DEVELOPING MOLECULAR MARKER USING INTERNAL TRANSCRIBED SPACER (ITS) REGION FOR *ZINGIBER* SPP. IDENTIFICATION

By

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DECLARATION

I declare that this thesis entitled "Developing Molecular Marker using Internal Transcribed Spacer (ITS) Region for *Zingiber* Spp. Identification" has been composed solely by myself that the work contained herein is my own except where explicitly stated otherwise in the text. All sections of this paper that describe an argument or use quotes or concept developed by another author have been referenced which including secondary literature used to show that this material has been adopted to support my thesis. This thesis has not been submitted for any other degree.

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APPROVAL

"I hereby declare that I have read this thesis and in my opinion this thesis is sufficient in terms of scope and quality for the award of the degree of Bachelor of Applied Science (Natural Resources Science) with Honours"

Signature :

Name of Supervisor I :

Date :

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Developing Molecular Marker using Internal Transcribed Spacer (ITS) Regions for *Zingiber* spp. Identification

ABSTRACT

In the order Zingiberales, the pantropic Zingiberaceae is the largest family with over 1200 species and 53 genera with varieties of medicinal and culinary uses. There is a very limited information exist using Internal Transcribed Spacer (ITS) regions as a molecular marker in the identification of *Zingiber* and *Etlingera* species. This study aims to develop a molecular marker for *Zingiber* and *Etlingera* species identification and differentiation. The Polymerase Chain Reaction (PCR) method was used in the identification of *Zingiber* and *Etlingera* species using Internal Transcribed Spacer regions in this study. The leaf samples of *Zingiber officinale* and *Etlingera elatior* were collected around Kelantan, Malaysia and Cetyl Trimethyl Ammonium Bromide (CTAB) method was used to isolate genomic DNA. Then the isolated DNA were used for ITS sequences amplification and analysis. The amplified ITS regions of *Zingiber officinale* and *Etlingera elatior* were sent for Sanger sequencing and the ITS sequences obtained were used to identify the species through Basic Local Alignment Search Tool. The sequencing results for isolated DNA *Zingiber officinale* showed 91% match with *Zingiber officinale* in National Center for Biotechnology Information Genbank (NCBI) database and 99% similar with *Etlingera elatior*. The molecular phylogenetic trees were constructed and indicated that *Zingiber* species and *Etlingera* species were separated into two cluster. Hence, it indicate that ITS can be used as the molecular marker in future for *Zingiber* and *Etlingera* species identification.

Membangunkan Penanda Molekul menggunakan Kawasan Spacer Transkrit Dalaman (ITS) untuk Pengenalpastian *Zingiber* spp.

ABSTRAK

Di dalam order Zingiberales, pantropis Zingiberaceae adalah keluarga terbesar dengan lebih daripada 1200 spesies dan 53 genera dengan pelbagai jenis kegunaan di dalam bidang perubatan dan masakan. Informasi yang sedia ada untuk penggunaan kawasan Ruang Transkrit Dalaman (ITS) sebagai penanda molekul dalam mengenalpasti *Zingiber* dan *Etlingera* spesies sangat terhad. Reaksi Rantaian Polimerase (PCR) telah digunakan dalam kajian ini untuk mengenalpasti *Zingiber* dan *Etlingera* spesies menggunakan kawasan Ruang Transkrit Dalaman. DNA daripada sampel daun *Zingiber officinale* dan *Etlingera elatior* telah diasingkan dengan menggunakan kaedah 'Cetyl Trimethyl Ammonium Bromide'. DNA yang telah diasingkan digunakan untuk penjujukan DNA dan telah dihantar untuk penjujukan Sanger dan jujukan Ruangan Transkrit Dalaman (ITS) telah digunakan untuk mengenalpasti spesies melalui kaedah alat carian penjajaran tempatan asas (BLAST). Keputusan mendapati 91% daripada jujukan adalah padanan dengan *Zingiber officinale* dan 99% padanan dengan *Etlingera elatior* di dalam pengkalan data 'National Center for Biotechnology Information Genbank' (NCBI). Pokok filogenetik molekul telah dibina dan menunjukkan *Zingiber officinale* dan *Etlingera elatior* berada di dua kluster berasingan. Maka, kawasan ITS dalam jujukan DNA boleh digunakan sebagai penanda molekul untuk mengenalpasti spesies *Zingiber* dan *Etlingera* di kajian masa hadapan.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

A	Adenine
BLAST	Basic Local Alignment Search Tool
C	Cytosine
CTAB	Cetyl Trimethylammonium Bromide
ddH_2O	Double Distilled Water
DNA	Deoxyribonucleic acid
ETDA	Ethylenediaminetetraacetic acid
G	Guanine
HCl	Hydrochloric Acid
ITS	Internal Transcribed Spacer
ITS F	Internal Transcribed Spacer Forward
ITS R	Internal Transcribed Spacer Reverse
KCl	Potassium Chloride
$MgCl_2$	Magnesium Chloride
mL	Milliliter
mM	Millimeter
NaCl	Sodium Chloride
ng	Nano gram
OD	Optical density
PCR	Polymerase Chain Reaction

RNA	Ribonucleic acid
rpm	Revolutions per minutes
spp.	Species
T	Thymine
TAE	Tris-acetate-ETDA
Ta	Annealing Temperature
Taq	*Thermus aquaticus*
uL	Microliter

LIST OF SYMBOL

%	Percent
℃	Degree Celsius
µL	Micro litter
ng	Nano gram

CHAPTER 1

INTRODUCTION

1.1 Background of Study

According to the scientific information available, approximately 141 species of genus *Zingiber* are distributed around tropical Asia. In general, the *Zingiberaceae* species is gaining importance in various aspects such as medicines, food and economic importance (Devi *et al*., 2017).

Molecular marker is known as the genetic marker where the fragment of DNA is related to the certain area inside the genome. There are two broad classes of molecular marker which is biochemical marker based on gene product like isozymes and marker relying on DNA assay (Semagn *et al*., 2006). The molecular marker is well known in various type of application. Jiang *et al*. (2006) have using polygenetic analysis and metabolic profiling in genus *Zingiber* for closely related species of ginger and confirmation of ginger species.

In recent years, to identify *Zingiber* species and *Etlingera* species, several species-specific DNA marker test have been developed and this provides new data of species identification for these two genera. The molecular marker is found to be the effective universal region in identifying plant species is the Internal Transcribed Spacer

(ITS) region. For instance, the sequence of data at the ITS region has been studied earlier to assess genetic diversity in barley cultivation (Petersen & Seberg, 1996). Therefore, this method is hoped to provide a set of specific primers by using the Polymerase Chain Reaction for *Zingiber* species and *Etlingera* species identification.

1.2 Statement of Problem

There is no prior information exist using Internal Transcribed Spacer regions as a molecular marker in the identification of *Zingiber* and *Etlingera* species. Besides, the usage of microscopy and chemo-profiling in identification and differentiation of the *Zingiber* and *Etlingera* species is very limited. In addition, most of *Zingiber* species are comparable in morphological characteristic with other *Zingiber* species. Hence, species members of these two genera are difficult to be differentiated by naked eyes during the non-blooming stage. Adding to that is the absence of logical and clinical information of *Zingiber* species and *Etlingera* species for better comprehension of the viability and security of the natural medication. Hence, this study aims to develop a molecular marker for *Zingiber* and *Etlingera* species identification.

1.3 Objective of Study

1. To optimize the Internal Transcribed Spacer (ITS) region based on molecular marker via Polymerase Chain Reaction (PCR) for *Zingiber officinale* and *Etlingera elatior*.

2. To develop the molecular marker for *Zingiber* species and *Etlingera* species identification.

1.4 Scope of Study

This study focus on a sampling of *Zingiber officinale* and *Etlingera elatior* around Kelantan, Malaysia. This study focus to establish the species-specific primer that suitable for identification of *Zingiber* species and *Etlingera* species. Shape, color, texture and odor of plant are the morphological characteristic which usually used as the principal indicators for classification of herbs. Using morphological features was reported to pose difficulties in identifying the species of genus *Zingiber* and genus *Etlingera* because the features are nearly similar. Hence, Internal Transcribed Spacer region in the genome sequence was used in this study as a tool for *Zingiber* and *Etlingera* species identification.

1.5 Significance of Study

Developing those molecular marker for *Zingiber* by using Internal Transcribed Spacer (ITS) has been uncovered a standout amongst those challenges encountered by the botanist. The result of Polymerase Chain Reaction (PCR) amplification had been designed the primers specific for species identification of *Zingiber* and *Etlingera*. In addition, the authentication results not influence by acknowledged traditional morphological characters of *Zingiber* and *Etlingera* species and had been shown a dependable result. At the end of the study, species-specific primers had been developed for *Zingiber* and *Etlingera* species identification. This study had been used DNA Internal Transcribed Spacer (ITS) region in the ginger genome to develop a molecular marker for *Zingiber* and *Etlingera* species identification.

CHAPTER 2

LITERATURE REVIEW

2.1 Family *Zingiberaceae*

Kai Larsen (1980) was the first person who proposed the taxonomic study of the family *Zingiberaceae* and the key to the genera of the Thai *Zingiberaceae*. The family *Zingiberaceae* is the largest families of the order Zingiberales that consists of 53 genera with approximately 1200 species (Kress, 1990; Angela, 2012).

Zingiberaceae generally known as the ginger family with monocotyledons flowering plant consist of aromatic perennial herb with creeping horizontal or tuberous rhizomes. Abundant species from this family are distributed mainly in southern Asia. Malesian region is one of the highest diversity of genera and species of *Zingiberaceae.* (Larsen *et al*., 1999; Angela, 2012).

The *Zingiberaceae* was divided into four tribes, namely *Alpinieae, Globbeae, Hedychieae* and *Zingiberaceae* were found mainly in the tropic throughout the world. The characters used to distinguish the tribes were morphological features based on both vegetative and floral characteristics (Larsen *et al*., 1999).

2.2 Genus *Zingiber*

Zingiber is the Latin term which was derived from Southern India-Tamil Nadu. Most of the Western European countries, the present-day name of ginger comes from this ancient term. For instance are *ginger* (English), *inkiyaari* (Finnish), *Ingyer* (German), *barlik ingyer* (Estonian), *gember* (Dutch) and *zingibro* (Esperanto).

An earlier perspective of some authors that the term *Zingiber* was derived from the ancient Indian Sanskrit, *singayera* (Watt, 1872; Rosengarten, 1969; Purseglove *et al*., 1981), that define as horn-shaped or antler-like that show the shape of the rhizomes. However, this is unconvincing nowadays in the region because Sanskrit was not easily seen.

There are examples of three *Zingiber* spp. in Malaysia showed in Figure 2.1 which are *Zingiber spectabile* by Steph (2009), *Zingiber officinale* and *Zingiber zerumbet*.

Figure 2.1 Example of *Zingiber* spp. From left *Zingiber spectabile*, *Zingiber officinale* and *Zingiber zerumbet* by Steph (2009).

(Source: Retrieved April 8, 2018 from http://www.alamy.com/stock-photo-zingiber-zerumbet-or-shampoo-ginger-zingiberaceae-family-rio-de-janeiro-56353402.html and http://www.alamy.com/stock-photo-zingiber-officinale-stem-ginger-showing-rhizome-and-flower-spike-138709351.html)

2.3 Origin and Distribution of *Zingiber* spp.

The genus *Zingiber* mostly distributed in tropical and subtropical Asia with the center of biodiversity of Southeast Asia. In 2013, referring to The Plant List, this genus has been published about 244 names, corresponding approximately to 100–150 species rewrite by Bai *et al*. (2015).

The largest distribution of genera and species are in Southeast Asia which judging by the family existing distribution was within the Indo-Malayan region (Holttum, 1950; Theilade 1996). In Peninsula of Malaysia, about seventeen of the nineteen species belong to genus *Zingiber* (Theilade, 1996).

Zingiber species undergrowth in the tropical forest. The plants are perennial and mainly grow in damp places. Furthermore, this plants frequently can be found in disturbed sites and secondary forest. In Peninsula Malaysia, a few *Zingiber* species can grow on high mountain ridges but commonly abundance of this species grow in low land and mid-mountain forest (Holttum, 1950; Theilade, 1996).

2.4 Morphological Characteristic of *Zingiber* spp.

In morphological features, genus *Zingiber* can be characterized by aromatic branched and tuberous rhizomes. *Zingiber* species are leafy and have pseudostems erect. *Zingiber* species have distichous leaves which are located in plane parallel to rhizome that are cushion-like and petiole swollen. The leaf blades are either oblong or linear. Conical inflorescence is clothed with scale-like sheaths and infrequently break through leaf sheaths without the peduncle and the conical inflorescence are arising from rhizomes on peduncle. The bracts closely overlapping and its color is green or other colors. *Zingiber* species usually a single flower, persistent and non-tubular bracteoles.

