas. Jalur yang diperkuat adalah tebal dan peramat yang menunjukkan integriti dan kekhususan primer. Usaha untuk jujukan semua kelima belas produk amplifikasi telah gagal kerana masalah semasa pengangkutan yang menyebabkan DNA menjadi terdegradasi. Hanya dua sampel Moris untuk Exon 2 dan Sarawak untuk Exon 3 berjaya dijujuk selepas menyelesaikan masalah. Jujukan ini dibandingkan dengan rujukan terpena sintase gen, berkenaan dengan exonnya, secara individu. Analisis jujukan gen menunjukkan polimorfisme yang rendah untuk Exon 2 di Moris tetapi polimorfisme jujukan tunggal yang tinggi untuk Exon Sarawak 3. Beberapa variasi asas tunggal yang dikesan menyebabkan mutasi bukan sinonim pada tahap protein yang menunjukkan perubahan yang berpotensi signifikan pada paras protein. Walau bagaimanapun, variasi ini dikehendaki memerlukan penyelidikan dan pencirian lanjut untuk kepentingannya untuk menyebabkan variasi utama pada tahap fungsian protein terpena sintase.

Kata kunci: *Ananas comosus*, terpene, kaedah CTAB, kaedah SDS, DNA

Assessment of Gene Variation in Terpene Synthase Gene among Varieties of Local Pineapple

ABSTRACT

Pineapple or *Ananas comosus* is an important horticultural industry throughout the area of the tropical and subtropical. Studies on pineapple compounds revealed more than 280 aroma compounds found the nature of pineapple flavors. Pineapple is widely consumed as fresh and canned fruit as processed juices and also use as ingredient in food due to its unique sweet flavor. In pineapple, the most abundant aroma volatiles that contributes to its flavour of pineapple are mainly from ester and terpene. Terpene synthase is an enzyme that responsible for the synthesis of sesquiterpenoid in aromatic plant. Identification of numerous variation in genes and analysis of their effect may lead to a better understanding of their impact on the gene function. In this study, optimization of extraction method using two different extraction methods produced different yield of DNA. SDS method produced higher amount of DNA yield as compared to the CTAB method. PCR amplification by using primer for Exon 2 and genomic DNA extracted using SDS method produced all specific single band as expected. While genomic DNA extracted using CTAB method failed PCR amplification for the same primer for two pineapple varieties, despite of its apparent band in gel electrophoresis. A total 15 bands were amplified using primer Exon 2, Exon 3 and Exon 6 for all five pineapple varieties. The bands amplified were thick and intense which indicates its integrity and specificity of the primer. Effort to sequences all fifteen amplification products were failed due to problem during transportation that cause DNA to be degraded. Only two samples Moris for Exon 2 and Sarawak for Exon 3 were successfully sequenced after troubleshooting. These sequences were compared with the reference terpene synthase gene, with respect to its exon, individually. Gene sequence analysis revealed low polymorphism for Exon 2 in Moris but high single sequence polymorphism for Sarawak Exon 3. Several single base variations detected caused non-synonymous mutation at the protein level that indicates their potentially significant change at protein level. Nevertheless, these variations detected required further investigation and characterization for its significance to cause major variation at the functional level of the protein terpene synthase.

Keywords: *Ananas comosus*, terpene, CTAB method, SDS method, DNA

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Pineapple or *Ananas comosus* is a member of *Bromeliaceae* order *Poales*, genus *Ananas* and species *comosus* (Bartholomew, R.E.Paull, K.G.Rohrbach. 2003). Pineapple is called as 'king of fruit' because it has crown of the leaves. Pineapple is an important horticultural industry throughout area of the tropical and subtropical. Pineapple as one of the tropical fruits are the third most important in the world after bananas and citrus (Bartholomew et al. 2003). Because of the sweet and its exotic taste demands for the fruit as fresh cut and canned fruits as well as processed juices and as exotic food ingredients (Tokitomo,Y. 2007). In Malaysia, pineapple is grown in an area of about 10,847 hectares with a production estimate of 272,570 tonnes in 2015 (Department of Agriculture (DOA), 2016). Malaysian Pineapple Industry Board (MPIB) stated that, pineapple industry is one of the important agricultural sector in Malaysia which play a role of country's earnings as one of the world pineapple suppliers besides the livestock industry, poultry industry and fishery industry.

The aroma volatile compounds are a vital factor for the fruits especially for fresh and processed fruit (Chang-Bin Wei, Sheng-Hui Liu, Yu-Ge Liu, Ling-Ling Lv, Wen-Xiu Yang & Guang- Ming Sun. 2011). An aroma compound is also known as odorant, aroma, fragrance and flavors.

Aroma compound consist of numerous chemical compound composition that contributes to the smell or odor of the fruit. Studies on pineapple compounds aged over 60 years old revealed more than 280 aroma compounds found to be the nature of pineapple flavors (Zhang X.M. et al. 2009; Tokitomo, et.al. 2005). Morais and Silva (2011) stated that ethyl hexanoate is the one of the most important compounds related to the pineapple flavor. According to Facundo (2009), it also stated that the compound ethyl hexanoate is related to the aroma that is described as 'pineapple'. Every type of varieties of pineapple have different aroma or volatile compound (Liu et al. 2008; Zhang X.M. et al. 2009). In the previous study, the analysis of pulp and core of pineapple shows the presence of 44 volatile compound, which are 18 esters, 17 terpenes and another 4 are alkenes (Wei et.al, 2011).

Terpenes is one of the largest secondary metabolite product of a various plants, mainly in conifers (major component of resin). Terpenes have a strong smell and insoluble in water and this important for helping plants to protect against parasites, some of the examples are camphor (*Cinnamomum*), menthol (*Mentha*), and carotenoids. Terpene are derived from the unit of isoprene that is produced biosynthetically. Terpene are classified by the chain of isoprene unit such as hemiterpene, monoterpene, sesquiterpenes, diterpenes, sesterterpenes, triterpenes and tetreterpenes. It is commonly known that the composition of the terpene compounds is one of the attributes of taste and flavour of fruit. Terpene is commonly attribute of flavoring and fragrances. The terpene synthase functions in the biosynthesis of sesquiterpenoid that responsible for the flavour profile citrus fruit (Wildermuth, 2006). The type of terpene that contribute to flavor or aroma is limonene. Limonene is a monocyclic monoterpene. Variety of crops that usually have high limonene are usually the fruit with strong citrus smells such as oranges, lemons, tangerines and limes.

For the caryophyllene, sesquiterpene was found in plants such as Thai basils, cloves, cinnamon leaves and black pepper, and in small quantities in lavender. Its aroma is like a peppery, woody or spicy. Terpineol have three types which are α-Terpineol, terpinen-4-ol, and 4-terpineol. It is related to the monoterpenoids. The aroma of terpineol is different to aroma of lilac and flower blossom. In pineapple, the most abundant compound that responsible for its flavours are from the group of ester and terpene (Wei et. al, 2011)

Genetic polymorphism in a gene may cause major phenotypic change between individual. The change of protein will lead to the change of phenotype. Genetic polymorphism refers to the appearance of two or more phenotypes in a certain population (in proportions that the rarest of the characteristics cannot be maintained just by recurrent mutation) (Ford E.B.1975). Polymorphism promotes diversity and persists over many generations because there is no single form that has an overall advantage or disadvantage over the others in terms of natural selection. A mutation is any changes in population and that the mutation changes this normal allele to a rare and abnormal variant.

Deletion is a one of the type of mutation that changes the DNA sequence and may lead to phenotypic change such as increase susceptibility to disease. The functional consequences of a mutation can be predicted based on the sequence change induced. Functional studies are valuable in confirming, refining, or changing of these predictions. Change of mutation may causes abnormal level of protein to be produced may prevent it from functioning properly. Its function may be partially or completely destroyed. Missense mutation is a type of mutation that change one DNA base pair. It results in the substitution of one amino acid for another in the protein made by a gene. Nonsense mutation also change in one DNA base pair. Instead of substitute one amino acid for another, the altered DNA sequence prematurely signals the cell stop building a protein. An insertion is the changes in the number of DNA sequence in a gene by adding base of DNA. So, the protein made by the gene may not function properly.

A duplication consists of a base of DNA that is abnormally copied one or more times. This type of mutation may alter the function of the resulting protein. Frameshift mutation occurs when the addition or loss of DNA sequence changes a gene's reading frame. A reading frame consists of groups of 3 bases that each code for one amino acid and change the codes of amino acids. The resulting protein is usually nonfunctional. Insertions, deletions, and duplications can cause frameshift mutations. Loss of function mutation also known as inactivating mutations, results in the gene product having less or no function (being partially or wholly inactivated).

Genetic polymorphism may occur either in the coding or non-coding part of the gene but more common in the non-coding region. This is because minor changes in DNA sequences that encode for proteins may cause detrimental effect on the function of the protein it encoded. Polymorphism in coding region may highly impact the organisms phenotype when they are non-synonymous (changing the amino acid which the codon codes). The discovery of single nucleotide polymorphisms (SNPs), insertions and deletions, which are the basis of most differences between alleles, has been simplified by recent developments in sequencing technology (Antoni Rfalski. 2002).

1.2. Problem Statement

Pineapple is widely consumed as a fresh and canned fruit as well as processed juices and as an ingredient in exotic food due to it attractive sweet flavor (Tokitomo,Y. 2007). It is very significant for fruit quality, selection and breeding, cultivation as well as industrial development to study characteristic attributes of pineapple aroma (Wei et. al, 2011). It is because the terpene synthase functioned in the biosynthesis of terpenoid compound that is responsible for the aroma and flavour of pineapple juice. In pineapple, the most abundant aroma volatile contribute to the flavour of pineapple are mainly from ester and terpene (Wei et. al, 2011).

Therefore, identification of numerous variations in genes and analysis of their effects may lead to a better understanding of their impact on gene function (Shastry, 2009). If, there are any detected polymorphism in terpene synthase gene, the variation may be responsible for different flavour profile of different pineapple variety. Thus, it is important to study about polymorphism in terpene gene among the different local varieties of pineapple.

1.3 Scope of study

Five varieties of pineapple was used for this study which are 'Sarawak', 'MD2', 'N36', 'Moris' and 'Josapine' which was obtained from Malaysian Pineapple Industry Board (MPIB). The total genomic DNA of pineapple was extracted for the amplification of the terpene synthase gene. Six different set of primers were designed based on the six exon of terpene synthase gene obtained in NCBI with gene ID of NC_033627. However, for this study only primers for Exon 2, Exon 3 and Exon 6 were used for PCR amplification and gene sequence analysis. The PCR product was sent for sequencing. The genetic polymorphism assessment was done by analyzed the protein level on the five different varieties of pineapples.

1.4 Significance of study

Pineapple is the third most important tropical fruit in the world after banana and citrus (Bartholomew et. al, 2003). Pineapple is widely consumed as a fresh and canned fruit as processed juices and also use as an ingredient in food due to its unique sweet flavor (Tokitomo,Y, 2007). In pineapple, the most abundant aroma volatiles that contribute to its flavour are mainly from ester and terpene (Wei et. al, 2011). It is widely known that the composition of the terpene compound are one of the determinants of the taste and flavor of fruit. Terpene synthase gene is an enzyme that responsible for the synthesis of

sesquiterpenoid in aromatic plant. Identification of numerous variation in genes and analysis of their effect may lead to a better understanding of their impact on gene function (Shastry, 2009). A polymorphism is a DNA sequence variation that is common in the population. Genetic polymorphism or mutation in gene may cause major phenotypic change between individual. If there are any detected of polymorphism in terpene synthase gene such as deletion of DNA sequence or loss of function, it may responsible for the different flavor profile of pineapple for the different varieties. The benefit of studying the polymorphism between the different varieties, would lead us to the characterization of the gene across the different varieties and the identification of the genetic variation observed may be associated with specific pineapple varieties, with its unique flavor profile. The unique polymorphism detected for each variety can also be utilized in marker-assisted breeding specifically for the flavour profile.