Calyx tubular are splitting on one side and with apex 3 toothed. *Zingiber* species have short filament which connective with elongate appendage and wrapped around style. The seeds are black and covered by aril.

Morphological characters of *Zingiber* spp. which are rhizome, leafy shoot, flowering shoot and structure of flower and an infructescence of *Hedychium* by Johnsen (2015) are showed in Figure 2.2.

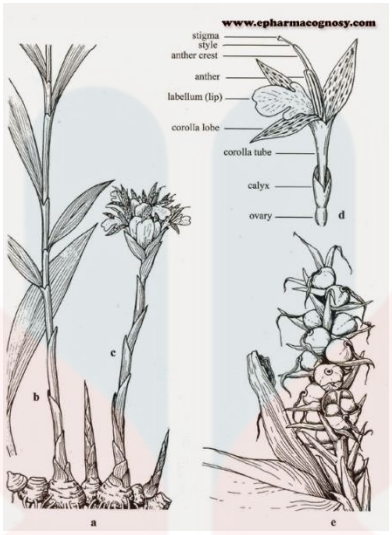

Figure 2.2 Morphological characters of *Zingiber* spp, which a. rhizome; b. leafy shoot; c. flowering shoot; d. flower; e. an infructescence of *Hedychium* by Johnsen, (2015).

2.5 Importance of *Zingiber* spp.

Plants of genus *Zingiber* which is belonging to the family of *Zingiberaceae* have a large range of uses and play a crucial role throughout the world as medicinal and food plants. This genus usually has grown in Africa, South America, Central and Asia and consist approximately 85 species of herbs (Sabulal *et al*., 2006). Plants of this genus have differed widely in therapeutic properties and pharmacological properties although members of this genus are comparable in morphological characteristic (Ghosh *et al*., 2011).

Furthermore, members of this genus represent varieties of traditional healing system which very popular in herbal remedies. In particularly, *Zingiber* species rhizome plants has a long history of ethnobotanical uses due to curative properties. A common ingredient in traditional medicines and a variety of physiological and pharmacological

effect possess by *Zingiber* plants. Rhizomes of *Zingiber* plants have medicinal value for some medical conditions which including a sore throat, common cold, wounds, muscular pains and high cholesterol (Shukla *et al*., 2007).

In the ginger root have phenolic compound especially the gingerols have revealed to have a chemopreventive effect and associated with anti-inflammatory and antioxidant activities (Shukla *et al*., 2007). Moreover, some of *Zingibe*r species has shown the presence of bioactive compounds in the rhizomes by the phytochemical investigation. For example are shogoals, phenylbutenoids, gingerols, flavonoids, sesquiterpenoids and diterpenoids (Sivasothy *et al*., 2011).

In addition, the *Zingiber* rhizomes have Essential oils (Eos) that consist of antioxidant and antimicrobial properties. The Essential oils have been used in a variety of food for preserving purpose in against microbial spoilage and autoxidation (Bellik *et al*., 2014). It have demonstrated many in vitro studies from the *Zingiber* plants extract which the plant has antimicrobial potential against Gram-positive and Gram-negative bacteria (Kumar *et al*., 2011). *Zingiber officinale* is the most popular spice which consumed worldwide as a flavoring agent and a spice that widely reported in the literature (Sabulal *et al*., 2006).

2.6 Developing Molecular Marker using Internal Transcribed Spacer (ITS)

For authentication and identification of plants or animal species, the most popular tools are DNA-based markers. This is because DNA-based polymorphisms assay are accurate, effective, reliable and sensitive technology for identifying and developing molecular marker (Zhang *et al*., 2007; Chun *et al*., 2009). For example, RAPD (Random Amplified Polymorphic DNA) technique methods have been successfully analyzed different adulterants and habitat of *A. villusum* (Wang *et al*., 2005; Xu *et al*., 2006; Chun *et al*., 2009). To study the phylogenetic relationships, species identification, population genetic and systematic DNA sequencing techniques can be applied (Wu *et al*., 2013).

The Internal Transcribed Spacer (ITS) are the sequencing-based technique which are the powerful tools for species identification and have been widely used (Cheng *et al*., 2004; Wang *et al*., 2005). The ITS1, 5.8S rDNA gene and ITS2 are the definition of the Internal Transcribed Spacer (ITS) region of rDNA which it can be amplified with specific primer by Polymerase Chain Reaction (PCR) and sequenced (Sun *et al*., 2011). Recently, various medicinal plant species originating from China and Korea have used nuclear ribosomal DNA Internal Transcribed Spacer (ITS) sequencing in developing molecular marker to identify the species (Yang *et al*., 2012; Han *et al*., 2016).

2.7 Advantages of Internal Transcribed Spacer (ITS)

The Internal Transcribed Spacer (ITS) are popular due to some advantages. For instance, the Internal Transcribed Spacer (ITS) are low functional constraint, intragenomic, simplicity, uniformity, universality, a high copy of number, and intergenomic variability (Wu *et al*., 2013).

In addition, earliest Internal Transcribed Spacer (ITS) primer such as ITS1, ITS4, and ITS5 for amplifying the Internal Transcribed Spacer regions shared high universality which has received a broad recognition for work with plant Internal Transcribed Spacer Region and fungal (Yan *et al*., 2013). Furthermore, Internal Transcribed Spacer region has done a lot demonstrated because it regions have high species discrimination, especially when used as herbal medicinal material authentication and region for barcoding (Yan *et al*., 2013).

2.8 Polymerase Chain Reaction (PCR)

In the 1980s, Kary Mullis has developed the Polymerase Chain Reaction (Bruce *et al*., 1999; Valones *et al*., 2009). By three simple step of cycling process in the PCR, specific DNA or RNA sequences can produce more than a million copies. The three simple steps in the PCR are denaturation, annealing and extension. The double-stranded DNA will denature in the initial steps in the PCR which to separate the complementary strands and the annealing process are the second step that DNA strands will dissociate because of the primers. The thermostable DNA polymerase will be catalyzed in an

extension reaction with participate primers and the cycle in the PCR will be repeated. The PCR reaction of target sequences to be amplified are one to the left (5') and one to the right (3') (National Laboratory of Enteric pathogens, 1991).

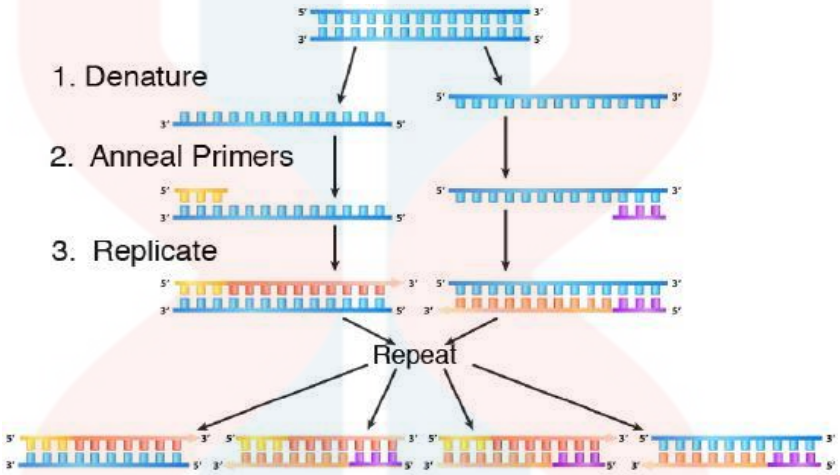

Figure 2.3: Illustration showing the step involved to produce lots of copies of DNA strands in Polymerase Chain Reaction (PCR) (Aleia, 2017)

2.8.1 Component of Polymerase Chain Reaction (PCR)

The template DNA, nucleotides, DNA polymerase and nucleotides are the components that requires for Polymerase Chain Reaction Assay. To form the PCR product, the key enzyme that helps individual nucleotides to links together is the DNA polymerase. The nucleotides which act as building blocks that are found in DNA include adenine, thymine, cytosine and guanine (A, T, C, G). The DNA polymerase will use these nucleotides to create the resultant PCR product (Garibyan & Avashia, 2013).

2.8.2 General Advantages of Polymerase Chain Reaction (PCR) in Amplifying DNA Samples

The product from the Polymerase Chain Reaction (PCR) process can be produced in millions of copies. Polymerase Chain Reaction is can be used for cloning, analysis and sequencing of DNA. This is because PCR is proven to be a highly sensitive technique in the analysis, cloning and sequencing with three simple steps which are involving denaturation, annealing and extension. PCR allows the study of conventional sequencing technique which PCR technique has the ability to do specifically *in vitro* amplification in small scale to the big numbers of DNA sequences. Furthermore, PCR can be used to identify microbes and figure out various disease state. In addition, using a variety of sources of DNA from tissues and organisms the PCR can be performed to analysis, sequencing and cloning PCR to produce millions to billions of copies (Garibyan & Avashia, 2013).

CHAPTER 3

MATERIALS AND METHOD

3.1 Materials and Apparatus

Casting tray, Zipper lock bag, Well combs, Media botel, Voltage source, Autoclave Machine, Gel box, Flask, UV light, Microwave, Beakers, Nanodrop spectrometer (Thermofisher, United States), Tris-acetate-ETDA (TAE), Agarose, Ethidium bromide, Tris–HCl, Sodium Chloride (NaCl), PCR machine, Ethylenediaminetetraacetic acid (EDTA), Cetyltrimethylammonium bromide (CTAB), PCR microtubes, Chloroform-isoamyl alcohol, Isopropanol, absolute Ethanol, Tris–HCl EDTA (TE), Distilled water, Tris-HCl, 6x gel-loading buffer and 1kb DNA molecular weight marker (Promega, United States).

The reagents that used in Polymerase Chain Reaction (PCR) were: $MgCl_2$ (Promega, United States), PCR Nucleotide Mix (dNTP) (Promega, United States), 5x Green Flexi buffer(Promega, United States), ITS1 and ITS4 forward and reverse primer and DNA *Taq* polymerase (Promega, United States).

3.2 Preparation of CTAB Buffer for DNA Extraction of *Zingiber officinale* and *Etlingera elatior*.

The preparation of all DNA extraction buffer was done following method by Vinod (2007). DNA extraction buffer includes Ethylenediaminetetraacetic acid (EDTA) buffer, Cetyltrimethylammoniumbromide (CTAB) buffer, Tris-HCl buffer and Tris-HCl-ETDA (TE) buffer.

One liter of Cetyltrimethylammoniumbromide (CTAB) buffer solution was prepared. For one liter CTAB buffer, 20.0 g CTAB powder was mixed with 860 mL sterile distilled water. The solution was added with 81.82 g NaCl, 100 mL of 1 M of Tris with pH 8.0 and 40 mL of 0.5 M ETDA (Ethylenediaminetetraacetic acid Di-sodium salt) with pH 8.0. The buffer was adjusted to pH 5.0 with HCl and 1000 mL distilled water. Then, the buffer solution was autoclaved (TOMY SX 500) to sterilize at 15 lbs pressure at 121 ℃ for 15 minutes and the sterilize CTAB buffer was stored at room temperature (±27℃).

Tris-HCl buffer was prepared by dissolving 121.1 g of Tris base in 700 mL of distilled water and was stirred to mix by using a magnetic stir plate. The prepared buffer was pH adjusted to 8.0 with 50 mL of HCl and then let to cool at room temperature (±27℃). The buffer solution was sterilized autoclaving (TOMY SX 500) at 15 lbs pressure at 121℃ for 15 minutes.

Tris-HCl-ETDA (TE) buffer was prepared by adding 10 mL of 1 M Tris and 2 mL of 0.5 M ETDA solution. The distilled water was added up to 1000 mL to the solution. The buffer solution was sterilized autoclaving (TOMY SX 500) at 15 lbs

pressure at 121˚C for 15 minutes. The sterile TE buffer was stored at room temperature (±27℃).

One liter of ETDA stock was prepared by adding 186.12 g of ETDA powder in 750 mL of distilled water. The prepared buffer was adjusted to pH 8.0 with NaOH pellets and 1000 mL distilled water was added. The prepared buffer solution was sterilized autoclaving (TOMY SX 500) at 15 lbs pressure at 121℃. the sterile ETDA buffer was stored at room temperature (±27℃).