1.5 Objectives

1. To amplify terpene synthase gene from five different pineapple varieties namely, Josapine, MD2, N36, Moris and Sarawak.
2. To identify polymorphism in the terpene synthase gene at protein level from the five different varieties of pineapple through gene sequence analysis.

1.6 Hypothesis

H_0 = There is no polymorphism in the terpene synthase gene among five different varieties of pineapple.

H_A = There is presence of polymorphism in the terpene synthase gene among five different varieties pineapple.

CHAPTER 2

LITERATURE REVIEW

2.1 Pineapple

Pineapple, [*Ananas comosus L. (Merr.)*] is a member of *Bromeliaceae* order *Poales*, genus *Ananas* and species *comosus* (Bartholomew, R.E.Paull, K.G.Rohrbach, 2003). Other than, bananas and citrus, pineapple also one of the most important tropical fruit in world production (Bartholomew et. al, 2003). It is an herbaceous monocotyledon that grown for a large cone-like fruit that normally consumed fresh or canned as sliced, chunks, tidbits, fruit cocktail and juice. Thailand, Indonesia and Malaysia are exception as the industry is run by local operators. The leading of production country in the world on 2005 is Thailand (2.1 million tonnes from 90,000 ha), then, follow by Philippines (1.8 million tonnes from 49,000 ha) then, follow by Brazil (1.4 million tonnes from 53,000 ha) (Anon, 2006). Malaysia, was once among the leaders in world for pineapple production in the 1970s. Canned pineapple is the major export earner for Malaysia, grossing about RM100 million annually (Chan, 2000).

The production of fresh pineapple have increased the interest for the introduction of new hybrids like ‘Josapine’ for domestic as well as for export markets.

2.2 Varieties of pineapple in Malaysia

2.2.1 Sarawak

‘Sarawak’ belong to the smooth Cayenne group which is the most popularly cultivated pineapple in the world. The origin of the cultivar name is not known but mostly certainly this cultivars did not have it root from the East Malaysia state of Sarawak [Malaysia Agriculture and Development Institute (MARDI)]. The ‘Babagon’ in Sabah is synonymous with this cultivar. Elsewhere in the world it is known as ‘Smooth Cayenne’, ‘Cayenne Lisse’, ‘Maipuri’, ‘Kew’, ‘Esmeralda’, ‘Claire’, ‘Typoon’, Saint Michel and ‘Hilo’ (Chan et. al, 2003). ‘Sarawak’ bears large ovoid fruit (2-3kg) on the short, strong peduncle. The eyes of the fruit are flat and shallow resulting in a thin skin that leads to good flesh recovery, but is vulnerable to bruise. The flesh is pale yellow, soft and juicy, with total soluble solids (TSS) of 13-19 °Brix. The production cycle for ‘Sarawak’ is longer than most of the other cultivars. The production cycle is 15.6 months from planting to harvest. ‘Sarawak’ is grown only for niche fresh fruit market and is price usually higher than other cutivars.

2.2.2 Moris

Moris belong to the Queen group and is the most popular fresh cultivar in Malaysia, but its dominance is rapidly losing out to the newer hybrids like 'Josapine' and 'N36' [Malaysia Agriculture Research and Development Institute (MARDI)]. A number of local variants exists, like 'Moris Slipping' and 'Moris Sungei Balang' as describe by Chan and Lee (2003) as well as 'Sarikei' was located in Sarawak. The 'Moris' plant is small, with short and very spiny, purple tingled leaves. The fruit is small (0.5-1.2kg), tapering and with many small bulging eyes. The production cycle for 'Moris' is fairly short compared with 'Sarawak'. The plant can be induced at 8 month after planting and 'red heart' follow after 30 days and takes another 110 days to fruit harvest. The production cycle is 12.7 month from planting to harvest.

2.2.3 Josapine

'Josapine' is a hybrid between 'Johor' and 'Sarawak' released by the Malaysia Agriculture Research and Development Institute (MARDI) in 1996 (Chan & Lee 2003). It fruit very early and cultivated on annual cycle in Malaysia. Fruit weight is between 1.1-1.3kg, cyclindrical, with dark purple skin and ripening to an attractive orange red. The flesh colour is deep-golden yellow with strong aroma and sugar content between 17-22 °Brix. The production of cycle for 'Josapine' is shortest among the commercial cultivars. The production cycle is only 11.2 month from planting to harvest. This making 'Josapine' the only cultivars that can follow an annual cropping cycle. It rapidly replaced the conventional 'Moris' cultivars as the premium fresh pineapple in Malaysia.

2.2.4 MD2

This is a famous export development by Del Monte also know in the market as ‘Golden Ripe’ or ‘Extra Sweet’ (Janick, 2003). It has its beginnings like ‘Maspine’ at the Pineapple Research Institute in Hawaii where it was known as hybrid member ‘73-114’. It is now extensively grown in Costa Rica. At Malaysia also extensively grown at Johor Bharu. It has medium to large (1.3-2.5kg) cyclindrical, square- shouldered fruit, with large flat eyes and an intense orange yellow colour. The clear yellow pulp is sweet, compact, and fibrous. It is high in sugar (15-17 °Brix) and ascorbic acid but lower in total acid than ‘Smooth Cayenne’.

2.2.5 N36

N36 is selected from the hybrid cross between 'Gandul' (Spanish) and 'Smooth Cayenne' by estate Peninsular Malaysia. It is a very strong cultivar and medium-sized fruit (1.5 - 2 kg) with a large crown. It has a high sugar content (14 ° Brix) and acid (0.6-0.8%) but somewhat pale flesh color. The fruit is quite tolerant with black heart problems but vulnerable to disease marbling.

2.3 Aromatic compound In Pineapple

The aroma volatile compounds are very important properties for fruits and vital factors to determine the attributes of fresh and processed fruit (Wei et. al, 2011). Pineapples known as unique sweet flavor, widely consumed fresh or canned or processed juices, and also as an ingredient in foods (Tokitomo, 2007). Pineapple contains good aroma, flavour, juiciness, sweetness, texture and high nutritional content such as vitamins, phenolics, fibre and minerals (Brat,Thi-Hoang, Soler, Reynes & Brillouet, 2004). In the previous study,thirty five volatile compounds was identified from headspace of the pineapple processing residue distillate and by chromatogram that captured by solid phase microextraction (SPME) (Lília Calheiros de Oliveira Barretto; Jane de Jesus da Silveira Moreira; João Antônio Belmino dos Santos; Narendra Narain & Raquel Anne Ribeiro dos Santo, 2013).

In same research study, among the identified components were esters (37%), alcohols (29%), aldehydes (9%), ketones (9%), acids (6%), and other compounds (11%) (Lília Calheiros de Oliveira Barretto et. al, 2013). Wei et al. (2011) identified forty four volatile compounds when characterizing pineapple pulp and core by headspace-solid phase microextraction (HS-SPME) and gas chromatography-mass spectrometry (GC/MS). Some of them are the same as those identified in the present study, in which pineapple residues were characterized: methyl hexanoate, ethyl hexanoate, methyl 3-(methylthio) propanoate, methyl octanoate, ethyl decanoate,tererpineol, nonanal, and decanal.

However, the ones with the highest odor activity values were ethyl hexanoate, nonanal, and decanal (Lília Calheiros de Oliveira Barretto et. al, 2013). Morais and Silva (2011) also emphasized that ethyl hexanoate is one of the most important compounds related to the pineapple flavor. According to Facundo (2009), the compound ethyl hexanoate is related to the aroma note described as 'pineapple'. Other same research study state, the analysis of the SPME extract of pineapple indicated the presence of thirty three volatile compounds (Liang-Yong Zheng, Sun, G.M., and Yu. G.L., Lv,L.L., Wen, X.Y., Wei, F.Z., & Chang, B.W. 2012). Nineteen of these compounds were esters, five were terpenes, three lactones, one ketone, one alcohol, one aldehyde, and two acids in the two pineapple varieties (Liang-Yong Zheng et. al, 2012). The five types of terpene present are (±) Dictyopterene A, 4, 9-Muuroladiene, α-Muurolene, (－) -Alloaromadendrene and 1, 6-Cyclodecadiene.

2.4 Terpene Gene

Terpenes is an enormous range of diversity in carbon skeletons and functional groups, with individual plants containing as many as 100 different terpenes as components of complex oils, resins, or volatile mixtures (Harborne & Turner, 1984). Terpene are classified by the chain of isoprene unit such as hemiterpene, monoterpene, sesquiterpenes, diterpenes, sesterterpenes, triterpenes and tetreterpenes. In plants, terpene synthases (TPSs) is responsible for the synthesis of the various terpene molecules from two isomeric 5-carbon precursor ‘building blocks’, leading to 5-carbon isoprene, 10-carbon monoterpenes, 15-

carbon sesquiterpenes and 20-carbon diterpenes (Feng Chen, Dorothea Tholl, Jorg Bolhman & Eran Pichersky, 2011). Sesquiterpene are the one type of common volatile terpenoids components of plants such as monoterpene and the various type of terpene synthase (TPSS). Terpene synthase genes in aromatic plants encode attendant enzymes in synthesis of plant essential oil (Bayrak, 2006). Essence oils including terpene are used as natural flavor additives for food, perfume, and aromatherapy and in traditional and alternatives medicine (Poyraz, 2007).

In some research study in grape, some of the most prevalent wine grape aroma constituents are terpenoids and this study represents a wide report about grape terpene synthase (TPS) gene transcript profiling in different tissues of two aromatic grapevine varieties, particularly flowers and developing berries, correlated with the accumulation patterns of free aroma compounds (Fabiola Matarese, Giancarlo Scalabrelli & Claudio D 'Onofrio, 2013)

2.5 Polymorphism:

DNA polymorphism is a variation difference of nucleotide sequences between individuals. The example of polymorphism is can either be differences in single base pair changes such as deletion, insertions, or any in the number of copies of a DNA sequences. SNP discovery in many crop species, such as corn and soybean, is relatively straightforward because of their high level of intraspecific nucleotide diversity, and the availability of many

gene and expressed sequence tag (EST) sequences (Antoni R. 2002). In the some research studies, a single nucleotide polymorphism only present in yellow root cultivars co-segregated rated with colour root in a breeding pedigree in *Cassava* (Ralf Welsch. 2010). Loss of function alleles of *Badh2* are also found in the indica genepools, and then transferred into japonica (Gaoneng Shao. 2012). In other research, the varieties of maize which are B73 and Delprim contain *tps4* and *tps5* alleles but their differences in terpene composition result from the fact that B73 has only a single functional allele of *tps4* and no functional alleles of *tps5*, whereas Delprim only has a functional allele of *tps5* and no functional alleles of *tps4* and lack of functional was shown to be attributable to frame-shift mutations or amino acid substitutions that greatly reduce the activity of their encoded proteins (Tobias G. Köllner, Christiane Schnee, Jonathan Gershenzon & Jörg Degenhardt. 2004).

In grapevine breeding programs, the sources of new varieties are derived from selection of somatic mutants and classical hybridization. Currently, there are many somatic mutant varieties that are important for commercial production (Moretti, 1983; Fregoni, 1998, 2000). Lately, new mutated grape varieties have been developed in Russia and in Italy. The mutant variety “Fikreti” is derived from “Marandi” in Russia. In Italy, several mutants were developed from Banarda, Regina Vigneti and Dolcetto cultivars (Maluszynski et al., 2000).

There some report of a genome-wide survey of DNA polymorphisms in the *Brasicca. rapa* genome based on the 557 bacterial artificial clone sequences of *Brassica. rapa ssp. pekinensis cv. Chiifu"* (Park S, Yu HJ, Mun JH & Lee SC. 2010). It identified and characterized 21,311 SNPs and 6,753 InDels in the gene space of the *Brasicca. rapa* genome by re-sequencing 1,398 sequence-tagged sites (STSs) in eight genotypes (Park S et. al. 2010).

2.6 Terpene Gene of Pineapple

Pineapple has attractive flavors and terpenes are one of the main volatile flavor compounds. Now the terpenoids reported in pineapple are mainly sesquiterpenes including selinene, caryophyllene, cadinene and copaene (Wei et al., 2011). In the database of pineapple genome there are twenty record of terpene synthase gene. Based on the table 1, terpene synthase contained seven homologs with size up to 4202 bp. Each exon has size range in between 139 to 308 bp.

Table 1: The sequence of terpene divided based on its exon.

Primer Name	*Exon Sequence*	*Base Pair*
Exon 1: NC_033627.1:13355413-13359778:683-944	GCGAGTCACAAATCACACAAATTGAGAAGTTGAAGGA GAAAGTGAAGCAGCTTATGTACAAGAAGGACGAACCA GCGGCTAAACTTAAGCTAGTTGATGCCCTGCAACGTCT CGGCATAGCTTATCACTTTGAGAAGGAGATCAAAAATA TGGTGAGCTCAATATCCATCGACGATGCAAAAGTTGCG TTCGAAGAGGATATTTTTTTGATGGCTTTATTATTCAGA TCGCTTCGAGAAAATGGCTTTTCGGTCTCCCAAG	261 bp
Exon 2: NC_033627.1:13355413-13359778:1082-1457	TCTCTTGAGTGGCTACAAGGATATCAAGGGTCACATCA AACCCTACCTTCAAAAGAAGGATATTTTGGGACTTCTCT CTCTATACGAAGCTTCATATTTTGGTTTTGAAGGAGAGA GGATATTGGATGAAGCAAGAATTTTCACGACCAAGCAT CTAAATGAACTTAAACCCTACATGGATCCAAACCTTAA GGCTGAAGTGGTTCATGCGCTAGAACTTCCACTACACT GGAGGCCTCCGCGATTAGAGGCGAGATCGTACATCGAA CAATACGAGAGAGATGAAGATGTTGAGCTCGTTGTTTT GCAACTAGCTAAGCTCGATTTTAACAGGGTGCAAATCA TACATCAGGAAGAACTTAAAAGAATTTCAAG	375 bp
Exon 3 : NC_033627.1:13355413-13359778:2529-2747	TGGTGGAGAGATGTGGCTCTTGCAGAAAATTTGTCGTT CGCCAGAGACCGCATAATAGAGTGCTTCTTTACCGCCG CTGGGGTTGTATTTGAGCCCCAGTTGGGACATTGCCGT GAGAGCCTGGCTAAAGTTTGCACTTTTATTACAGTCATA GACGATGTTTACGATGTCTATGGAACTGTCGATGAACT TGTGCTATTCACAAGTGCCGTCGAAAG	218 bp
Exon 4: NC_033627.1:13355413-13359778:2878-3016	TGGGAGAATGATGCAGTGGAAGGGCTTCCAAATTACAT GAAAACACTGTATTCCACTCTATACAACACAACTAATG AAATGGCATGTCACATGCTTAGAGAAAAGGGTTGGGAT GTACTCCCTTATCTTAAGAATGCG	139 bp
Exon 5: NC_033627.1:13355413-13359778:3485-3733	GGCATGATCTATGCAAGTCATTTCTAGTGGAGGCAAAA TGGCATCACAATGGCTACAAACCGACTCTACGCGAATA TTTGGAGAACGGTTGGATGTCGTCGTCGGGGAATGTTA TACTTCTCCATGCATTCGCGTTGACGGGGGAGAAGGTA GCGATAGAGACTTTACGGAAATCGGAAAACTACCAAG GACTAGTCCGGTCGTCTTCGTCGATTCTTCGACTTTGCA ACGACTTAGCGACTTACACA	248 bp

Exon 6: NC_033627.1:13355413-13359778:3894-4202	*CGGAGCGGGAGAAAGGTGACGCTCCATCATCGATAGATTG CTACATGCAAGAGCACGACACAAACGAGGAAGAGTCTCGC GAAGCCGTAAGAGATCTAATCGTCGAGACATGGAAGAAGAT GAATAAAGATGCTTACGATCGATGTCGTTTGCCTCGATCCT TCACGAATTCGGCGATGAATCTCGGTCGTATATCGCATTGT CTCTACCAGAATGGGGATGGCATAAGTGCTCCAAACCAAGA GAAGAAGTATCAGATCAATTCCTTGTTTTTGGAACCCATACT TGTGAAAGGAACTGAAAATTAA*	*308 bp*
Exon 7: NC_033627.1:13355413-13359778:79-315	TGGTAAATAGTACTAGTGCATTGTATGGGTGCTATTCAC CGTGCAGTGTGTTGAGCAATGGCTCGGCCAAAGCAGGC CGTGTCCGCTGTACTGCTTCTGCCTCACCTCCGACGTAT GTGCGCGCACGTGTGTCACCACCGCCACCGCCCCAACC ACAACCGATGCGGCGGTCGGGGAATTACCAACCTAACT CGTGGGATTACAAGCTGATTCGATCACTGAAGGGCGGT TATTTG	236 bp

CHAPTER 3

METHODOLOGY

3.1 Plant Materials

The five different varieties of pineapples which were the ‘Sarawak’, ‘N36’, ‘Moris’, ‘MD2’ and ‘Josapine’ were obtained from Malaysian Pineapple Industry Board (MPIB). Only the leaves part was collected from each variety. The leaves were stored in -20°C until further use.

3.2 DNA extraction

3.2.1 CTAB Method

DNA extraction was performed by using a modify CTAB method based on the protocol of (Doyle.1987). The leaves sample of each variety of pineapple was dried and grinded to powder form with liquid nitrogen by mortar and pestle. The powder was added into a centrifuge tube with addition of 500µl of CTAB extraction buffer. After that, the mixture was incubated in the water bath at 65 °C for approximately 30 min and the content was mixed by inverting the tubes. Then, the content was cooled and then 750 µl of chloroform-isoamyl alcohol (24:1) was added and the contents was mixed for about 1 min by shaking. The sample was centrifuged for 5 minutes at 10,000 rpm. Without disturb the bottom layer, the aqueous (top) layer was pipetted carefully out into new sterile 1.5 ml micro centrifuge tube that contains 2/3rds of a volume of ice-cold isopropanol. The contents was mixed gently by a little inversions. White threads of DNA was become evident at this stage. At this point, the sample can be kept in a -20 °C freezer until analysis.

To pellet the DNA, the sample was centrifuged for 5 minutes at 10,000 rpm and the supernatant was discarded. Then, 70% of ethanol was added in amount of 500µl to wash DNA. The tube was tapped gently and allowed to stand to air dry for a few minutes. The tube was centrifuged for 5 minutes at 10,000 rpm to re-pellet the DNA and any remaining supernatant was discarded gently by pouring away or by clean paper towel at the edge of paper. The tube was left open upright for 30 min for remaining liquid to evaporate. Then 100 µl of 1 X TE buffer was added and the DNA was suspended in buffer prior to use. Letting tubes stand for several hours and tapping them occasionally aids re-suspension. DNA was

stored at 4 °C. DNA quality was checked on 1% agarose gels using the same technique as for PCR product visualization. The concentration compared the DNA standards against 10 ng/µl, 20 ng/µl and 100 ng/µl lambda DNA.

3.2.2: SDS METHOD (Dellaporta, 1983)

The 3cm leaves sample of each variety of pineapple that has been frozen (was cut to a small cube) was grinded with 5ml extraction buffer in mortal and pestle. 66.6 ml of 20% of sodium dodecyl sulfate (SDS) and 50 µl of β-mercaptoethanol and was added into the mortar and pestle. After that, the mixtures was transferred into the 15ml tubes and was incubated in thermomixture for 950 rpm at 60 °C for approximately 20 min and the content will be mixed by shaking the tubes. Then, 2 µl of RNase was added into each tube. After that, once again the samples were incubated in thermomixture for 500 rpm at 37 °C for approximately 1 hours. Then, 333 µl of 5M potassium acetate was added and gently inverted to mix. The samples was incubated on ice for 30 minutes.

The samples were centrifuged for 20 minutes at 10,000 rpm. The aqueous (top) layer was pipetted carefully out into a new 15 ml tubes. 3333 µl of isopropanol was added into the tubes and gently inverted to mix. The samples was incubated overnight at -20 °C. To pellet the DNA, the sample was centrifuged for 30 minutes at 10,000 rpm and the supernatant was discarded. The pellet was dried. After the pellet was dried, the pellet was redissolved in 50 µl of TE buffer. 75 µl of 3M sodium acetate and 500 µl of isopropanol were added into the

tubes. After that, the sample was mixed well and incubated in -20 °C for 4 hours. After that, the sample was centrifuged at 10 000 rpm for 20 minutes. Then, 80% of ethanol was added to wash DNA. The tube was centrifuged for 5 minutes at 10,000 rpm to re-pellet the DNA and any remaining supernatant will be discarded gently by pouring away or by clean paper towel at the edge of paper. The tube was left open upright for 30 min for remaining ethanol to evaporate. Then 100 µl of TE buffer will be added and the DNA was suspended in buffer prior to use. Letting tubes stand for several hours and tapping them occasionally aids re-suspension. DNA was stored at 4 °C. DNA quality was checked on 1% agarose gels using the same technique as for PCR product visualization. The concentration compared the DNA standards against 10 ng/µl, 20 ng/µl and 100 ng/µl lambda DNA.

3.3 Determine of Quality and Quantity of DNA

3.3.1 Quantification of DNA

Spectrophotometer was measured the concentration of DNA in solution. The dilute sample with volume of 1 ml was measured at 260 and 280. The 260nm reading was as indicator of DNA concentration and the 280nm reading is an indicator of the protein contamination. The ratio of $A_{260/280}$ of pure DNA should be above 1.8.

3.3.2 Determination of DNA Quality

Electrophoresis of an aliquot of sample in 1.5% agarose gel was determined the degree of DNA degradation. Large molecular weight DNA should appear as a sharp band while partially degrade DNA formed as a long smear from large to small fragments.

3.4 Primer Design

Primer was designed by using Primer 3 Plus browser. The terpene synthase gene obtained from NCBI were uploaded to the sequence field and of the other parameters were kept at default. From the Primer 3 output, list of primers were suggested by the software. Primer that has same melting temperature for both forward and reverse for each exon were selected. Moreover, the primers were also selected to range from the earliest starts of the exon to the latest end of the exon. This is to ensure that the sequence amplified expands through exon region. The seven primers of pineapple was designed. However, only three primers were used for this research which were Exon 2, Exon 3 and Exon 6. These three primers were chosen because these three primer were for the largest exons as compared to the other four exon.