3.3 Collection of *Zingiber officinale* and *Etlingera elatior* Plant Sample and Storage for DNA Isolation

Plant sample of *Zingiber* species and *Etlingera* species which are *Zingiber officinale* and *Etlingera elatior* was collected from around Kelantan, Malaysia using coordinate data by Sarmila (2018). Fresh and young leaves with a minimum of three replicates of *Zingiber* sp. and *Etlingera* sp. were collected and their surfaces were wiped with 70% of absolute ethanol. The plant samples were labeled and were kept in a clean A4 size zip lock bag. The plant samples were then stored -10℃ freezer in laboratory BAP 1.1, Universiti Malaysia Kelantan, Jeli Campus until further use.

3.4 DNA Extraction from *Zingiber officinale* and *Etlingera elatior* using CTAB Method

The DNA extraction from *Zingiber* sp. and *Etlingera* sp. was done following method by Devi *et al*. (2013). The prepared DNA extraction buffer for *Zingiber* sp. and *Etlingera* sp. containing 500 µL CTAB buffer for 100 mg of *Zingiber officinale* and *Etlingera elatior* samples was preheated in the water bath at 60℃ for about 15 minutes. The one gram of *Zingiber officinal*e and *Etlingera elatior* tissue was chopped into a paste using a clean single edge razor blade. The *Zingiber officinale* and *Etlingera elatior* leaf tissues were ground together with 1% PVP (Polyvinylpyrrolidone) using pestle and mortar. The ground leaf samples were transferred into 2 mL centrifuge tube and were incubated in a water bath at 60℃ for one hour. Then, the sample with the tubes was centrifuged at 12,000 rpm for 10 minutes and the clear supernatant was collected in 1.5 mL microcentrifuge tubes using a wide bored tip. The 1 mL supernatant was added to equal volume of chloroform and isoamyl alcohol (24:1) and was mixed by inversion method. Next, the tubes was centrifuged at 12,000 rpm for 10 minutes and the 1 mL supernatant was collected in 1.5 mL microcentrifuge tube. An equal volume of chloroform and isoamyl alcohol (24:1) was added to clear supernatant and was mixed by inversion and place back on ice for 3 min. The tubes with the mixture were centrifuge at 12,000 rpm for 10 minutes and 1 mL supernatant was collected. The supernatant was added 540 µL of ice cold isopropanol and was incubated in ice (-20℃) for 30 minutes. Then, the tubes were centrifuged at 10,000 rpm for 10 minutes and the pellet was collected. The pellet was washed with 500 µL 70% ethanol (HmBG) and was air dry in

room temperature (±27℃) for 20 minutes. The TE buffer was added 50 µL to the air dried DNA pellets and was stored at -20℃ for further use.

3.5 Quantification of Extracted Genomic DNA of *Zingiber officinale* and *Etlingera elatior*

The concentration and purity of the purified DNA were identified by measuring the absorbance value at 260nm and 280nm. The Nanodrop Spectrophotometer (Thermofisher, United States) was first blanked with 2 µL of TE buffer. A 1 µL of extracted DNA was pipetted onto the pedestal. Both absorbance readings at 260nm and 280nm were measured and recorded.

3.6 Agarose Gel Electrophoresis Analysis of Extracted *Zingiber officinale* and *Etlingera elatior* Genomic DNA

The agarose gel electrophoresis of extracted *Zingiber officinale* and *Etlingera elatior* was done following method by Appalasamy (2018). A 50x of Tris-acetate-ETDA (TAE) buffer stock was prepared in 1000 mL of distilled water. A sufficient electrophoresis buffer (normally 1x TAE) was prepared to fill the electrophoresis tank and cast the gel. A 1% of agarose solution was prepared in electrophoresis buffer. The one gram of agarose powder in 100 ml of 1x TAE buffer was mixed in a microwavable flask and was microwave for approximately 2 minutes. The 2.5 µL of RedSafe DNA Stain (Intron Biotechnology, South Korea) was added into the mixture once the molten

gel has cool down. The gel solution was mixed thoroughly by gentle swirling. The agarose was poured into a gel tray with the well comb in place. The warm agarose solution was poured into the mold and allowed it to sit at room temperature (±27℃) for about 30 minutes until it completely solidified.

The loading buffer was added to *Zingiber officinale* and *Etlingera elatior* DNA samples. The agarose gel was placed into the gel box once it solidified. The box was filled with 1x TAE until the gel is covered. The 1 µL of 1 kb DNA molecular weight marker (Promega, United States) was loaded into the first lane of the gel. The 5 µL of DNA of *Zingiber officinale* and *Etlingera elatior* was mixed with 1 µL of a 6x gel-loading buffer by using 10 µL micropipette. The samples were loaded into the well of submerged gel. The gel was run for 45 minutes with 90 V. The DNA fragments was examined by using UV illumination transilluminator (Appalasamy, 2018).

3.7 Polymerase Chain Reaction (PCR) with Internal Transcribed Spacer Region Specific Primers for *Zingiber officinale* and *Etlingera elatior* Identification

The Polymerase Chain Reaction (PCR) was done following the method that described by White *et al*. (1990). By using forward ITS1 and reverse ITS4 primers, the amplification of nuclear ITS (ITS 1, 5.8S, ITS 2) regions was accomplished following method reported by White *et al*. (1990). The ITS1 and ITS4 nucleotide sequences that were used in PCR were stated in Table 3.1.

The date and samples name were label on the PCR microtubes. The components that shown in Table 3.2 and Table 3.3 were mixed gently in a sterile 0.5 mL microcentrifuge tube. The PCR amplification was carried out in a total reaction volume of total 25 µL of 25 ng DNA samples of *Zingiber officinale* and *Etlingera elatior*.

The Table 3.4 and 3.5 are showing the conditions and temperature profile for the process in PCR for *Zingiber officinale* and *Etlingera elatior*. The Table 3.4 and 3.5 are showing forty five cycles was repeated for Process V, IV and VI followed method by White *et al*. (1990).

Table 3.1 Primer Sequences for (PCR) of *Zingiber officinale* and *Etlingera elatior*

Name of primer	Primer sequence	References
ITS1 F	5'-TCCGTAGGTGAACCTTGCGG -3'	White *et al.*, (1990)
ITS4 R	5'-TCCTCCGCTTATTGATATGC-3'	

Table 3.2 Component for PCR in 0.5 mL Microcentrifuge Tubes for *Zingiber officinale*

Component	25 µL Reaction	Final Concentration
5X Green GoTaq@ Flexi Buffer	2.5 µL	1X
$MgCl_2$ Solution, 25 mM	2.0 µL	2.5 mM
PCR Nucleotide Mix (dNTP), 10 mM	0.5 µL	0.2 mM
ddH_2O	18.3 µL	-
Forward Primer, 10 µM	0.4 µL	0.5 µM
Reverse Primer, 10 µM	0.4 µL	0.5 µM
GoTaq@ DNA Polymerase (5 u/µL)	0.5 µL	1.5 unit
Template DNA	0.4 µL	25 ng

Table 3.3 Component for PCR in 0.5 mL Microcentrifuge Tubes for *Etlingera elatior*

Component	25 µL Reaction	Final Concentration
5X Green GoTaq@ Flexi Buffer	2.5 µL	1X
$MgCl_2$ Solution, 25 mM	2.5 µL	2.5 mM
PCR Nucleotide Mix (dNTP), 10 mM	0.5 µL	0.2 mM
ddH_2O	16 µL	-
Forward Primer, 10 µM	0.5 µL	0.5 µM
Reverse Primer, 10 µM	0.5 µL	0.5 µM
GoTaq@ DNA Polymerase (5 u/µL)	0.5 µL	1.5 unit
Template DNA	2.0 µL	25 ng

Table 3.4 Process, Temperature Profile and Conditions of PCR of Primer ITS1 Forward and ITS4 Reverse for *Zingiber officinale*

No	Process	Conditions	Cycles
I	Pre-denaturation	94 ℃ for 4 minute	1
II	Denaturation	94 ℃ for 1 minute	45
III	Annealing	58.8 ℃ for 1 minute	45
IV	Polymerisation	72 ℃ for 2 minute	45
V	Final extension	72 ℃ for 4 minute	1

Table 3.5 Process, Temperature Profile and Conditions of PCR of Primer ITS1 Forward and ITS4 Reverse for *Etlingera elatior*

No	Process	Conditions	Cycles
I	Pre-denaturation	94 ℃ for 4 minute	1
II	Denaturation	94 ℃ for 1 minute	45
III	Annealing	58.9 ℃ for 1 minute	45
IV	Polymerisation	72 ℃ for 2 minute	45
V	Final extension	72 ℃ for 4 minute	1

3.7.1 Agarose Gel Electrophoresis Analysis Amplified Internal Transcribed Spacer (ITS) Region for *Zingiber officinale* and *Etlingera elatior*

The 1% Agarose gel was done prepared following the method by Appalasamy (2018) as was detailed written in section 3.5. The agarose gel electrophoresis analysis was following method Manokar *et al*. (2017). A 5 µL of PCR amplicons was stained with 0.1 µL 5X Green GoTaq$^{@}$ Flexi Buffer and was resolved on 1% agarose gel and 1x TAE buffer gels. Simultaneously, 1 Kb ladder (Promega, United States) was loaded to identify the size of the PCR amplicons. The gel was examined by Ultraviolet illumination transilluminator at research laboratory Faculty of Earth Science, Universiti Malaysia Kelantan, Jeli Campus.

3.8 DNA Sequencing of *Zingiber officinale* and *Etlingera elatior* and BLAST Analysis

The sequencing was performed by the commercial company, First Base Sdn. Bhd. The sequence for the PCR products from section 3.6 was obtained in FASTA format and was used to Basic Local Alignment Tool (BLAST) again with the ITS sequences in National Center for Biotechnology Information (NCBI) database. The phylogenetic tree for both genera was constructed using MEGA 7 software at https://www.megasoftware.net/ using the neighbor-joining method.

CHAPTER 4

RESULT AND DISCUSSION

4.1 Quantification of Extracted Genomic DNA that Isolated from *Zingiber officinale* and *Etlingera elatior*

Table 4.1 shows the reading of optical density for the six DNA extractions of *Zingiber officinale* which is collected around Kelantan, Malaysia. The purity for all the six genomic DNA samples of *Zingiber officinale* and one genomic DNA sample of *Etlingera elatior* were in the range of 1.17 to 1.89.

The lowest purity of extracted genomic DNA from *Zingiber officinale* leaves was 1.17 which the value is low compared to expected ratio ~1.80 of A260nm/280nm is generally accepted purity for DNA. The low 260nm/280nm ratio of extracted DNA could indicate the contamination in the DNA samples of *Zingiber officinale* and *Etlingera elatior*. For instance, the presence of residual guanine, phenol, protein in the DNA sample or the contamination on reagents that were used in the extraction protocol for *Zingiber officinale* and *Etlingera elatior* which result in reducing the 260/280 ratio. However, the highest purity ratio of extracted DNA of *Zingiber officinale* was 1.89 which the 260nm/280nm ratio is not higher than 2.0 that shows the RNA contamination in extracted DNA (Wilfinger, William & Karol, 1997).

Furthermore, the DNA extraction of *Etlingera elatior* showed the purity ratio of 1.56 which the 260nm/280nm ratio does not exceed 2.0. Normally, the RNA contamination in extracted DNA showed the result of 260nm/280nm reading exceed 2.0 (Wilfinger, William & Karol, 1997).

To detect the contamination of polysaccharide in extracted DNA, A260/A230 ratio is used because usually polysaccharides absorb at the wavelength of 230 nm. This step is important as the contamination in extracted DNA can influence in PCR process (Linacero, Rueda & Vázquez, 1998).

In assessing the purity of the extracted genomic DNA of *Zingiber officinale*, the six DNA samples of extracted *Zingiber officinale* and one sample of extracted *Etlingera elatior* has been measured by using ThermoFisher NanoDrop™ 2000c Spectrophotometers with the wavelength of 260nm. A 260 nm and 280 nm are the ratio of absorbance used in assessing the purity of DNA and RNA. Generally for DNA at ~1.80 of A 260/280 ratio is normally produce pure nucleic acids and ~2.0 A 260/280 of RNA ratio (Desjardins & Deborah, 2010).

To assess protein contamination in extracted DNA, the A260/A280 ratio was used because absorbance maximum of protein is at 280 nm (Page, Andrew & Monica, 2005). The commonly poor ratio shows a value less than 1.6 while 1.8 ratios is considered good. Protein absorption is mainly the result of the aromatic amino acids phenylalanine, tyrosine, and tryptophan. Contamination with organic solvents or protein will obviously lower this value and will prevent accurate nucleic acid quantification from the OD260 reading (Coleman & Tsongalis, 2006).