Table 2: List of the primer design from terpene synthase 10 of pineapple

NAME	SEQUENCE	LENGTH	TM (° C)
EXON_1_F	ATAGTACTAGTGCATTGTATGGGTGCT	27bp	60.6
EXON_1_R	CAAATAACCGCCCTTCAGT	19bp	57.7
EXON_2_F	GCGAGTCACAAATCACACAAAT	22bp	60.0
EXON_2_R	CCGAAAAGCCATTTTCTCG	19bp	60.7
EXON_3_F	TCTTGAGTGGCTACAAGGATATCAA	25bp	61.3
EXON_3_R	TGAAATTCTTTTAAGTTCTTCCTGATG	27bp	60.4
EXON_4_F	TGGAGAGATGTGGCTCTTGC	20bp	61.5
EXON_4_R	CTTTCGACGGCACTTGTGA	19bp	61.0
EXON_5_F	TGGGAGAATGATGCAGTGG	19bp	60.6
EXON_5_R	CGCATTCTTAAGATAAGGGAGTACA	25bp	60.1
EXON_6_F	GCATGATCTATGCAAGTCATTTCTAGT	27bp	61.2
EXON_6_R	TGTGTAAGTCGCTAAGTCGTTGC	23bp	61.7
EXON_7_F	GGAGCGGGAGAAAGGTGA	18bp	61.3
EXON_7_R	TAATTTTCAGTTCCTTTCACAAGTATG	27bp	59.1

3.5 PCR Amplification

Five samples from the pineapples varieties was prepared. PCR mastermix was standardized to 100 ng/ul DNA template, 1.5 Mm/ml $MgCl_2$, 10 Mm dNTP, 100 mM PCR buffer, 1.25 U *Taq* polymerase and 1 uM primer was prepared. The PCR program was set at 94 °C for 2 minutes for initial denaturation, then 30 cycles of denaturation process and the temperature was set at 94 °C for 30 seconds. After that, followed by annealing temperature ranging from 48 °C to 52 °C for 30 seconds and at several number of cycles. Last steps in PCR techniques, the temperature was set at 72 °C for 40 seconds for extension process. After the extension process, the temperature was reduced to 4°C for termination of PCR process and before the sample was collected.

3.6 Agarose Gel Electrophoresis

Gel electrophoresis was used in order to inspect the DNA during the processed. The quantity of agarose was made either 1%, 1.5% or 2.0% depending on the size of DNA fragments inspected. The agarose was weighed into a conical flask with suitable size for the volume and concentration of gel. The agarose powder was mixed with 1 × TE buffer in weight or volume ratio depends on the desired concentration and swirl the content. The gel was casted in different size of wells depending on the volume of DNA loaded into the gel

and the number of sample in one run. Before it is casted, the mix of agarose and 1× TE buffer was heated in the microwave until the solution become clear, and then, the flask was swirled to mix.

The agarose which has been cooled was poured into the prepared gel tray, to a depth of around 5 mm. Any obvious bubbles in the gel was pushed to the side of the tray by using a disposable pipette tip. Each end of tray was prepared by involving sealing at the each end with tape, the gel combs was placed into their allocated positions. The number of teeth was used in the gel combs depends on how many samples need to be run on the gel, and the volume of material that needs to be loaded in each well. The gel was allowed to make the gel become solid for 30 minutes and the tape of the ends of the tray was removed and then, the gel was placed just below liquid level in an electrophoresis tanks that contains 0.5 × of TBE. The tanks was added with more 0.5 × of TBE as the gel must be immersed completely. The prepared samples was loaded into a well include markers at the start and at the end of each series of the wells by using pipette. The prepared samples was mixed with $1/5^{th}$ volume of 6 × of agarose gel loading dye.

The voltage 80V was used depending on the size of the gel, the bigger the gels able to run at higher voltages. As a rule of thumbs, the gels was run at the voltage of 5 × the distance in cm between electrodes. The gel normally run until the first dye markers have be travelled approximately three-quarters of length of the gel to be resolve PCR product. Samples can either be shorter such example for DNA extraction or long such example if it is known as PCR polymorphisms difficult to resolve distances. The power will be turned off; florosafe solution was used to remove and stain the gel for 45 minutes in sealed plastic

container. 50 µl of stock solution to a liter of 1 × TBE was added to prepare stain. The gels was viewed on a UV transilluminator and the result was captured and recorded by using camera.

3.7 DNA Sequence Analysis

The PCR product from the amplification of terpene gene from the five pineapple samples was sequenced by service provider. The same primer used for PCR amplification was used for the sequencing purpose. From the sequencing result obtained, the sequences were in abi format file which first converted to fasta format by using Sequence Conversion browser for further analysis in MEGA software. All of the sequences data were combined into one file using MEGA software for variant analysis in MEGA software, the sequences were inspected for its primer sequence to decide if it needs to be reverse complemented to match other sequence in alignment. The analysis in MEGA was done per each exon for all pineapple varieties subsequently. The sequence was translated to protein and any variant that exist among the varieties were recorded. Any variant that existed at protein level was translated back to the gene level. If there is no variant at protein level, then the gene will be inspected at gene level for synonymous mutation.

CHAPTER 4

RESULT AND DISCUSSION

4.1 Determine of Quality and Quantity of DNA

4.1.1 Quantification of DNA

Based on the table 3, the DNA amount of five different types of pineapple which were Moris, N36, Sarawak, MD2 and Josapine were 68.056 µg, 64.064 µg, 276.903 µg, 528.675 µg, and 278.057 µg respectively. For the DNA purity as accessed by 260/280 was 1.66 for Moris, 1.50 for N36, 1.75 for Sarawak, 1.95 for MD2 and 1.76 for Josapine. The variation of the DNA yield indicated that only the Sarawak variety yielded higher amount than the other varieties. The variation of the DNA yield indicated that only Moris and N36 varieties yielded the lowest amount.

Then, the DNA amount of five different types of pineapple for SDS method which were Moris, Josapine, Sarawak, MD2 and N36 were 534.219 µg, 304.314 µg, 300.543 µg, 300.665 µg, and 301.346 µg respectively. For the DNA purity access by 260/280 was 1.96 for Moris, 1.89 for Josapine, 1.84 for Sarawak, 1.84 for MD2 and 1.85 for N36

Variation of the DNA yield indicated that all five varieties of pineapple were yielded higher amount compared to the previous method that yielded the lowest amount of DNA.

According to table 3, the variation of the DNA yield indicated that CTAB method yielded lower amount while the variation of the DNA yield from the SDS extraction method yielded higher amount. The contamination of nucleic acid solution also can makes spectrophotometric quantitation inaccurate. Low ratios also could be by protein and phenol contamination. The ratio A260/280 were lesser than 1.5, this was below than optimum limit which was 1.8 – 2.0 (Sambrook & Green, 2012) and made the extracts no amenable for molecular study.

Table 3: DNA yield and purity access by 260/280 resulting from five different types of pineapple

SAMPLE NUMBER	SAMPLE NAME	A260/A280		CONCENTRATION (µG/ML)	
		CTAB METHOD	SDS METHOD	CTAB METHOD	SDS METHOD
1	Moris	1.56	1.96	68.056	534.219
2	N36	1.50	1.85	64.064	301.346
3	Sarawak	1.75	1.84	276.903	300.543
4	MD2	1.95	1.84	528.675	300.665
5	Josapine	1.76	1.89	278.057	304.314

4.1.2 Qualification of DNA

Based on the figure 1, electrophoresis analysis revealed the presence or absence of DNA in the sample for each DNA extraction of five different types of pineapple. The DNA extraction of sample number 1 indicated for Moris variety showed smear band. For DNA extraction of sample number 2 indicated for N36 variety showed absence or no band. Then, for sample number 3, 4 and 5 indicated for Sarawak, MD2 and Josapine respectively revealed presence of the band. Based on the figure 2, electrophoresis analysis revealed the presence or absence of DNA in the sample for each DNA extraction of two different types of pineapple. All of the pineapple variety extracted producing thick intense band, which indicate its integrity of the extraction process.

DNA extraction is a cell structure disruption to create lysate, isolation of soluble DNA from cell debris and other soluble substances and DNA purification appealing from soluble proteins and other nucleic acids. Different extraction methods produce different yield and purity of DNA. In this study, there was many difference to encounter from the first step of cell lysis to DNA separation in supernatant reaction when tested on CTAB extraction method. Highly viscous pellets were difficult to manipulate and the low A260/280 indicated contaminated by protein, polysaccharides and phenolic compound (Moriera & Oliviera, 2011). The extracts obtained from CTAB method may contain debris, polysaccharides, protein and other components that was interfered with DNA and was difficult to eliminate.

According to the figure 1, the smear band occurred at sample number 1 and no band at sample number 2 but the other three samples had thick band for CTAB extraction method. Based on the figure 2, by using the SDS extraction method all five sample of pineapple showed presence of the band. For figure 1, there were smear band that may cause by DNA in the process of degradation or the DNA already cut in small pieces or called DNA fragmented. The smearing at the lowest end of the gel may cause by RNA. DNA may become fragmented while in extraction procedure, giving smeared appearance. For figure 2, the sample of DNA was revealed a sharp band. This is because the DNA well transformed in gel electrophoresis. This may cause by a good extraction procedure and giving a sharp appearance.

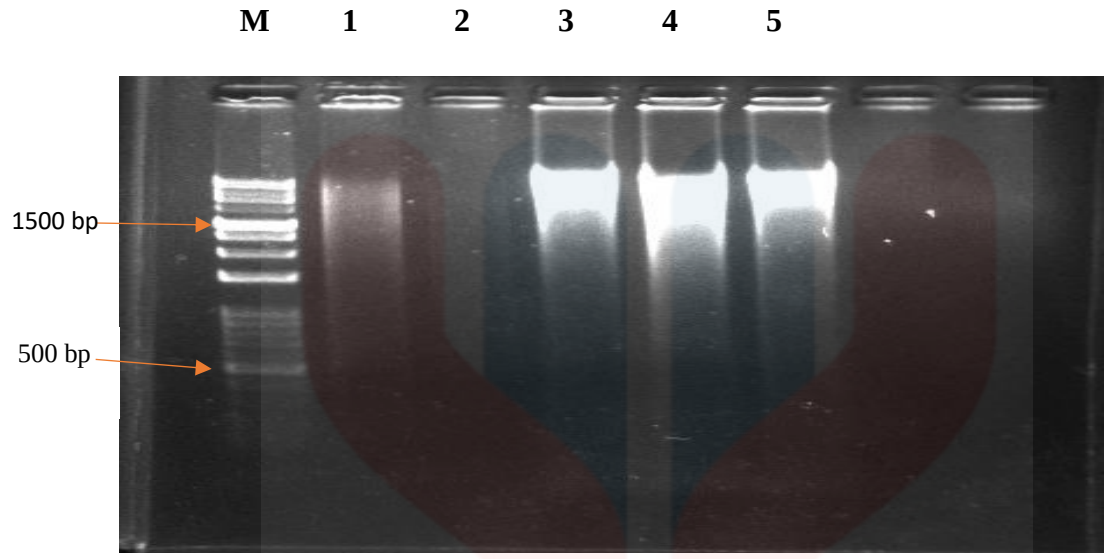

Figure 1: DNA extracted from five different types of pineapple M= DNA ladder, 1= Moris, 2= N36, 3= Sarawak, 4= MD2 and 5= Josapine.

Figure 2: DNA extracted from two types of pineapple, M= DNA ladder, 1= Moris, 2= Josapine, 3= Sarawak, 4= MD2 and 5= N36

From the result of DNA quantification and qualification, it showed the different result between the two extraction method which were CTAB method and SDS method. The different between the two method was the used of chloroform for CTAB method while the used of sodium acetate for SDS method. SDS extraction method usually used TRIS buffer to set up pH at 8 to stabilize the DNA. EDTA was used to chalet Mg and Ca and inhibits nuclease (DR.F.Shokouhifar, n.d). Then, the high NaCl concentration function for the salting out Histone protein (DR.F.Shokouhifar, n.d). SDS buffer act as detergents to disrupt cell membrane and the heat at 60 °C will help the detergent in its action. Potassium acetate was used to precipitate the polysaccharides.