Table 4.1: Optical Density of DNA Extraction of *Zingiber officinale* and *Etlingera elatior* Around Kelantan, Malaysia

No.	DNA Extraction	Concentration (ng/µL)	A260	Purity (260/280)
1.	UMKSZO1_DNA	46.117	0.9223	1.66
2.	UMKSZO2_DNA	29.990	0.5998	1.17
3.	UMKSZO3_DNA	42.728	0.8546	1.80
4.	UMKSZO4_DNA	549.959	10.9992	1.86
5.	UMKSZ05_DNA	125.250	2.5050	1.89
6.	UMKSZO6_DNA	177.731	3.5546	1.85
7.	UMKEE01_DNA	54.222	1.0844	1.56

4.2 Agarose Gel Electrophoresis Analysis of Extracted *Zingiber officinale* and *Etlingera elatior* Genomic DNA

The DNA extraction method described by Devi *et al*. (2013) with some modifications made yielded high quality genomic DNA from *Zingiber officinale* dry leaves. The extracted DNA for *Zingiber officinale* has been analyzed and the presence of DNA was examined by agarose gel electrophoresis followed by the method of Appalasamy (2018). The DNA was loaded into 1% of agarose gel and been run for 45 minutes with 80 V.

All six extracted genomic DNA for *Zingiber officinale* and one extracted genomic DNA for *Etlingera elatior* were loaded into the wells. An amount of 5 µL of each seven extracted DNA samples were tested in agarose gel electrophoresis. The DNA band appeared on the gel after the power to the gel box is turned on. The DNA are negatively charged as it molecules contain phosphate group and it will move toward the positively charged. These steps are important to determine the size of the DNA fragment for the extracted genomic DNA. A 1kb DNA marker was loaded into the well to determine the size range for expected DNA fragment of *Zingiber officinale* and *Etlingera elatior*. A 10,000 kb to 250 bp is the size range ladder for 1kb DNA marker (Promega, United States).

Figure 4.1 showed the agarose gel electrophoresis result for all six extractions from genomic DNA samples of *Zingiber officinale*. All lane in Figure 4.1 showed the smearing of *Zingiber officinale* DNA from line 1 to line 6 between the expected base pair value and does not contain visible bands. Figure 4.2 showed the agarose gel

electrophoresis analysis for *Etlingera elatior*. Smearing of genomic DNA on agarose gel electrophoresis could be caused by the overloading of extracted DNA in the agarose gel electrophoresis well and the gel was run at high voltage (Biotium, 2018). The pore of the matrix will be blocked due to high concentration of protein. This resulted in smearing as the proteins bleed slowly into the gel instead of entering as a compact band. In addition, other factors that caused the smearing of DNA occurred due to the poor quality product as contamination of protein and high salt concentration in the DNA sample. Furthermore, degradation of DNA sample resulted in smearing to occur during gel electrophoresis (Melissa, 2018).

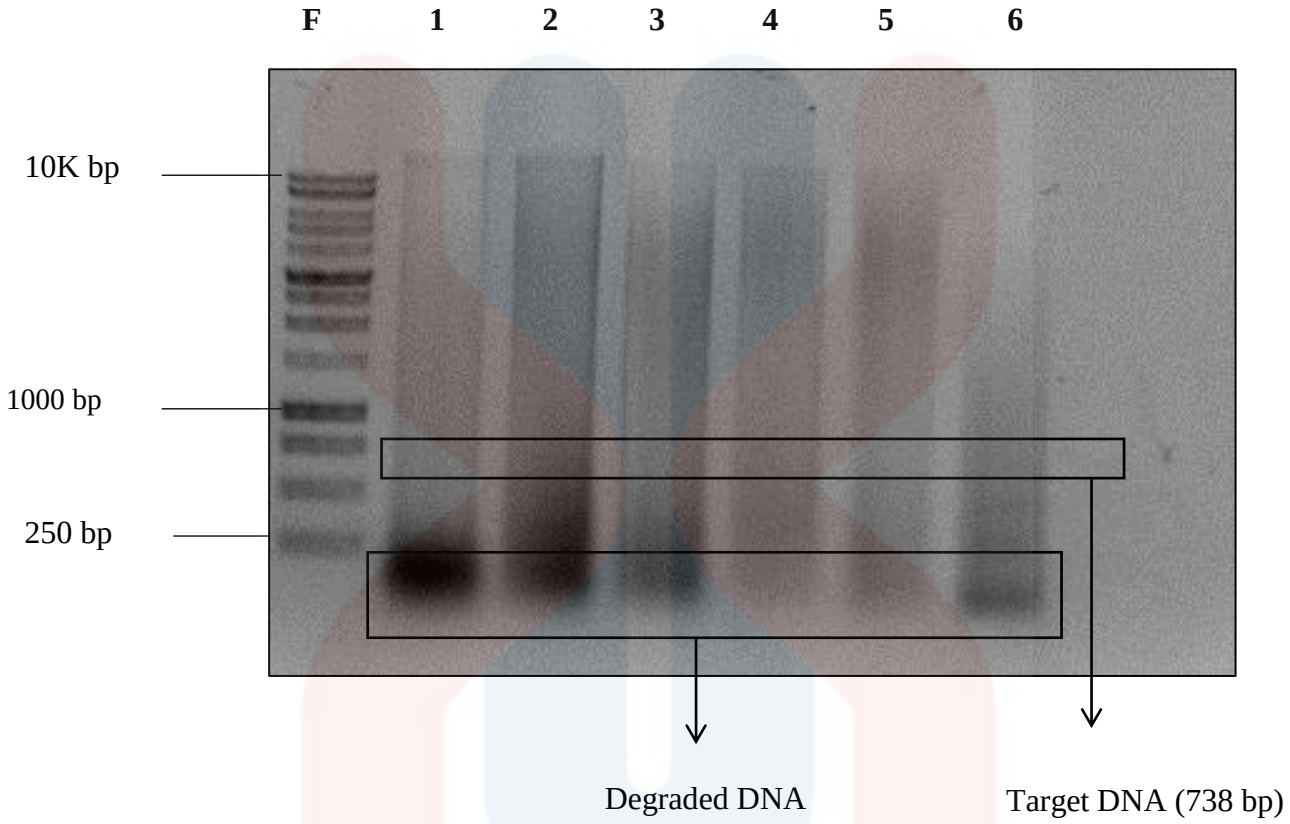

Figure 4.1: Gel electrophoresis of DNA extraction from six sample leaves of *Zingiber officinale*. The figure shows that there was DNA smearing appear in Lane 1, 2, 3, 4, 5 and Lane 6 after being visualized under UV transilluminator of 1% agarose gel at 80V for 40 minutes. Lane 1: UMKSZO6_DNA, Lane 2: UMKSZO5_DNA, Lane 3: UMKSZO4_DNA, Lane 4: UMKSZO3_DNA, Lane 5: UMKSZO2_DNA and Lane 6: UMKSZO1_DNA. Lane F are 1kb DNA marker (Promega, United States).

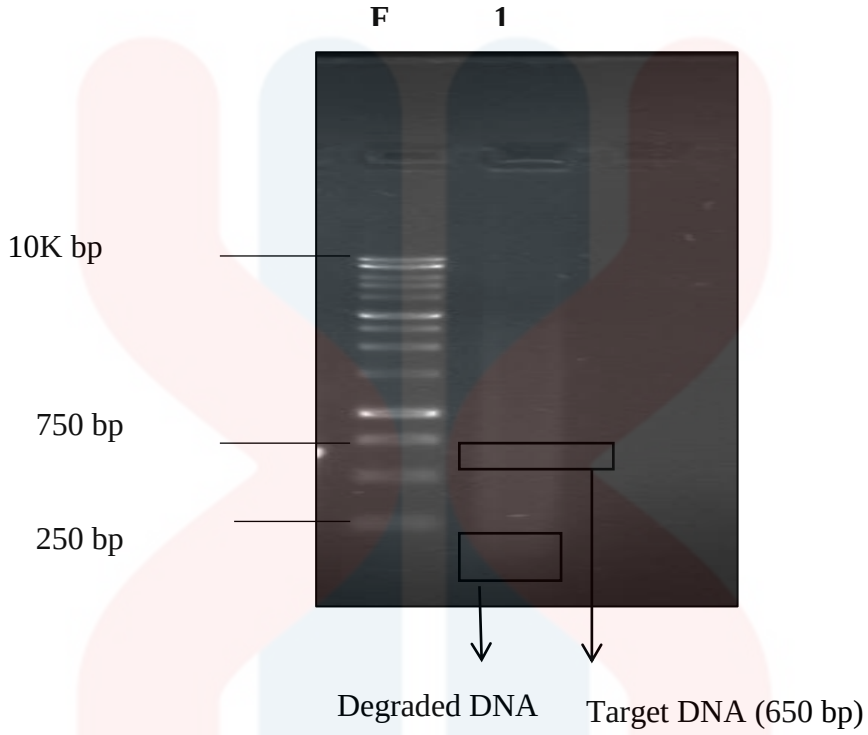

Figure 4.2: Gel Electrophoresis of DNA Extraction of *Etlingera elatior*. The figure shows at lane 1 DNA smearing appear after being visualized under UV transilluminator of 1% agarose gel at 80V for 40 minutes. Lane 1: UMKSEE01_DNA and Lane F is 1kb DNA marker (Promega, United States).

4.3 Polymerase Chain Reaction (PCR) with Internal Transcribed Spacer Region Specific Primers for *Zingiber officinale* and *Etlingera elatior* Identification

4.3.1 Optimization of Polymerase Chain Reaction (PCR)

DNA extraction of *Zingiber officinale* and *Etlingera elatior* were prepared for amplification of ITS regions. The PCR specificity depends only on the gene specific primer. Gene specific primers that are found to be attached with non-specific amplification products are called 'off-target'

The specificity of the PCR in these applications depends solely on the gene-specific primer. Accordingly, they are often plagued with non-specific amplification products derived from so-called 'off-target' (Miura, Uematsu & Sakaki, 2005). Annealing temperature (T_a) is one of the test variables in optimizing the Polymerase Chain Reaction (PCR) (Rychlik, Spencer & Rhoads, 1990). Optimization to obtain the best annealing temperature is crucial so that the primers would anneal at correct region and relatively yield better results of DNA synthesis (Rychlik, Spencer & Rhoads, 1990).

DNA extraction from *Zingiber officinale* and *Etlingera elatior* leaves were used in the PCR optimization. Gradient PCR was performed for both *Zingiber officinale* and *Etlingera elatior* DNA samples. The amount of synthesized DNA from *Zingiber officinale* and *Etlingera elatior* were determined by agarose gel electrophoresis (AGE). AGE was being used to check the success and integrity of the PCR product from *Zingiber officinale* and *Eltingera elatior* DNA samples (Rychlik, Spencer & Rhoads, 1990). Figure 4.3 showed the agarose gel electrophoresis of gradient PCR of *Zingiber*

officinale DNA and Figure 4.4 showed the agarose gel analysis for gradient PCR of *Etlingera elatior* DNA.

Figure 4.3 showed the optimization of gradient PCR of *Zingiber officinale* with the temperature gradient of PCR 52°C to 62°C. Line F is 1kb DNA ladder loaded into the well. Lanes 1 to 6 showed DNA bands at the temperature between 52°C to 61.3°C. At 58.8°C, amplification product can be seen without the presence of primer dimers which indicate the temperature is suitable and specific enough as for optimum annealing primers for ITS1 and ITS4 regions amplification.

At lane 11 and 12 there are no bands observed and only smearing occurred which indicate that the optimum temperature should not exceed more than 61.9°C. Amplification was not detected for samples loaded at lane 11 (61.9°C) and lane 12 (62°C) which could be caused by several factors. This could be occurred due to high annealing temperature which disabled the primers to bind to the DNA template. In addition, the contamination in distilled water used during the PCR master mix preparation or the annealing temperature is too high could also contribute to the absence of amplification in the DNA sample (Bio-Rad Laboratories,2018). All of the PCR products for *Zingiber officinale* DNA showed low molecular weight smearing which could be due to a few factors. The smearing could happen due to a low or high temperature of annealing or denaturation, cycling times and PCR components (Bio-Rad Laboratories, 2018)

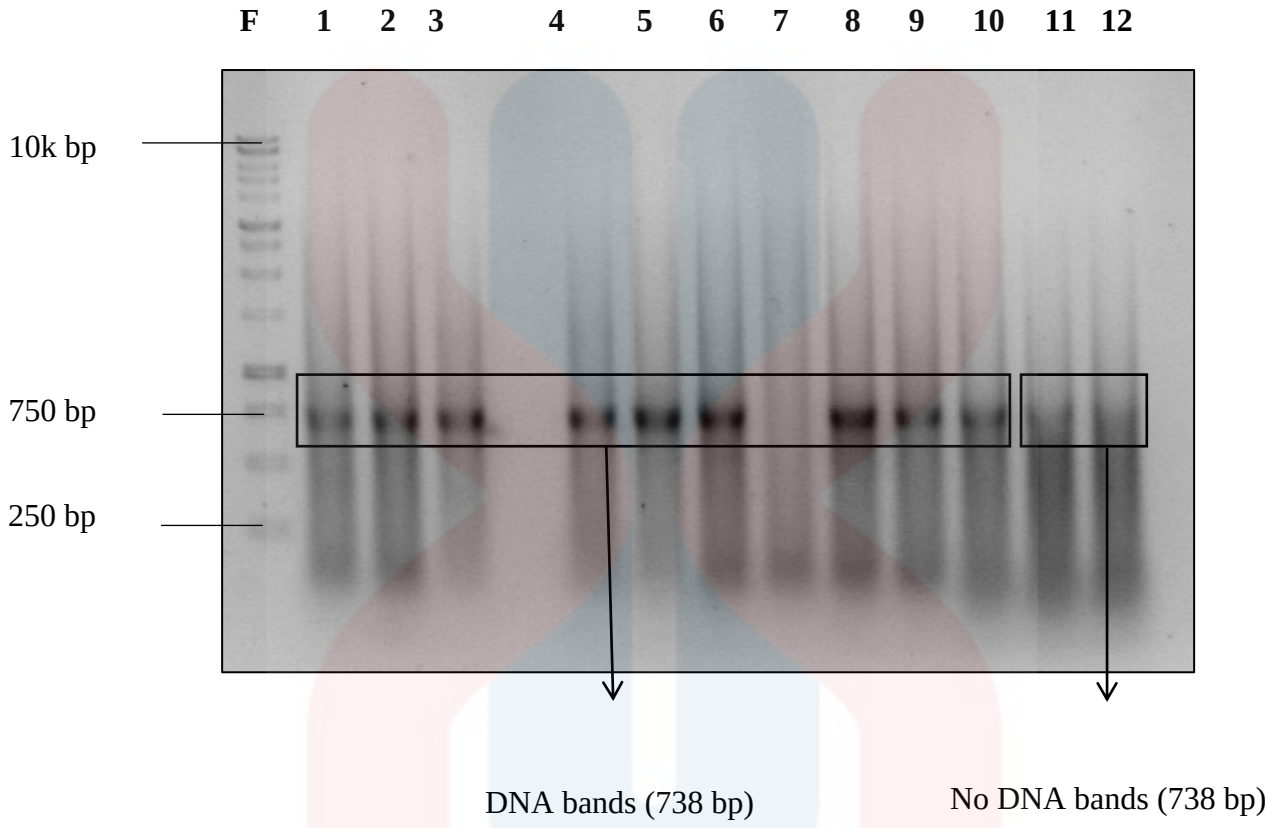

Figure 4.3: Optimization of PCR conditions of *Zingiber officinale* DNA samples with gradient temperature of PCR at 52 to 62°C. Lane F was 1kb DNA ladder; Lane 1 to 12 contained PCR products and electrophoresed on 1.2% agarose gel at 80 volts for 45 minutes. Lane 1: 52.0°C, Lane 2: 52.3°C, Lane 3: 52.9°C, Lane 4: 53.9°C, Lane 5: 55.3°C, Lane 6: 56.4 °C, Lane 7: 57.7°C, Lane 8: 58.8°C, Lane 9: 60.3°C, Lane 10: 61.3°C, Lane 11: 61.9°C, Lane 12: 62.0°C and Lane F: 1kb DNA ladder (Promega,United States).

Figure 4.4 showed the optimization of gradient PCR of *Etlingera elatior* with a temperature gradient of PCR 52°C to 62°C. The primer dimers were observed at lanes 1 to 7 at the PCR products on agarose gel electrophoresis. The primer dimers or also known as nonspecific bands occurred due to the low annealing temperature (Ta) which

caused the primers to bind nonspecifically to the DNA template. In addition, a high concentration of primers used during the PCR master mix preparation could also lead to primer dimers formation. Percentage of primers binding to nonspecific sites on the DNA template increases if using a high concentration of primer in PCR reactions (Bio-Rad Laboratories, 2018).

Lanes 10 and 11 showed absence of primer dimers and only low molecular weight smears were observed at the PCR product on the agarose gel electrophoresis. This may occurred due to a few factors such as longer extension time, primer temperature (Tm) was inaccurate and excess DNA template added to the PCR preparation (Bio-Rad Laboratories, 2018). Lane 8 (58.9°C) showed the correct amplicon band (650 bp) and there was absence of primer dimers observed which indicated the temperature used was suitable and specific enough for optimum annealing temperature for ITS1 and ITS4 amplification in *Etlingera elatior*.

Optimizing the annealing temperature (Ta) in the amplification of DNA regions is important to ensure the success and increase the specificity of a PCR product (Roux, 2003). The calculated temperature for primer melting point Kelantan in the PCR usually started at 5°C below (Prezioso & Jahns, 2000). If the annealing temperature (Ta) is too low, the multiple bands on the agarose gel would appear due to the amplification of non-specific DNA fragments. Furthermore, high annealing temperature (Ta) used could lead to poor annealing of primer and sometimes the purity of the PCR product is reduced and the yield of the desired product also reduced (Rychlik, Spencer & Rhoads, 1990).

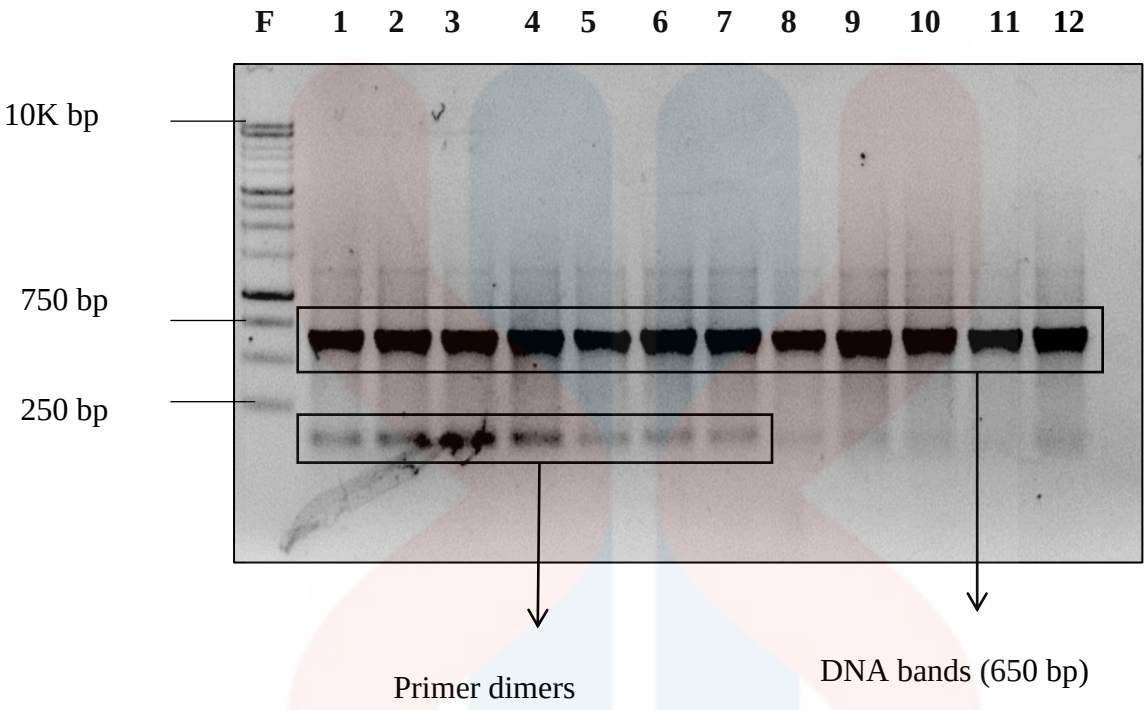

Figure 4.4: Optimization of PCR conditions of *Etlingera elatior* with gradient temperature of PCR at 52 to 62°C. Lane F was 1kb DNA ladder; Lane 1 to 12 contained PCR products and electrophoresed on 1.2% agarose gel at 80 volt for 45 minutes. Lane 1: 52.0°C, Lane 2: 52.3°C, Lane 3: 52.9°C, Lane 4: 53.8°C, Lane 5: 55.1°C, Lane 6: 56.4 °C, Lane 7: 57.6°C, Lane 8: 58.9°C, Lane 9: 60.2°C, Lane 10: 61.1°C, Lane 11: 61.7°C, Lane 12: 62.0°C and Lane F: 1kb DNA ladder (Promega, United States).

In addition, the other components used in PCR were adjusted to optimize the concentration. For examples are the concentration of primers, Mg^{2+}, DNA template and dNTPs (Miura, Uematsu & Sakaki, 2005). For instance, the decrease in the concentration of free Mg++ available will increase the concentration of dNTPs which result in influencing the polymerase function (Roux, 2003). Moreover, multiple

undefined and unwanted products will be produced in un optimum conditions and no PCR product will be produced at extreme conditions (Roux, 2003).

Figure 4.5 showed the PCR product for *Zingiber officinale* used a different concentration of primer, Mg^{2+} and template of DNA with a fixed temperature of 58.8°C. Total 45 cycles for *Zingiber officinale* extracted genomic DNA was conducted for denaturation, annealing, polymerization and extension with fixed temperature (58.8°C).

Two different concentrations from *Zingiber officinale* DNA template which were 0.2 µL and 0.4 µL with the total DNA concentration of 25 ng were used for optimization. From Figure 4.5, 0.2 µL of DNA template was used at lanes 1 and 2. At lanes 3 and 4, 0.4 µL DNA template was used. The presence of primer dimers can be seen appeared at lanes 3 and 4 of the amplification products which indicated nonspecific products have occurred. Meanwhile, lanes 1 and 2 showed an absence of primer dimers at low concentration of amplification products. The amplicon specificity will decrease as the DNA concentration is higher particularly when running at a high number of PCR cycles (BioLabs, 2018). Genomic DNA with higher complexity templates was suggested at 50 µL reaction only used one ng to 100 ng of DNA concentration per PCR reaction (PrimerDigital, 2018). The optimization of the quantity DNA is important as the relevant unit for successful PCR are the number of molecules besides the quality of the template DNA from the optical density measurement (Todd, 2012).

Optimization of PCR was continued by adjusted two PCR components which were DNA template and concentration of $Mgcl_2$. Lanes 5 and 6 showed high molecular weight smearing. The smearing could be occurred due to the high concentration of DNA

template used in the PCR product. In addition, the high concentration of Mg2+ will increase the yield of PCR product and could also contribute to decreasing the DNA polymerase fidelity and specificity. Furthermore, specificity resulting in undesired PCR products decrease and can stabilize annealing primer in the presence of too much Mg2+ (Todd, 2012).

Next, the optimization of PCR for *Zingiber officinale* by adjusted the concentration for ITS1 forward and ITS4 reverse primers. From Figure 4.5, primer dimers were observed at lanes 7 and 8 at the PCR product on agarose gel electrophoresis. Non-specific products are the primer dimers which occurred when the template-independent primer interaction take place (Brownie, 1997). Moreover, this primer dimers occurred due to other primers in the PCR reaction were anneal or self-anneal to the other primers (Todd, 2012). In addition, the spurious amplification products were observed when the concentration of primer is higher and lead in increasing the secondary priming (PrimerDigital, 2018).

At lanes 9, 10, 11 and 12 showed there were smears occurred can be observed at the PCR products on the agarose gel electrophoresis in Figure 4.5. Furthermore, there were an absence of primer dimers at lanes 9, 10, 11 and 12 of the PCR products on agarose gel electrophoresis. The optimization was continued by adjusted the concentration of $MgCl_2$ and another concentration of three PCR components which were DNA template, primer concentration and $MgCl_2$ concentration.

The PCR optimization was continued by adjusted the concentration of $MgCl_2$ for *Zingiber officinale* as can be seen at lanes 9 and 10. The DNA template, both

forward and reverse primers with $MgCl_2$ concentrations were adjusted at lanes 11 and 12 on the agarose gel electrophoresis. The accumulation of non-specific amplification products cause by the excess amount of magnesium that regarded as multiple bands on the agarose gel, while inadequate magnesium causes the productivity of the desired PCR product reduced (Grunenwald, 2003).