Cooled isopropanol was added prior to centrifuge, to concentrate and precipitate DNA. TE buffer to resolve precipitated DNA. Sodium acetate to raise the salt concentration, and therefore cause the nucleic acid (here DNA) to precipitate out of solution. For CTAB extraction method, CTAB buffer function was same with SDS buffer which was act as detergent to disrupt cell membrane (Doyle.1987). For chloroform it solubilize lipids and a lot of protein to remove them from the DNA (Doyle.1987). Cooled isopropanol and centrifuge it to concentrate and precipitate DNA. TE buffer to resolve precipitated DNA. Ethanol was used to separate DNA out of water based solution.

For pineapple, the usage of high concentration of NaCl combination with SDS, works best to extract good quality and quantity of DNA from pineapple. The ratio of A260/A280 way much better using this method and the CTAB method caused the residual organic solvent used to be extracted with the DNA at the end of the process. In SDS method, there is no

strong organic solvent used instead it relies on the high concentration of NaCl to precipitate the protein. Removal of polysaccharide was assisted with the used of potassium acetate

4.2 PCR Amplification of Five Different Types of Pineapple.

Initially, the PCR was carried out using DNA extracted using the CTAB method. As seen in figure 3, only sample Josapine and MD2 were amplified for Exon 2. The band was observed at 300bp. The DNA obtained with the CTAB method was very not well PCR amplified when using primer Exon 2. There were three samples with no amplification. This could be due to the presence of contaminated polysaccharides co-existed with the DNA extracted. The contaminated by polysaccharides makes the DNA unamplified by inhibited of *Taq* polymerase activity during PCR process (Fang et all., 1992).

For the genomic DNA using SDS method, all of the PCR amplified product produced expected band for all sample and for all exons. In total there were fifteen PCR products. All at the PCR product produced the expected size which were 250 bp, 350 bp and 250 bp for Exon 2, Exon 3 and Exon 6 respectively (figure 4). The intensity of the band for all the PCR product was also bright with no presence of other unspecific band. Presence of such single bright band indicated that the primer was specified for the targeted region.

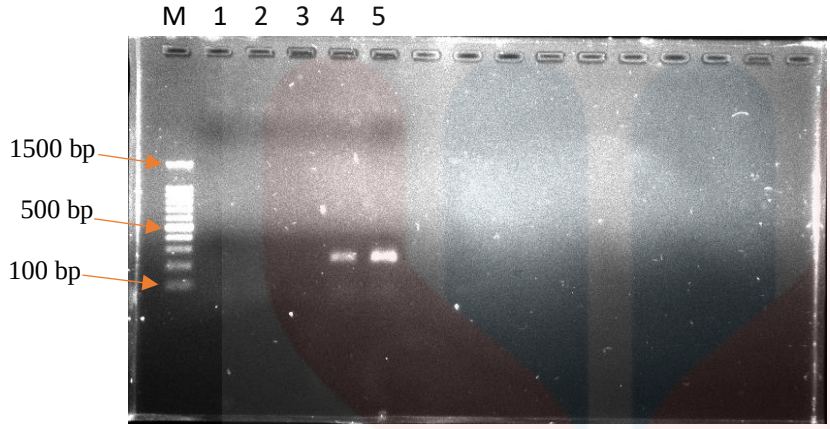

Figure 3: PCR amplification of Exon 2 of DNA from five different types of pineapple M= DNA ladder, 1= Moris, 2= N36, 3= Sarawak, 4= Josapine and 5=MD2.

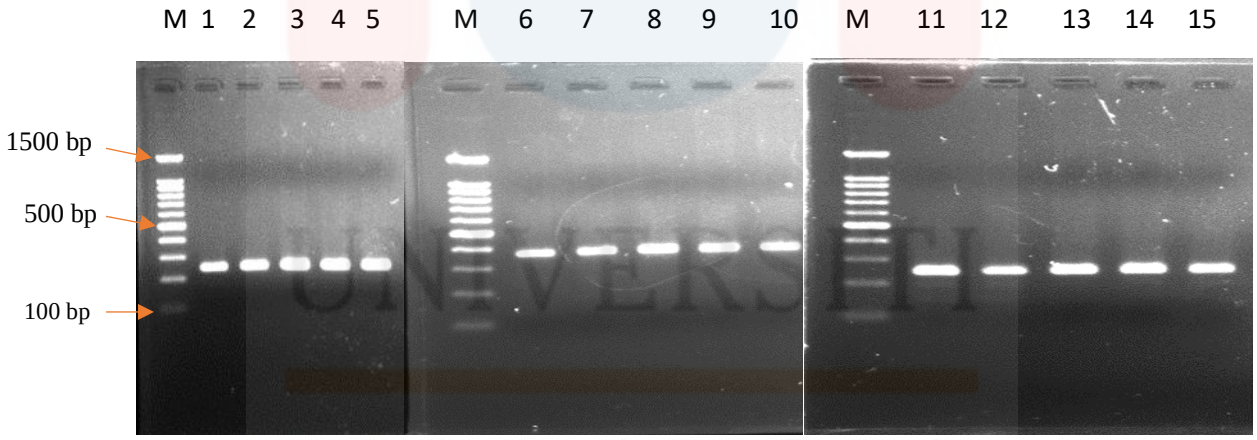

Figure 4: PCR amplification of DNA from five different types of pineapple for exon 2, M= DNA ladder, 1= Moris, 2= N36, 3= Sarawak, 4= Josapine and 5=MD2, exon 3 M= DNA ladder, 6= Moris, 7= N36, 8= Sarawak, 9= Josapine and 10= MD2 and exon 6, M= DNA ladder, 11= Moris, 12= Sarawak, 13= N36, 14= Josapine and 15= MD2.

4.3 DNA Sequence Analysis

Fifteen PCR product of five pineapple varieties for the each of the three exons was sent for sequencing. Sequencing was performed on forward and reverse. Thus, for each PCR product there were two sequences overlapping. This is important to ensure that accuracy of sequencing is achieved and to eliminate the possibility of data loss due to low quality ready at the start of sequencing. After inspection of the sequence through MEGA software, the data showed that the sequence produced consist of mainly runs of adenine (A) and Thymine (T) repeat. This is different than the expected result. All of the sequences received were facing the same result (Appendix B).

The result was referred to the service provider for troubleshooting purposes. A personnel at the service provider randomly inspected the DNA intensity of the DNA sample sent. Figure 5 showed that the DNA sample that was used for sequencing was degraded and this explained the error of sequencing data obtained. Furthermore, during the transportation the PCR product may not in the optimum temperature. The ice pack used in order to maintain the temperature of the PCR product was melted during transportation.

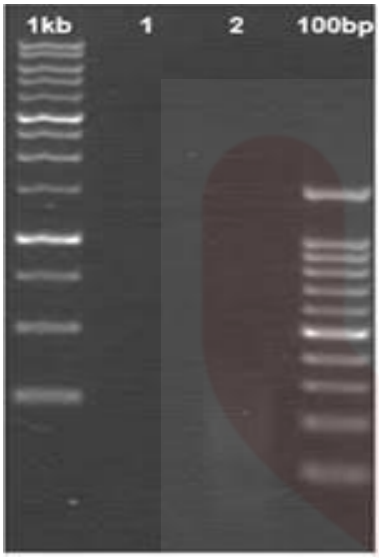

Figure 5: The agarose gel electrophoresis of DNA sample.

Due to the unsuccessful sequencing data, we have failed to answer the second objective which was the presence of the polymorphism of the terpene gene among the different varieties of pineapples cannot be identified and characterized due to the error result of DNA sequencing. The PCR amplification for two sample which were Moris variety for Exon 2 and Sarawak variety for Exon 3 have been repeated for re-sequencing.

4.4 DNA Re-sequencing Analysis

After troubleshooting, the service provider successfully sequenced two PCR products that have been resubmitted. The two sequences were Moris for Exon 2 and Sarawak for Exon 3. Subsequently, the sequences were compared to the references sequences of hybrid pineapple obtained from the whole genome sequencing study (NC_033627.1:13355413-13359778) individually for each exon.

Based on figure 6, the protein sequences analysis showed that the variation detected for Moris variety was substitution. This is because it has a change of lysine to methionine at 689 amino acid and change of tryptophan to cysteine at 735 amino acid. The chemical properties of amino acid at 689 amino acid of lysine was basic and methionine was non-polar. The chemical properties of amino acid at 735 amino acid of tryptophan was non-polar and cysteine was polar. So, their chemical properties was different. Thus, the different variation of the chemical properties of the amino acid may cause structural change to the protein produced, that may explained its variation of fruit quality between different varieties.

Based on the figure 7, the protein sequences analysis showed all of the variation detected for Sarawak variety were only on one of the sequence (i.e. non-consensus). This indicated that the variation may either be a sequencing error or allelic variation. The amino acid had a missense at base 1104 while tyrosine amino acid located at forward sequence and the amino acid missense at base 1174 while threonine amino acid located at reverse sequence. The amino acid change of Proline to Histidine at 1140 amino acid at reverse sequence. The

amino acid also has a change of leucine to proline at 1148 amino acid at reverse sequence. It also has a change of methionine to isoleucine at 1152 amino acid at reverse sequence and change of cysteine to serine at 1184 amino acid at reverse sequence. In comparison to the Moris variety at Exon 2, Sarawak variety at Exon 3 had more variation, but the variation was only for either one of the sequences, whereas variation of Moris at Exon 2 was consensus for both forward and reverse. This may indicate more polymorphism is expected at Exon 3 terpene synthase gene in comparison to its Exon 2. More sequencing need to be carried to confirm this hypothesis.

Figure 6: Protein sequence of Moris Varieties

Figure 7: Protein sequence of Sarawak Varieties

CHAPTER 5

CONCLUSION AND RECOMMENDATION

As s conclusion, the different extraction method showed different amount of DNA yield. From DNA quantification and qualification, it showed different result between the two extraction method which were CTAB method and SDS method. For the CTAB method, the variation of the DNA yielded indicated that only one sample which is Sarawak variety yielded higher amount compared to the N36, MD2, Moris and Josapine variety. While for the SDS method, the variation of the DNA yield indicated all five samples of pineapple were higher amount. Then, for DNA qualification of CTAB method the gel electrophoresis analysis revealed that the sample 1 which was Moris smear band and for sample 2 which is N26 absence or no band (Figure 1). While for another three samples showed presence of the band (Figure 1). For SDS method, all of the pineapple variety extracted producing thick intense band (Figure 2). From the result obtained, using SDS extraction method better compared to the CTAB extraction method.

The amplified terpene gene from five different pineapple variety show that the SDS extraction method was very well PCR amplified when using the primer which are Exon 2,

Exon 3 and Exon 6 due to the sharp band revealed for all sample of pineapple during electrophoresis analysis (Figure 3). However, CTAB extraction method was not well PCR amplified when using the primer Exon 2 due to the sharp band showed only for two samples which were Josapine and MD2 while for another three sample were showed missing band (Figure 4). The extraction method play an important role in PCR amplification. This is because different extraction method yielded different amount of DNA and purification of DNA.