Therefore, lane 11 was showed the correct amplicon b and (738 bp) as there were an absence of primer dimers and smears can be observed on the agarose gel electrophoresis which indicate the PCR components used were suitable and specific enough for ITS1 and ITS4 amplification for *Zingiber officinale*.

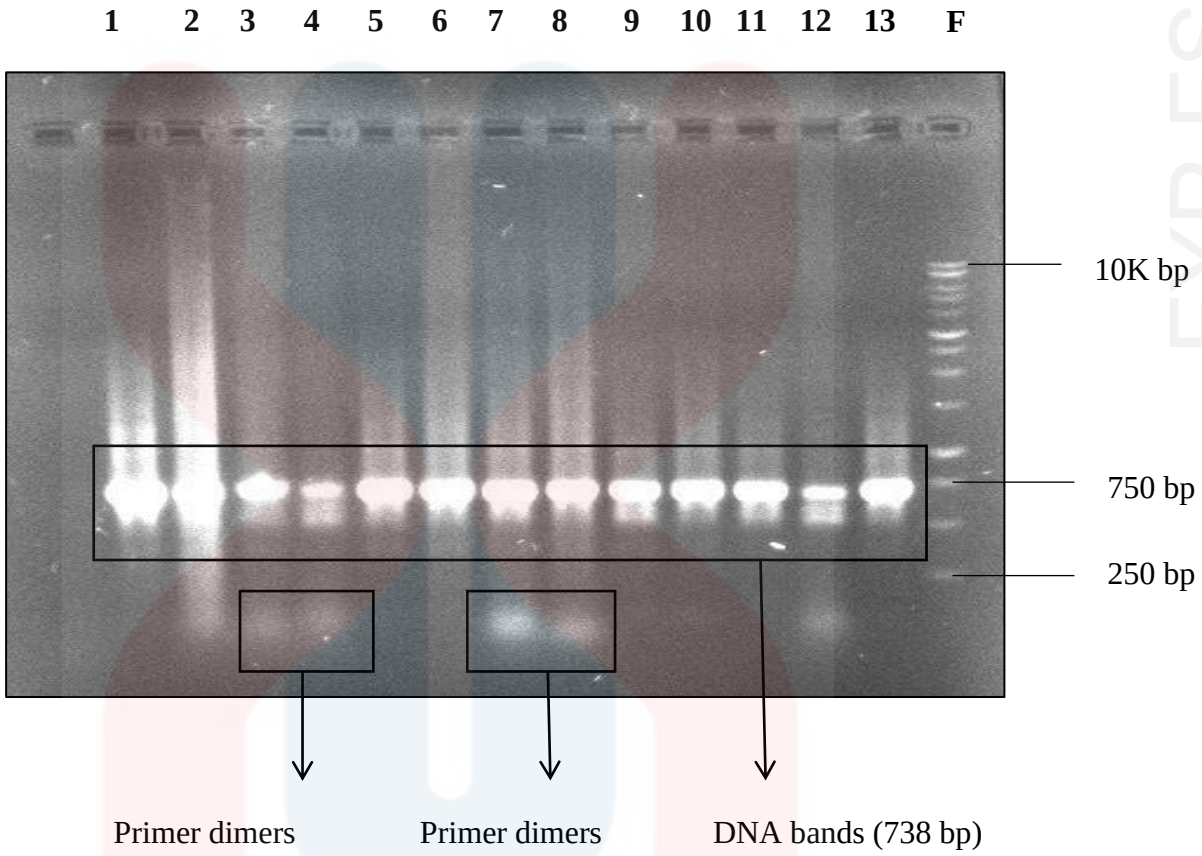

Figure 4.5: Optimization of PCR components for *Zingiber officinale* by applying 45 cycles in the denaturation, annealing, polymerization and extension processes respectively with different concentration of $Mgcl_2$, primers and DNA template. Lane F was 1kb DNA ladder (Promega, United States). Lane 1 to Lane 13 contained 5µL of PCR products being electrophoresed on 1.2% agarose gel at 80 volt for 45 minutes.

4.3.2 Amplification of ITS (ITS1, 5S, ITS2) Region of *Zingiber officinale* and *Etlingera elatior*

In this study, two sets of primers were used for PCR amplification at nuclear ITS regions. The accuracy of the amplified PCR product for genomic DNA sequences are done by using the National Center for Biotechnology Information (NCBI) Primer Basic Local Alignment Search Tool (BLAST).

Both of the samples of *Zingiber officinale* and *Etlingera elatior* were amplified using nuclear ITS regions which are ITS1 forward and ITS4 reverse primers as reported by White *et al*. (1990).

For *Zingiber officinale* the ITS1 and ITS4 primers were used to amplify the ITS region at annealing temperature (Ta) 58.8°C. The optimized temperature is the result of the gradient PCR which done by using a PCR machine. Figure 4.6 showed the outcomes of the PCR amplification for *Zingiber officinale* DNA extraction with the annealing temperature of 58.8°C.

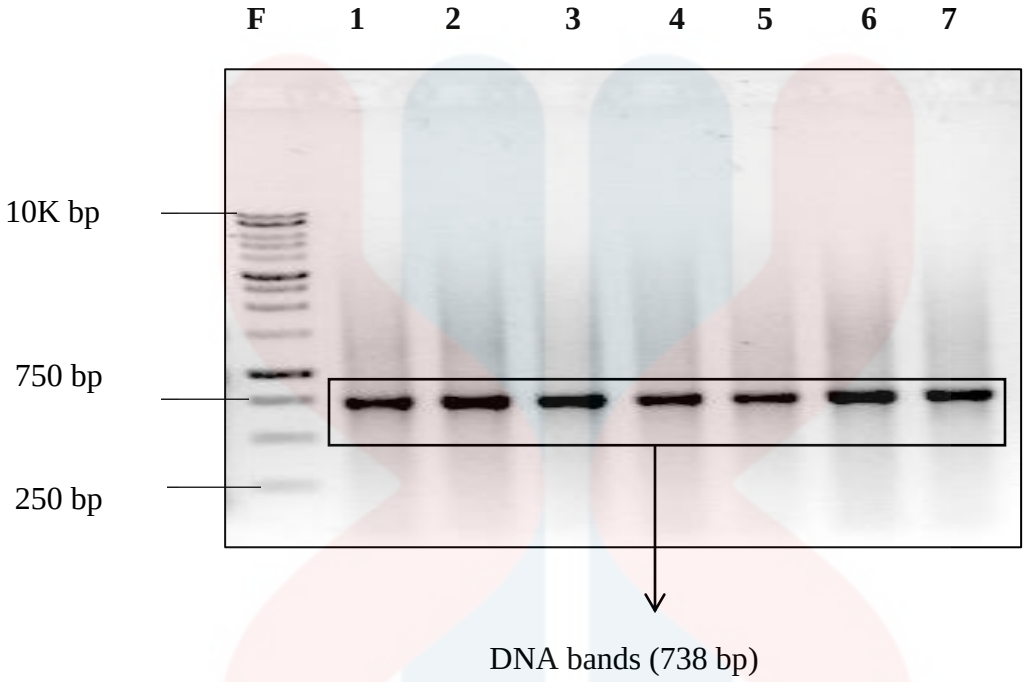

Figure 4.6: Amplification of ITS region using ITS1 and ITS4 primers for *Zingiber officinale*. Lane 1 to Lane 7 contained 5 µL of PCR products of *Zingiber officinale* was electrophoresed on 1.2% agarose gel at 80 volts for 45 minutes. Lane F: 1kb DNA ladder (Promega, United States).

DNA from *Etlingera elatior* was amplified using ITS1 forward and ITS4 reverse primers at 58.9°C. The optimized temperature was the result of the gradient PCR which done by using a PCR machine. The gradient for *Zingiber officinale* and *Etlingera elatior* showed minor differences which *Etlingera elatior* showed high annealing temperature compared to *Zingiber officinale*. Figure 4.7 showed the outcomes of the PCR amplification for *Etlingera elatior* DNA extraction with the annealing temperature of 58.9°C.

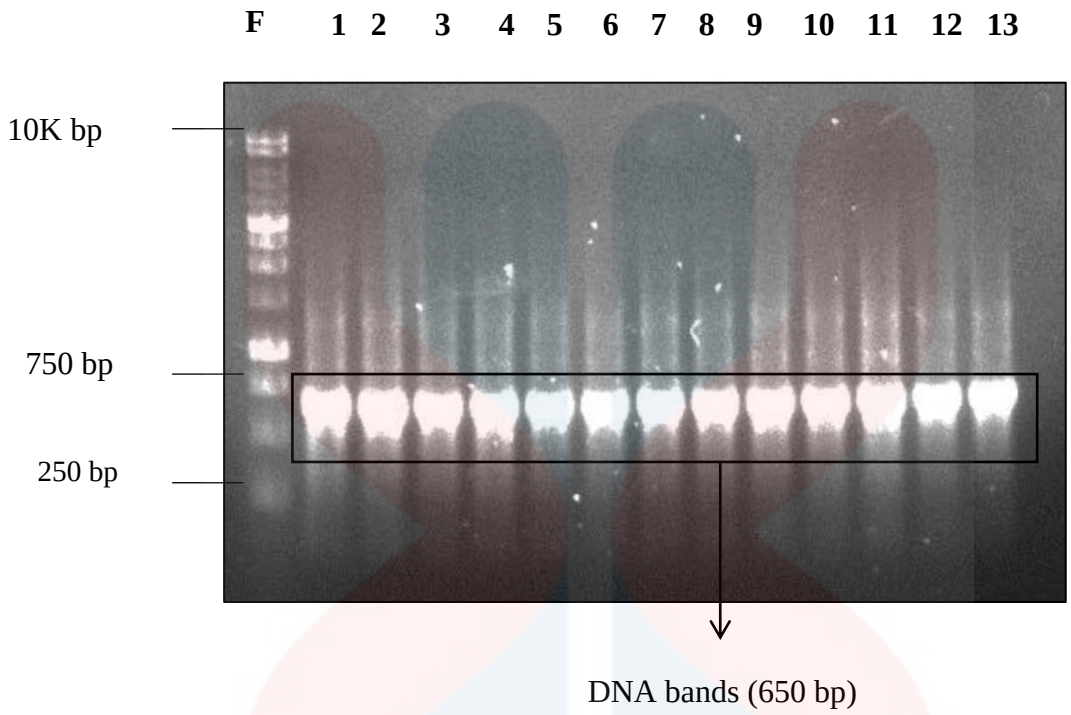

Figure 4.7: Amplification of ITS region using ITS1 and ITS4 primers for *Etlingera elatior*. Lane 1 to Lane 13 contained 5 µl of PCR products of *Etlingera elatior* was electrophoresed on 1.2% agarose gel at 80 volts for 45 minutes. Lane F: 1kb DNA ladder (Promega, United States).

4.4 DNA Sequencing of *Zingiber officinale* and *Etlingera elatior* and BLAST Analysis

Both unpurified PCR samples of *Zingiber officinale* and *Etlingera elatior* were sent for sequencing at First Base DNA Sequencing Service, Malaysia. Basically, the results of the sequencing run showed four different colors of the chromatogram. The chromatogram analysis revealed the quality of sequencing. Chromatography is the biophysical technique that important to enables the identification, purification, and separation for quantitative and qualitative analysis of the components of a mixture (Ozlem, 2016).

Both forward and reverse sequencing of *Zingiber officinale* and *Etlingera elatior* contained the baseline noise at the chromatogram. For *Zingiber officinale*, the chromatogram contained baseline noise at the beginning until the end of the sequences. This could be occurred due to residual of primer dimers in PCR products lead the primers amplification during the reaction (Katia & Jennifer, 2018). The chromatogram for *Etlingera elatior* contained minimal baseline noise at the center of the sequences with only one color and only contained high baseline noise at the front and back of the sequences. Hence, it showed good sequencing result for *Etlingera elatior* (APPENDIX D).

In addition, there is the base caller or usually indicate by sign N at the only front of *Etlingera elatior* sequences which was absent for *Zingiber officinale* chromatogram. Factors the chromatogram having high baseline noise due to the low sample concentration (BioMedical, 2018). The contamination present in the sample such as

detergent, residual primers and proteins could also influence the results of the chromatogram (Katia & Jennifer, 2018).