Initially, all of the fifteen PCR products that was sent for sequencing received sequences with erroneous error. Sequencing was performed on forward and reverse. Thus, for each PCR product there were two sequences overlapping. Almost all of the sequences were consist of repeats of two bases which were adenine (A) and Thymine (T). This is different than the expected result. This error occurred due to problem during transportation of the PCR product which have caused degradation of DNA.

After resubmission of the sequences Moris for Exon 2 and Sarawak for Exon 3, these sequences were obtained as expected. Upon comparison to reference, Moris variety for exon 2 had a two amino acid changed at amino acid 689 and 735. Both changes are from amino acid with different properties. Thus, significant change at protein level is expected. In comparison to reference, Sarawak variety for exon 3 had five amino acid changed. However, all of the variations were only on a single sequence (either forward or reverse). This variation need validation. Alternative hypothesis is accepted as there is polymorphism detected in two varieties of pineapple for two different exons.

For the recommendation, choosing a good extraction method to get a higher yielded of DNA amount. This is because different extraction method showed different amount of DNA yielded. SDS extraction method is a good choice for extraction method due to the usage of high concentration of NaCl combination with SDS, works best to extract good quality and quantity of DNA. The extraction method also play an important role in PCR amplification. This is because different extraction method yielded different amount of DNA and purification of DNA. The optimization in PCR process also will produce a well PCR amplified. To get the better result of DNA sequencing this study can be further evaluate if the samples are in optimum temperature and sample need to purify before sent to sequence in order to avoid degradation.

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APPENDICES

Appendices A

Figure 1: The DNA pellet of pineapples

Figure 2: the student pour a chemical in beaker

Figure 3: The students observed the pellet in microcentrifuge tube.

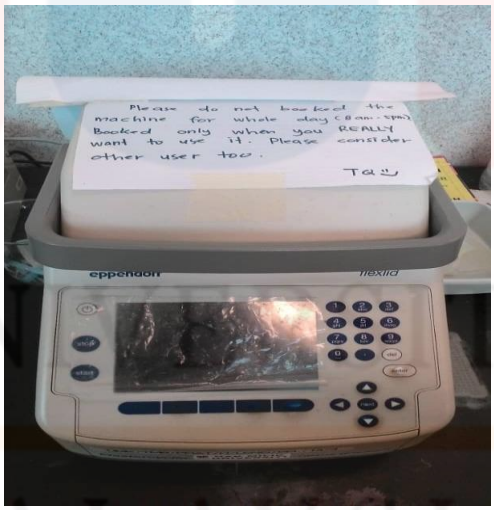

Figure 4: The PCR machine.

Appendix B

Table 4: DNA sequence analysis of five different types of pineapple

SAMPLE	DNA SEQUENCE
3360375_SARAWAK_-_EXON_6_PRIMER_EXON_6_-_R	GGGGGCGATATGCGTAGATATCGGGCCGCGGCTACCTCCCTCT ATATCTCTCTACATGGGGATATACATATAGGGCGATGGATGTA GGTATATATATGGACATATATAGATATGGTACATATGGATATG GTACATATATATGTATATATATGTATATGTATATATATATGCCT AGATCCATATATATATATCTATACATAGATATATATATATGCAC CTACGCCCATATATATATATATATATATATATATATATATATAT ATATATATATATATATATATATATATATATATATATATATATAC ATATATATATAGATATATATATCTATATATATATATATATATAT CTATATACCTATATACATATGTATATATATATATATATATATAT ATATATATATATGTATATATATATATATATATATATATATAT ATATATACATATATATATATATATATATACCTATCTATATATAC ATATATATATATATATACATATATATATATACATATATCTATAC ATATATATCGAGATATATCTACATATGTAGATACATCTGTATAC ATATATATATATGCATATATAGATATATATACGGCTAGAGATAT ATAGAGATATACATACATCTATACATAGATACATATATGTAGG TATATATAGAT
3342693_JOSAPINE_-_EXON_6_PRIMER_EXON_6_-_R	ATATATATATATAGATATCTATATAGATATATATAGATATAGAT ATATATATATATATATATCTATATATAGATATATATACATATAT ATATATATATATATATATATATACATATAGATATATATATATATAT ATATATATACATATACATATATATATATATATATATCTATATGT ATCTGTATACACATATATATATATCTATAGAGGTATATATATAT AGATATATATATATGTATATATCTACATATACATAGATATATAT ATATATATATATAGATATATATATAGCGGGTATGGATATATATG TATATGTATATATATATATAGATATATATATATATAGATAGCCA TATCTAGATAGAGATATAGATATGCATATATAGATATAGGTAT

	ATCTATACATCTCGCGATAGATATATATGCATATATATATCTAT ATATATATAGATATGTATC
3342692_JOSAPINE_-_EXON_6_PRIMER_EXON_6_-_F	AGGAGTAAAGAATATTAAATCTATATGTATCCTCCGTAAGCCG TCTGCTCTATCTCTAGAGACATCCTGCCCGCTGCCACATGTATA TACAATTATGTTCCTCATAATCCACACATCTTTAGAGACGGAAT ATATATTGATAAGCCTGGGGAGCCTGATGAGATATATACTTAT ATTAATTGCATGCGCTATATATATATATATATAGATAGAACCTG TAGAGATATATACATAAATGAATCTACCAATATATGTAGATAT GCAGATTGATAATTGGGCGCTCTTCCGCTTCTATATATACTGAT ATATAGCGCTCGGATATATATATGTATATAGATATATCTATATA CTCATATGTATAAATACATATATATATATATATATGTATGTA CATATATATATATGTATATATATATATATATATGTATATATATA TATATATATATATATATATATATGGAGTTATACATATATCTCCACC CCCCTGTCGATCTATATATATATCTACACTCATATCTAAAGAGA CAAATCCCGATATATCTATAAATATACATAGATATATATCC
3342691_MD2_-_EXON_6_PRIMER_EXON_6_-_R	ATATATATGTATATATATAGATATATATATATACCTGTATATAT ATGTATATATATATATATACCTATATATATATATATATATATAT ATATATGTATATCCAGATATGCCTATATGTATATATATATATAT ATATATATATATATATATATATATATATATATATGTATATATAT AGATATGTATATATATATATATATATATATATATATATATATAT ATATATATATATATATATATATATATCTATATCTACATATATAT ACACATATATATATATATATATATATATATATATATATATATAT ACACATATAGGTATATATATAGATATATATATATATATATATAT ATATATATATATATATATATATATATATATATATATATAGACAT GTATATATATATATATATATATATATATATATATATATATATAT ATATACCTATATATATATATATATATATATATATATATATATAT ATATATATATATATATATATGTATATATATATACATATATATAT ATATATATATCTATATATGCGTACATCTGTATATATATATATAT ATATATGTATATATGTATATATATATATATATATATATACATGT ATCTATATATATATATATATCTGTATATATCTCTATATATATATA

	GATCTATCTATCCATATATAGATAGATAGCTCTATACCTCTCTA TCTATATATACATATGTATATATATCTATATATATACATCTAGA TATATATCTATATATATCTA
3342690_MD2_- _EXON_6_PRIM ER_EXON_6_- _F	ACATATATATGGATCTAGCTGTATATACCTATATAGATATATAT ATATAGATATATATATATATATATCTATATATATATATAGATAT ATATAGATATATATATATATATATACATATATCCATATATATAT ATATATATATATATATCTATATAGATATGTATAGACAGATATAT GTATATACATATATATATAGATATATATATATATATATATATAT ATAGATAGATCTATATATATATATATATACATATGTATCTATAT ATACATATATATATATATATATATATATATAGAGCTCTATCTAT ACATCTCTATATGTATATCTATATATATATATATATATATATAT ATATGTATACATATATATATATATATATATATATATATATAT ATATATATATATATATACATATATATATGTATATACATAGATAT AGATATATATATATATATATATATATGGCCATATAGATATATAT ATATATATATATATATATATATATATGTATGTATATATGTATATAT CGCTCGTATATATATATATATATATAGATATGTATATATATATA TATGGCGGACCATATATATACATCCACATGCGGGCGGCATATA TACCCGATCTAGTACAGATATCTATACCGCCATACATCCATCGA TATGGTGGATGTGTATACATATATATATATATATA
3342689_N36_- _EXON_6_PRIM ER_EXON_6_- _R	CGAAATAAGTGCTCCGACCTTCTCCTCTGCTCCCCCCAAGCCAG TGAAAAGAGTAAGATTCAGAGTCTGTATTGCTCTCACGATAGG TTTTTGGTTATGAATTTCCGAAGCCCAAAATACAACGGAACTA CTGACGCCGGGGGCCAA
3342688_N36_- _EXON_6_PRIM ER_EXON_6_- _F	GAAAAAAAAGTCCGGTTTTGTACTAACTATATCATCCGTAAAA ACTATCCGCTCACTCTAAAGACAGAGCTGTCACAGATATTTTG GATACAGGGCTGGTCCTACTAACATGGCATCGAACAAACGAGT GTGATCCAGGGTATATTGTCTTACTTTCGAATTCCACACAAACA CGACCCGTAAACTTAACACGTACCAACGGGGAGCCTGACCAGC AGCGAACA

3342687_MORIS_-_EXON_6_PRIMER_EXON_6_-_R	ATATATACAGACCTATAGGCAGAGATATGTGGGCGCACCGCTA TATATCTGGAGGTATATATATATACATACATATATATATATCTG TATCGATATGGGTATATCCCGTATATATATACGTATATATATAT ATATGGATATATACCTAGATAGGTATATAGATATATGTGTATAT ATACCTGCCTATGGGTACATATATCTATATATCTATATATATAT GTGGGCATATATGGACATATATATCTATATATATAGGTATACAT ATATCTATCTAGATATATATATATATATATATATATATATAT ATATATATATACATATATATATATATATATATATATATATAT ATATATATATATATATATATATATATATATATCTATATATATAT ATATACCTATATATATATATATATATATATATATATACATAT ATATATATATATATATACATATATATATATATATATATATAT ATATATATATCTATATATATACATATATATACATATATATATAT ATATACATATATATATATATACCTATACATATGCATATATATAT ATATATCTATATATATATATATATCTATATATATATATATATAT ATATATATATATATATATCCCCCCATATATATATATATATATCT ATATATATATATATATATCTATATATCTCTATATATATGTGTCTA TCTACATATATATGGGTAT
3342686_MORIS_-_EXON_6_PRIMER_EXON_6_-_F	ANNNNNNNNNNNTNTNTCCGTTTNNNNCCATATNTCCTATATA TATATATATATATATATATATATATATATATATATATATATATATA TATATATATATATATATATATATATATATATATATNTATATATATATA TGTATATATATATANATATATNTATATATATACAGATATATATA TATATATATATANATATATATATATATATATATATATGGATATA TATATATATATATATATATATATATATATATATATATATATATA TATATATATAGATATATATATATATATATATATAGAGATGTATA TATATATATATATATATATATATATATATATATATATATATATA TATATATATATATATATATATATATATATATATATATAGATATA TATATATATATATATATATATATATATATATATATATATATATA TATATATATATATATATATATATATATATATATATATATATATA TATATATATATATATATATATATATATATATATATATATATATA TATATATATATATATATATATATATATATATATATATATATATA

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	TATATATATATATATATATATATATATATATATATATATATATA
3342685_SARAWAK_-_EXON_6_PRIMER_EXON_6_-_R	CCCATATATATATGCATATATATGTATATATATATATATATATC
	CCGATATCTATATATATATATGGGATATATACCATGCGTACAG
	ATATAGAGGGACAGCGATAGGTACCCATATATATGTGTGTGTG
	GATGCATATGCCGATGTCGCTGTGGCTCTATACGCGGTGGGGG
	CTGGGGGGCTGTGTACATCCAGCTAGGGATATATATATATATA
	TGTACATGTATATATATATATATGTAGGTATATGCATATGGGTA
	GGTAGGTAGATATGGGTGTGGCCACCGCGACCGGCGATATATC
	TATCCGTATATATATGCAGATATAGAGACGTATAGACATACCT
	ATCTATATATGCCTATATATCTCCCCCACCCGTGGAGATACATA
	TGCATACACATATATCGCCTCTCTATCTCCTCCATATCTACGTA
	TATGTCGCGTAGATACACATATATATATATATATGTATATATAT
	ACATCTACCCCTATATAGCGCGATATATAGCTGTGTATATATGT
	AGACATAGCCCCTATGTCGACGTATCTACATATATCCATGTCTA
	CCTATCTACATATAGATGCATATATCCACATCCATGGGCCCATA
	CAGCTGCGCTACTACATACGCTCACAGATACGACACCTACCGA
	TATCTGGTATGCCTAGTATAGCATGGATGTACCTCCGTATATAC
	TAGCTATGCTGTGCGTATATAT
3342684_SARAWAK_-_EXON_6_PRIM	NNNNNNNNNNNNNNNNNNNNNNNTATAGTATATATATATATAT
	ATATATATATATATATATATATATATATATATATATATATATAT
	ATATATATATATATATATATATATATATATATATATATATATAT
	ATATATATATATATATATATATATATATATATATATATATATAT

ER_EXON_6_- _F	ATATATATATATATATATATATATATATATATATATATATATAT ATATATATATATATATATATATATATATATATATATATATATAT ATATATATATACATATATATATATATATATATATATATATATAT ATATATATATATATATATATATATATATATATATATATATATAT ATATATATATATATATATATATATATATATATATATATATATAT ATATATATATATATATATATATATATATATATATATATATATAT ATATATATATATATATATATATATATATATATATATATATATAT ATATATATATATATATATATATATATATATATATATATATATAT ATATATATATATATATATATATATATATATATATATATATATAT ATATATATATATATATATATATATATATATATATATATATATAT ATATATATATATATATATATATATATATATATATATATATATAT ATATATATATATATATATATATATATATATATATATATATATAT ATATATATATATATATATATATATATATATATATATATATATAT ATATATATATATATATATATATATATATATATATATATATATAT ATATATATATATATATATATATATATATATATATATATATATAT ATATATATATATATATATATATATATATATATATATATATATAT ATATATATATATATATATATATATATATATATATATATATATAT ATATATATATATATATATATATATATATATAAATAATTATAAAT
3342683_JOSAPI NE_- _EXON_3_PRIM ER_EXON_3_- _R	CATACAGCCGTCGGCCTCTCTCCGCATGGGGGATATATCTGTAT GCCGGCGTGGGTCCCCCGAGCTATCCCCACATCTCTATACCTAC CCGGGTGCATCTCCACATGCATACACATACCTCGGCATCGATA GATCCCCCCATAGATCTACATGGATACCTATCCGTACCCATGG GTCTATATCTATATCTAGGTAGATCTAGATCGAGATGCCTCCAC ATATGTCCCTATCTGGATATACAGCCCCCGTATACTACATACCG CCCACAGGCCCCCGGTCCCCGGGATATATACATACTATATATA CAGGTACGCCCAGATGTATATATATATAGCATATACATGTCCA TGATATCTATAGATATGTATATGTGTACCCTATATATAGAGATA TATATATACATATGTACCAGGGCTATATGTCCATGCTATCCGCC TATCTATGTGTAGATGTACATATGTATATATATATATATATATA TATATGTAGATATATATATATATATATAGATATATGTATATA TATATATATAGATAGGTGTATGTATATATATATATATATGGT

	ACATATATACATATATGTATATATAGATATATAGATATGTAGAT GTAGCGGCAGCGATCTGTATCCATGTAGCTGCGTCTCCACCGC GCTGTCCACCTATGTATCTATACATAT
3342682_JOSAPINE_-_EXON_3_PRIMER_EXON_3_-_F	ATCCCCCCTTCCTTCCCGCTAGGCCACCCGCCCCCCGACATCTC CCCTGCCGGACCCGGTACCGCGGGGTCCCTCGCGCCGCTGGAC CGCGCCCCCGGTGTGGGCGAGGGCCCCGCCCCCCTCCCTGCCC CCGGATCGGCGGCGCGCGCGGAGGCATGCACGCGCCATGCATA CAGGCTATATATACACCGCGCCCAGGGGCCGTGCGATGCGGCA TGTGCCGCCGCTCTACGCCGCTATGTATACCATATATACGCCCA GCGATATGCGGCCGGGAGATGGATATAGATGTATATAGATATG GATCCACATATCCGTCTCCCCACCCCTATATGTAGATATATATA TACCCCCCTACCCATATATATATATAGATATATATATATGTATA TATATATATAGATATAGATATACATATCTATATATATGTACGGC CCGCCGACCTACCCCCCGCGCCCCCACATATAGCTGCGCCCCC CCTATCTCTCTATCTCTCTATCTATAGCCCCCATCCACACCCAT ACATCCCCCCCCCCCCCTCTCCCCCGCCCCCGTATCTGCATCCAT CTATATGTATATCTCTCTATGCATCTATCTCTGCCCCCTCTGTGT ATCTCCCGCCTCCATATAT
3342681_MD2_-_EXON_3_PRIMER_EXON_3_-_R	CCCCCCCCGTGGGGCCCCAACCCGATACATCTGTACAGACATCG ATACATATACACATATATATATATGGATATACCTGCGGCCATA CCTGTCCGAAGGTCTGCATATAACATATCTACCTCCATATCGAC ATGGCTAGGCATATGCATACCGGCGTGTACCCCCTATATATCTG TATATATGGATACACCTATGTGCCGAAGCATAGGCATCCCCCT ATATATATGCCCGGAATACACATATAGACATGTCGATATCTAG GTATATATAGACATATGTATATATAGCTCCATATATATATATCT AGATGCTATATATATATATATATATATGCCATCCACATATATCTCT ATATATACCTCTATACATATATATATCTATATATAGATGTATAT ATATATATATATATATATATCTATATATATATATATATCTCTAT ATATATCTATATATATATATCTATATATCTATATGTATCTATATA AATATCCATCCGCATCTATATATATATGTGGATATCTATATCGA

	TCTATCGGTCCTATCTATATGTATGTCCCCCCCCCCCCTCTGGG GGCCTCCCGAACCCC
3342680_MD2_- _EXON_3_PRIM ER_EXON_3_- _F	CCACCCCCCCCTACCATATATATATATATCTATATATATATATA TATATATATATATATATATATATATATACATATATATATATATC TATAAATATATATATATCTATATCTCCACCCCTATATATACGTG TCTTTATTCGTATATATATATATATCCATCTATATGTATACGTAT GAATGGACCCGCCAAGGGCATAGCCCAGGGCGACCGGTCTTCA TCTATGTCCATACGACCGATACGTGGCTAGATGTTTGTACGGA AGATTTATCTATATGAATGCACACACACTGACTACATGCGTGG ATCGCTGGGTGGCACTTCCTCGCCCGGACCCCGGACCGAACT
3342680_MD2_- _EXON_3_PRIM ER_EXON_3_- _F	CCACCCCCCCCTACCATATATATATATATCTATATATATATATA TATATATATATATATATATATATATATACATATATATATATATC TATAAATATATATATATCTATATCTCCACCCCTATATATACGTG TCTTTATTCGTATATATATATATATCCATCTATATGTATACGTAT GAATGGACCCGCCAAGGGCATAGCCCAGGGCGACCGGTCTTCA TCTATGTCCATACGACCGATACGTGGCTAGATGTTTGTACGGA AGATTTATCTATATGAATGCACACACACTGACTACATGCGTGG ATCGCTGGGTGGCACTTCCTCGCCCGGACCCCGGACCGAACT
3342679_N36_- _EXON_3_PRIM ER_EXON_3_- _R	CNNNNNNNNNNNNNNNNNNNTATATACCATATATATCTATAT ATATATATATATATATATATATATATATATATATATATATATATAT ATATATATATATATATATATATATATATATATATATATATATATAT ATATATATATATATATATATATATATATATATATATATATATATAT ATATATATATATATATATATATATATATATATATATATATATATAT ATATATATATATATATATATATATATATATATATATATCTATAC ATATATATATATATATATATACATATATATATATATATATATAT ATATATATATATATATATATATATATATATATATATATATATATAT ATATATATATATATATATATATATATATATATATATATATATATAT ATATATATATATATATATATATATATATATATATATATATATATAT ATATATATATATATATATATATATATATATATATATATATATATAT

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	ATATAT
3342678_N36_-_EXON_3_PRIMER_EXON_3_-_F	ANNNNNNNNNNNNNTANNTATATATCTATATATATAATATAT
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	ATATATATATATATATATATATATATATATATATATATAAAT
	ATATATATATATATATATATATATANATATAACATATATATATTAC
	ACTTTATCTATCTAACTNTAATNAAAAAAA
3342677_MORIS_-_EXON_3_PRIMER_EXON_3_-_R	NNNNNNNNNNNNNNGTNNNNTATNNNATACATNNNGTATAT
	ACNTACTANNTCTCTCTATATATATATACTATATATATATATAT
	ATATATATATATATATATATATATATATATATATATATACATATAT
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	ATATATATATATATATATAANTATATATATAAATA
3342676_MORIS	AACGCTCATACACCGTTACTTAAAAATTTTAAAAAAAAAAAAA
_-	AAATAAAAAAAAATTTTTTTTTTTTTTTTTTTTTTTTAAAAAA
_EXON_3_PRIM	AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
ER_EXON_3_-	AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
_F	AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
	AAAAAAATTTTTTTTTTTTTTTTAAAAAAAAAAAAAAAAAATTT
	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT
	TTTAAAAAAAATTTTTTTTTTTTTTTTTTTAAAAAAAAAAAAA
	AAAAAAATTTTTTTTTTTTTTTTTTAAAAAAAAAAAAAAAAATT
	TTTTTTAAATAAAATAAAAATTTTTAAATTTTATATTATATATTA
	ATATACTATCCTATACAAAATCTAATTAAAATATAAATATCTAT
	AATTAATCATTGCCCCACCACTTCCCACTAACACCCACTCGAAA
	ACCCATACACCCACACCCCCCCCAAAACAACACG
3342675_SARA	ACCGGCCACCGCCCACGGGCCCTGCCACCTGCCTGTACACGCC
WAK_-	TCCATATGGGTATAGGCGGCCATGGGTATGCAGATGTACCCGC
_EXON_3_PRIM	CCGCCCGAGGGGGGCTATATATACACCCCGCGGCCCCCCCGCG