All organisms on earth are genetically related as the evidence from biochemical, gene sequence data and morphological and by vast phylogenetic trees, the genealogical relationships can be shown (Biro, 2015). In constructing a phylogenetic tree, there are four methods can be used which are Unweighted Pair Group Method with Arithmetic Mean (UPGMA), Maximum Parsimony (MP), Neighbour-Joining (NJ) and Maximum Likelihood (ML) (Biro, 2015).

By comparing with *Zingiber* species and *Etlingera* species sequences available in the NCBI GenBank, the sequences boundaries of ITS1, 5.8S and ITS2 of *Zingiber officinale* and *Etlingera elatior* were determined. Then, the alignment of the sequences for both *Zingiber officinale* and *Etlingera elatior* were done using ClustalW and used in constructing the phylogenetic tree using MEGA 7 software.

The hierarchical clustering of the alignments of ITS1, 5.8S rDNA and ITS2 were used for constructing the phylogenetic trees and produced by the neighbor-joining method and the bootstrap value based on 100 replicates were presented at the above the branches. The neighbor-joining method is a method which is used for reconstructing a phylogenetic tree from evolutionary distance data (Saituo & Nei, 1987). The advantages of using neighbor-joining compare to others method are it suitable for data sets with large varying rates of evolution and allowing making a correction for multiple substitutions (Saitou & Nei, 1987; Kuhner & Felsenstein, 1994).

For *Zingiber officinale*, the sequences obtained in FASTA format were used to compare with other *Zingiber* sequences in the NCBI Genbank database. The *Zingiber officinale* sequences showed 91% identity with the ITS sequences of *Zingiber officinale* (Genbank number: KJ872253.1) in the Genbank. Meanwhile, sequences obtained for *Etlingera elatior* were used to compare with other *Etlingera* sequences in the NCBI Genbank database. The *Etlingera elatior* sequences showed 99% identity with the ITS sequences of *Etlingera elatior* (Genbank number: AB097230.1). The results indicated that identification of species will show variations if using different methods (Wu *et al*., 2013). Table 4.2 showing the closest match for isolated DNA of *Zingiber officinale* and *Etlingera elatior* from the nucleotide BLAST database for species identification by comparing the percentage of similarities.

Table 4.2: The Identification of Isolated DNA of *Zingiber officinale* and *Etlingera elatior* with Closest Match by Comparing the Similar Percentage

DNA samples	Closest match from nucleotide BLAST	Accession number	Similarity %
UMKSZO4_DNA	*Zingiber officinale*	KJ872253.1	91%
UMKEE1_DNA	*Etlingera elatior*	AB097230.1	99%

The ITS sequences of four Species of *Zingiber* available from NCBI GenBank (*Zingiber officinale*, *Zingiber zerumbet*, *Zingiber wrayi* and *Zingiber pellitum*) were used to performing phylogenetic analysis. As for outgroup in this analysis, *Kaempferia*

parviflora and *Kaempferia elegans* used due to the genus is one node below the *Zingiber* (Kress *et al*., 2012). The group comprising the genus *Zingiber* which including *Zingiber officinale, Zingiber zerumbet, Zingiber wyari, Zingiber pellitum* is strongly supported as monophylogenetic with bootstrap value 100% sister to isolated *Zingiber officinale* (UMKSZ04_DNA).

The *Zingiber* species and outgroups data with sequences accession number from NCBI Genbank database were listed in Table 4.3. Figure 4.8 showing the phylogenetic tree of *Zingiber* sp. based on neighbor-joining method using the Internal Transcribed Spacer (ITS) data.

Table 4.3: The *Zingiber* Species and Outgroups Data with Sequences Accession Number from NCBI Genbank Database

No.	Species	ITS length (bp)	Genbank accession number (ITS)
1.	*Zingiber wrayi*	603	HM2386155
2.	*Zingiber wrayi*	673	AF478802
3.	*Zingiber wrayi*	605	DQ064583
4.	*Zingiber officinale*	654	KC582873
5.	*Zingiber officinale*	662	KJ872250
6.	*Zingiber officinale*	665	KJ872253
7.	*Zingiber pellitum*	603	DQ064574
8.	*Zingiber zerumbet*	621	KU215128

9.	*Zingiber zerumbet*	645	KJ872295
10.	*Kaempferia parviflora*	399	KU958987
11.	*Kaempferia elegans*	701	KJ872200
12.	UMKSZO4_DNA	647	NR3334616

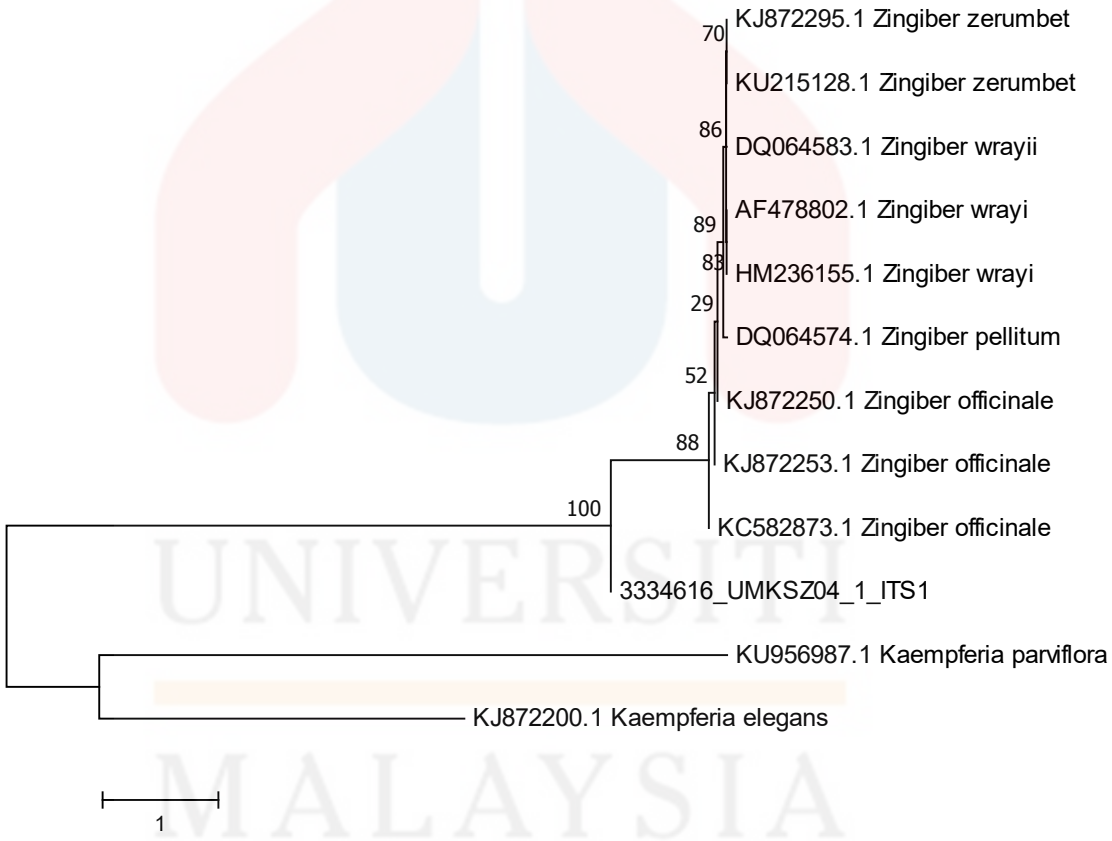

Figure 4.8: The phylogenetic tree of *Zingiber* sp. based on neighbor-joining method using the Internal Transcribed Spacer (ITS) data.

Zingiber officinale or commonly known as ginger often consumed worldwide for the medical and culinary purpose (Mbaveng & Kuete, 2017). *Zingiber officinale* consists a variety of uses such as medicinal uses in reducing inflammatory eilossanoids, lowering fevers and the ability in preventing blood clothing (Yogeshwa, 2017). Most of Western Malesia *Zingiber* species have long or short peduncles, tightly imbricate bract and the labellum joining with lateral staminodes (Ardiyani, Newman, Poulsen, 2017). In morphological characteristics, *Zingiber officinale* having pseudostem with leaves, fibrous root and underground rhizome. In addition, the *Zingiber officinale* inflorescence in colour white to pale yellow with purplish lips and borne on radical (Baker 1894; Schuman 1904; Theerakulpisut *et al*., 2012). Moreover, the pollen of *Zingiber officinale* is spherical with cerebroid exine sculpturing. The phylogenetic analysis demonstrated that *Kaempferia parviflora* and *Kaempferia elegans* clearly separated from the *Zingiber* species.

The ITS sequences of five species of *Etlingera elatior* available from NCBI GenBank (*Etlingera hemisphaerica, Etlingera elatior, Etlingera yunnanensis, Etlingera linguiformis,Nicolaia elatior* and *Etlingera sessilanthera*) were used to performing phylogenetic analysis. As for outgroup in this analysis, *Zingiber officinale, Zingiber zerumbet* and *Zingiber pellitum* used to differentiate between two genera. The group comprising the genus *Etlingera* which including *Etlingera hemisphaerica*, *Etlingera elatior*, *Etlingera yunnanensis*, *Etlingera linguiformis,Nicolaia elatior* and *Etlingera sessilanthera* is strongly supported as monophylogenetic with bootstrap value 100% sister to isolated *Etlingera elatior* (UMKEE1_DNA). There are 446 positions total in the final data set.

The *Etlingera* species and outgroups data with sequences accession number from NCBI Genbank database were listed in the Table 4.4. Figure 4.9 showing the phylogenetic tree of *Etlingera* species based on a neighbor-joining method using the Internal Transcribed Spacer (ITS) data.

Table 4.4: The *Etlingera* Species and Outgroups Data with Sequences Accession Number from NCBI Genbank Database

No.	Species	ITS length (bp)	Genbank accession Number (ITS)
1.	*Etlingera hemisphaerica*	779	AF414507
2.	*Etlingera hemisphaerica*	784	AF414470
3.	*Etlingera elatior*	777	AF414465
4.	*Etlingera elatior*	644	AF478749
5.	*Etlingera yunnanensis*	622	AB097230
6.	*Etlingera yunnanensis*	767	AY769846
7.	*Etlingera linguiformis*	608	KJ872128
8.	*Etlingera sessilanthera*	777	AF414510
9.	*Etlingera sessilanthera*	783	AF414473
10.	*Nicolaia elatior*	644	AF478739
11.	UMKEE01_DNA	640	NR240645

Figure 4.9: The phylogenetic tree of *Etlingera* sp. based on a neighbor-noining method using the Internal Transcribed Spacer (ITS) data.

Etlingera elatior or known as torch ginger having a variety of uses in medicinal and culinary purpose (Choon & Ding, 2016). The different part which is the leaf, inflorescence and rhizome of *Etlingera elatior* contain essential oil used as a therapeutic agent. In addition, it pharmacological and phytochemistry properties have been extensively studied (Mohamad *et al*., 2005; Lachumy *et al*., 2010; Abdelmageed *et al*., 2011). *Etlingera elatior* can be propagated asexually through rhizome and sexually through seeds. In morphological characteristics, *Etlingera elatior* inflorescence consist of two type of bract which are involucral bracts and floral (Choon & Ding, 2016). The stem in horizontal known as a rhizome. Spider-hunter bird is believed to be an important pollinator for *Etlingera elatior* (Sakai *et al*., 1999).

The molecular phylogenetic tree that was constructed based on Internal Transcribed Spacer (ITS) region indicated that *Etlingera elatior* and *Zingiber officinale* were separated into two clusters. The phylogenetic trees based on ITS regions were generated using neighbor-joining methods. The two samples of *Zingiber officinale* and *Etlingera elatior* were identified as two groups. The length of the complete ITS sequences were 640 bp for isolated DNA of *Etlingera elatior* and 647 bp for isolated DNA of *Zingiber officinale*.

The *Zingiber officinale* had a longer ITS length compared to *Etlingera elatior*. Both short length of sequences and single nucleotide differences of alignment of both *Zingiber officinale* and *Etlingera elatior* proved there were deletions or insertions in ITS regions. Identification of different plant species was used the hypervariable nucleotide sequences of ITS region between 18S and 28S rDNA (Wu *et al*., 2013).

Phylogenetic analysis appeared to agree that Internal Transcribed Spacer (ITS) regions can be used to recognize and differentiate between the genus *Zingiber* and genus *Etlingera* based DNA isolated DNA from *Zingiber officinale* and *Etlingera elatior*. Recently, to explore the phylogenetic relationships within family Zingiberaceae as well as within several genera, several papers have used molecular data (Searle & Hedderson, 2000; Wood, Whitten & Williams, 2000).