ER_EXON_3_-_R	AGGGCTGGGGCCAGGCCGCCGGGGAGCCTCCCCGCGGATGCC GACAGAGGGGTAGCTAGGTCGAGGGAGATCCCCATATGTGCCC ATACATCTATATGCATGTGAAGCTAGATACGTGGCTAGATGGA TAGACATACTGATCTACAGAGATGCCTGCACAGGTCTCTGTCT ATAGATATGTATATATACGCGGAGACCTATCCCTATCCCGTGC ATACGCGATGCGCGCGCCCCACAGCTATACCAAAGATGTAGCT ACATATATATATATATATATATATGGTATATATACATCCCCGAT GTAGATATATATATATATATGTATATATATGGATAGTCTATCTA TATATATATATATATATATAGATACATATATATGTATATATATG TATATATATAGATATATATATATATATATATATATATGTATA TCTACATATATATATATATATATCTATATATATGTATATATCTAT AGCCACCCTATATATATATCCAGATATATCCTTGGTATCCTATA TCATCTAGCTGTGCTATCTGTGTCTAGGGTGTCGAGTGATGTCG
3342674_SARAWAK_-_EXON_3_PRIMER_EXON_3_-_F	ACCCGCCCAACCCCCAGATACTACATCATATACCTCTCTCCACA TATATCGGGATCGATACATCTAGAGCTATATGCAGAGACCCCG GCGACAGAGAGCCATATACACATCGCTACCCCCCCCCCACGTG GAGATAGCCGTATCTGCATGCAGGGATACCTAGATATATACAT ATATATGGTACCCCCCGGGAGGACGGTAGGGGATCTATACATA TGTAGATGATCGTACCCCCCCCAGGATATAGCAGCCAATATAT ACTACTATATAGCGCTATAGCTACATTTATATATGCATCTATAT ACCTATATGTATACTAAAGAACTCTAACCCTCTATATCTATCAA TCCCCAAAAATCATCGCCAGGATGTAGGATATCTACACTATAT ATATGTTTGTATGCGATCCACCATATATTGGGTATAGGCGGAG GATATAAATAAAAAAAGCGACG
3342673_JOSAPINE_-_EXON_2_PRIMER_EXON_2_-_R	CGTCTAAGAAAGTTATACGTACAGATCACCGTGTAGGCATCTG CACATCTCTATGGCTATGGCTGTCCATACATATATCTACATGCA TATGCACCCAGATATATATATATATCTGTCTCCATCTATCTACG TCTACAGGTATAAACCGCCGGAGATATAGACATATATATCTAT ACATATGTATCTATATATATCGATATATATGTATATATAGATAG ACCAGGAACATATAGATATATCCGATACATATATATATATCAT

	AGATGTATATATGTATATATAGCTATATGTATGTATATATATAT ATATATGTATATCTATATATATATATATATATATATATATATAT ATATATATATGGATATATATCTATATATATATATATCTCTATAT GTATATATATATATATATATATATATATATCAGATATATACATA TATATATATATATATATATATATATATAGATATATATATATATATA TATATATATATATATAGATACATACATAGATATATATATACATA TATATATCTATATATATATCTATACATATCTATATATATATATAT ATATATGTATATATCTATATATATATATATATATATATATATAT ATACATATATACATATATATATATATATATAGATATACATATAT ATCCATATATATATATATATATATATAGATATATATATATCATCAT ATATATATATCTATATATATATATCATATAGTATATATATATGA CATATCTATATGATATATATATATATATGATCATATATATATCGAT ATATCCCATATGTGCGATACATATGACATGATCATCATATCTCA CCCAATATATAATCATAGATGCCGCTATATAATATCATTACCAT AATCATCAATCATCACGAATCAACTCCTTAAAGTTGAGGCAAC CATGACGA
3342672_JOSAPINE_-_EXON_2_PRIMER_EXON_2_-_F	TGGAGGTAGCGGAACAATCAGAAGTGAGCATCCGGTGAGGCA TCCGCTCTCTCGATAGGTATGGCTGTACATATATATACATACAT ACATATATACATAGATCTATATATATATATATATATATATATCT ACGCTATATATATATCCATCCGGAGATCTAGACGGATATACAT ATATAGAGGCGGCATATAGCCATATATCTATAGGGATATGTAC ATAGATGGAGACATCTATATATAGCTATACATATCTAGCCCTG CATACATCTATATGTATATGGCGGGTATATATATGTAGATAGA CATATATATATATGTATATCGCTATGTATATATATCGATAGATC TGTACATACATATCTACAGGGACGTACATATCTATATATATATC TATATATATATATATATATACACATCTATATATATACATACATATA TATATATATATGTATCTACATCTATGTATATACATACAGATATA TCTATACATATATGTACATATATATATATAGAGATCGCTATATA GATATGTATCTATATATATATCCCCGCATAGATATATACATATC TCTATATCGCTATATCGGTAGAGACACATATACGTATATATAG

	ATATATATATATGTATATATATATCTATGTATATATAGAGCTAT CTCTAGATATATATCTATATATATATAGGTAGATAGATAGATAG ATATATATATATGTATACAGATATATATCTCTATACATATATAT ATATACACATATATATAGACATATACATATATCGTCTAT
3342671_MD2_- _EXON_2_PRIM ER_EXON_2_- _R	CGGCTTCTTTCCGCCTTGATATATATGTATATCTGTCTATATATA TATATATATATATACATATATATATTTCTATATATATATATATC GCATAGATATATATTGACATATATATGGACACATAAATATATA TTTATATATATATATATATATAGATTTCTATCTATAGATATATTT AGATGTAGCTATCTATATAGGTATCTGCATATGTGTCCGGACA GGTCTCGCATACGATGATACATCCGTGGACAAAGGGTAGGCTG ATGCGCCCGCTCCCCTGAGGGCTATGGCCTCTGTCCGTGATGCG TGGCCCCCCACCGGTGCCCCTCCCTCTGCCAGACCTCGAATTAT AGG
3342670_MD2_- _EXON_2_PRIM ER_EXON_2_- _F	CCACCCCCCCCCCCCGAAGGCTATATATCTATACTATACATATA TATATATATATATATATATATATATATATATATATAAATATAGATA TATATTTATCCATCTATATATTTATAAGATATCGACATCTGGAA GTAACTCTTGCCCCGGCGGGGGCCCCAGACACGACCTCTATAC ATTTTTGCCCCCCTTGCTATTACTATTGGTACAATTGTGGAGGG GCTCTGCAAAGACCCTACAGATGTCGAAAAAGGTTCCTCCCAG TGCGCCGGGTTCTCTAAGGACTAAAGCCCCTTTCCCCGAACCG GCCCCAC
3342669_N36_- _EXON_2_PRIM ER_EXON_2_- _R	GGGTAGAAGCAGCCCGGTGTATATACTATACACATATACTATA TATACTATATATATATATATATATATATATATATATATATATAT ATATATATATATATATATATATATATATATATATATATATAT ATATATATATATATATATATATATATATATATATATATATAT ATATATATATATATATATATATATATATATATATATATATAT ATATATATATATATATATATATATATATATATATATATATAT ATATATATATATATATATATATATATATATATATATATATAT ATATATATATATATATATATATATATATATATATATATATAT

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	TATATATATCAATCTCTTACATAGATCTATGTCTGTATCTATAT
	AGTACAGAATAGTATGA
3342668_N36_-_EXON_2_PRIMER_EXON_2_-_F	NNNNNNNNGNNNNNNNNNNNNNNANGNGAGCAGCNTNGAN
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	ATATATATATATATATATATATATATATATATATATATATATATATAT ATATATATATATATATATATAAATTATATATATATATATATATATATA TATATAAAAAATATATATATATATATATATATATATATATATATATAT ATATATATATATATATATATATATATATATAATATATATATATATATA TATAATATATATATATATAATATATATATATAAAAAATATATATAA ATAATATATAAAAATTATATANNNAAAATATAAAAATTATATA ATATATAAAAAATATAAAATATATATAATATAAATATAAATTA TATAATATTTATAATATATATATAAAAATAAAAAATATAAAAA AAAAAAAATATATAAATTATAAAAAATAAAGAATATA
3342667_MORIS _- _EXON_2_PRIM ER_EXON_2_- _R	CGGTTATTATATCTGTTCTATCCATCATATATATCCATATATAT ATATATATATATATATATATATATATATATATATATATATATATATAT ATATATATATATATATATATATATATATATATATATATATATATATAT ATATATATATATATATATATATATATATATATATATATATATATATAT ATATATATATATATATATATATATATATATATATATATATATATATAT ATATATATATATATATATATATATATATATATATATATATATATATAT ATATATATATATATATATATATATATATATATATATATATATATATAT ATATATATATATATATATATATATATATATATATATATATATATATAT ATATATATATATATATATATATATATATATATATATATATATATATAT ATATATATATATATATATATATATATATATATATATATATATATATAT ATATATATATATATATATATATATATATATATATATATATATATATAT ATATATATATATATATATATATATATATATATATATATATATATATAT ATATATATATATATATATATATATATATATATATATATATATATATAT ATATATATATATATATATATATATATATATATATATATATATATATAT ATATATATATATATATATATATATATATATATATATATATATATATAT ATATATATATATATATATATATATATATATATATATATATATATATAT ATATATATATATATATATATATATATATATATATATATATATATATAT ATATATATATATATATATATATATATATATATATATATATATATATAT ATATATATATATATATATATATATATATATATATATATATATATATAT ATATATATATATATATATATATATATATATATATATATATATATATAT

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	GTATATATACATATATATATATACATATATAT
3342666_MORIS	CGCTGCTCGGATTTATATCTATCGAGCCGTCCGAGAGGCTACA
_-	GCTCTATCATATATATATGCATATCTATATACATACCTCTATCT
_EXON_2_PRIM	ATCGATACGTATATATATATATATACATATATATATATATATAT
ER_EXON_2_-	ATATATATATATATATATATATATATATATATATATATATATAT
_F	ATATATATATATATATATATATATATATATATATATATATCTCTAG
	ATATATATATATATATATATATATATATATATATATATATATATAT
	ATATATATATATACATATATATATATATATATCTATAGATATAT
	ATATATATATATATATATACATATATATATATATATATATATAT
	ATATATCTATATATATATATATATATATATATATATATATATAG
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	ATCCCCCCCCCCCCCCGCGCGCCCCCCCCCCCGCGCATAGGG
	GGTGTGGGGTATCTAGATGTATATAGAAGATGTGTATGT
3342665_SARA	CCCCTTTTTCCTTTCCCCCTGCCTTTCTCCTCTCTGTCGACCTTG
WAK_-	GCCGGTACACATGGCCCTATGGGCACCCATAGGTAGGGATGGA
_EXON_2_PRIM	TAGCTATATACTAGATATGTATGCGCGGTCTACCGATAGAGGA
ER_EXON_2_-	ATATATATATACCGATGGATGGGGACCTATATGTATGCCCATA
_R	TATATACAGATATATATATATACCGCCGCTAGATATATGTATAT
	ATATATATATATATATATATATATATATATATATATATATCCAT
	ATACGCCGGGATATATATCTATATATAGGTATATATATATAGC
	GGTATCCCTATCTATATACCCCCCTGTATATACAGATCCACATA

	TATCTCCATCCCCCCCCCACATATATATCTGTCTATATATATAT ATATATATAGATACATATATATATATATATATATATATATATAT ATATATATATATATATATATATATATATATATATGTATATGACACA CATATATATATATATATATATCTACATATATATATATATATATG ACTATATCTATATATATATATATCCCTGTATCTATATGTGTCTAT CTAGATATGTATGCGTGGGCATAG
3342664_SARAWAK_-_EXON_2_PRIMER_EXON_2_-_F	CCGCCCCCCCCCCCCCCTACAGCGAGCCGCATCGACAGTCCGG TCTATAGGTACATATATATATATGGATAGATCGCTGGAGATAC ACGGGGGGCACAGAGATATGCCGTCTGTATACGGCGGGGGGG TAGGCACATATCTATATATACAGATATATCTGGATCCTAGAAT ATAAATATATATATATATAGATCTATAGGGGAGTACATATATA TATATATATATGTATATATAGATATGGATATATATATATATATA TCTGTATAGATATATATAGATATATGTATATATATATATATGGA TGTATCTATATAGATCCCTCGCTGTGCGCGGGGTGTATGTATTT GGGGGTGCGGCTCTCATAGGTGGACGTATAGGGAGGAGTGTGA AT