The important mechanisms in angiosperms are polyploidization and hybridization. In the past, most of the studies indicated that the rule in the evolution of polyploidy complexes is the recurrent formation of autopolyploid and allopolyploid plant species (Arnold, 1997). All the angiosperms thought to be of the hybrid origin

approximately 35-50% (Masterson, 1994; Koch, 2003). The study of the ITS sequences for *Zingiber officinale* and *Etlingera elatior* provided the idea of identification and classification of the genus. Moreover, the result for *Zingiber officinale* and *Etlingera elatior* phylogenetic tree indicated that genetic evolution was independent for both genera.

Figure 4.10 showed the phylogenetic trees of the genus *Etlingera* different with genus *Zingiber* based on a neighbor-joining method using Internal Transcribed Spacer (ITS) data.

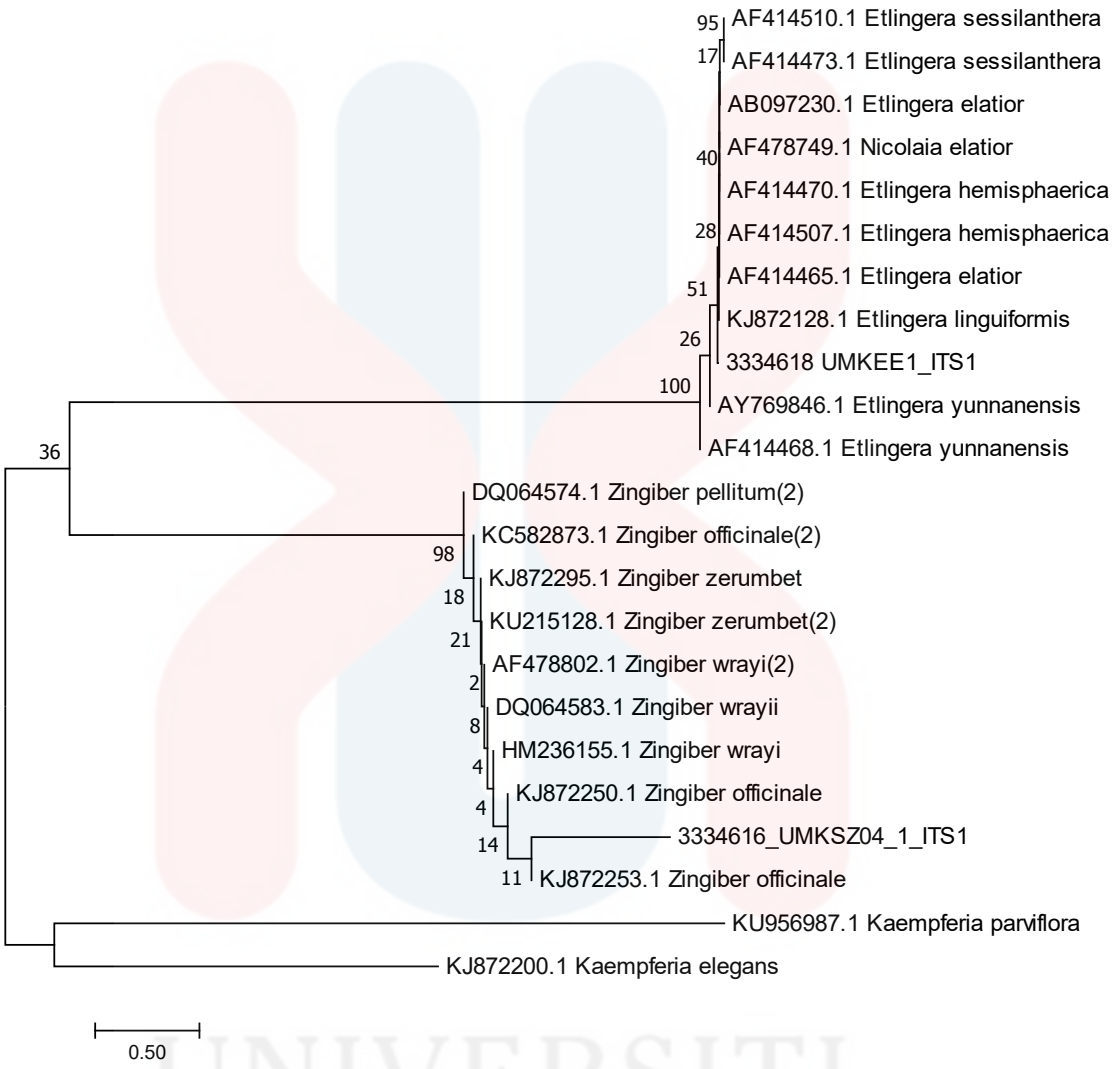

Figure 4.10: The phylogenetic trees of genus *Etlingera* differentiate with genus *Zingiber* based on a neighbor-joining method using Internal Transcribed Spacer (ITS) data.

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

The optimization of PCR conditions for *Zingiber officinale* and *Etlingera elatior* were done successfully using Internal Transcribed Spacer (ITS) region. Different variables were used in optimizing the PCR product of isolated *Zingiber officinale* and *Etlingera elatior*. Both isolated DNA samples with good purity ratio were chosen for molecular identification using the Internal Transcribed Spacer (ITS) region. The identification and differentiation of both *Zingiber officinale* and *Etlingera elatior* by comparing the database sequences at the Basic Local Alignment Search Tool (BLAST). The phylogenetic analysis was constructed using MEGA 7 software for both *Zingiber officinale* and *Etlingera elatior*. Hence, identification of species in the plant can be done successfully by the DNA-based method. In this study, the primers used to target the ITS region can be used in assessing botanical identity and can be used for further study.

5.2 Recommendation

There is need a further study on molecular biology for *Zingiber officinale* and *Etlingera elatior* as its lack of information on these species. This Polymerase Chain Reaction (PCR) is a faster way to differentiate between *Zingiber officinale* and *Etlingera elatior* which can be used for further studies. For agarose gel electrophoresis of *Zingiber officinale* and *Etlingera elatior* DNA extraction, in preventing smearing of DNA down the surface of the gel is to avoid overfilling the gel wells. In addition, using a low percentage of agarose gel in higher molecular weight DNA is better in separating the DNA (Biotium, 2018).

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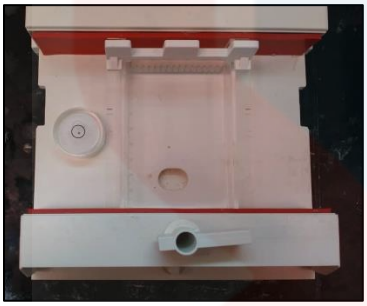
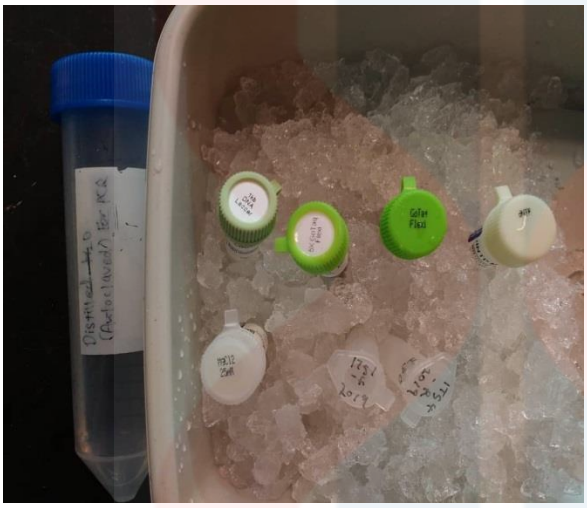
APPENDIX A

Raw Material	Description
	Agarose Gel Casting Tray • Image of agarose gel casting Tray consist of three part which are the comb, the tray and the support. The tray to give the shape to the gel as it polymerizes.
	UV Transluminator Machine • Image of Ultra Violet (UV) transluminator used for viewing the target DNAs and proteins from the agarose gel electrophoresis product.
	Polymerase Chain Reaction (PCR) Machine • Image of Polymerase Chain Reaction (PCR) used for amplification of ITS regions.

APPENDIX B

Chemical	Description
	PCR Component • Image of PCR component used that are 5X Green GoTaq@ Flexi Buffer, 25 mM MgCl2 Solution, PCR 10 mM Nucleotide Mix (dNTP), ddH2O 10 µM Forward Primer, 10 µM Reverse Primer, GoTaq@ DNA Polymerase (5 u/µL) and template DNA

APPENDIX C

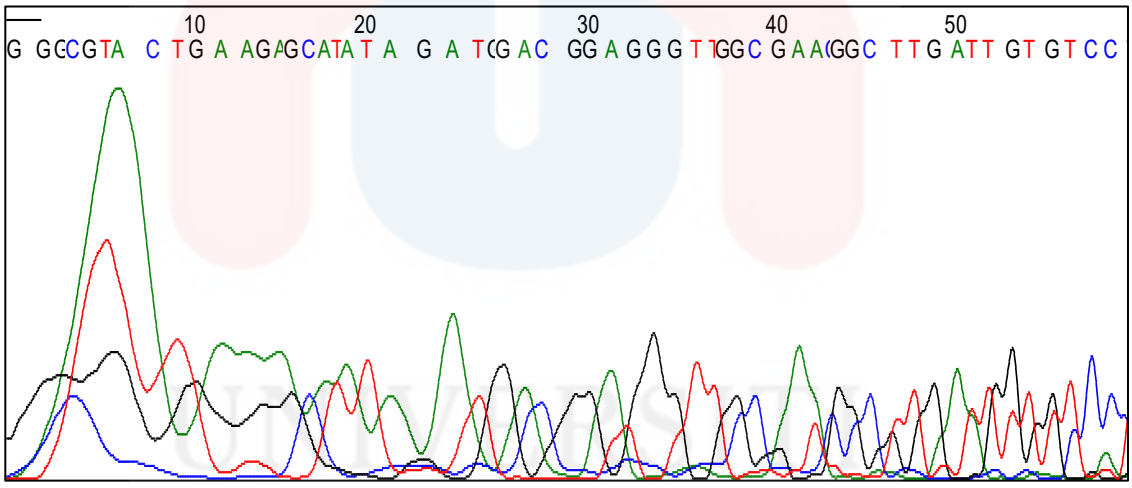
Field work	Description
	Collecting Plant Samples • Image collecting plant samples at Hutan Lipur Bukit Bakar, Machang Kelantan.
	Laboratory Work • Image using micropipette for pipetting chemical used in DNA extraction at Universiti Malaysia Kelantan, Jeli Campus Research Laboratory.

APPENDIX D

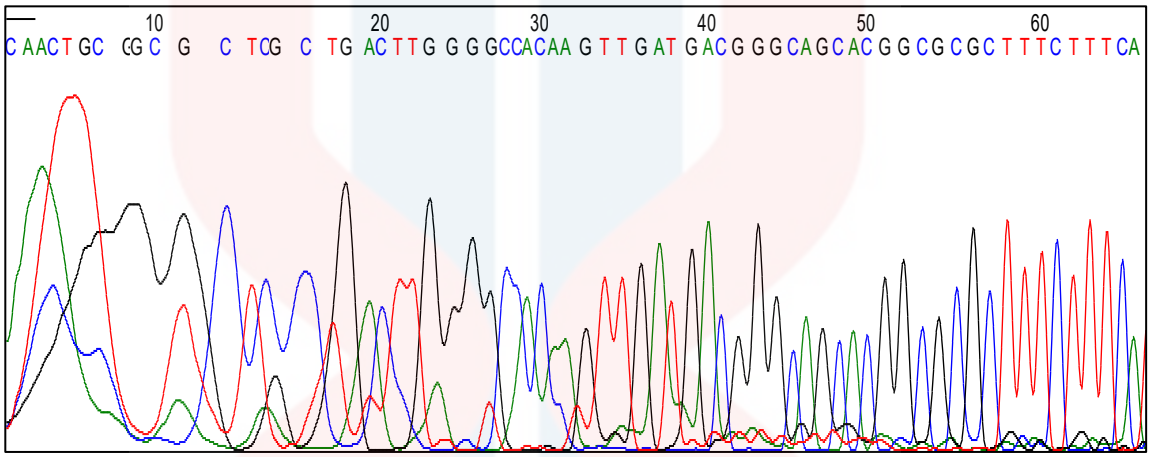
Image of *Zingiber officinale* and *Etlingera elatior* forward and reverse sequences chromatogram for Internal Transcribed Spacer (ITS) Region.

Note: Only a portions of ITS forward and reverse which 1 to 59 nucleotide sequences shows for *Zingiber officinale* and *Etlingera elatior* in the appendix D.

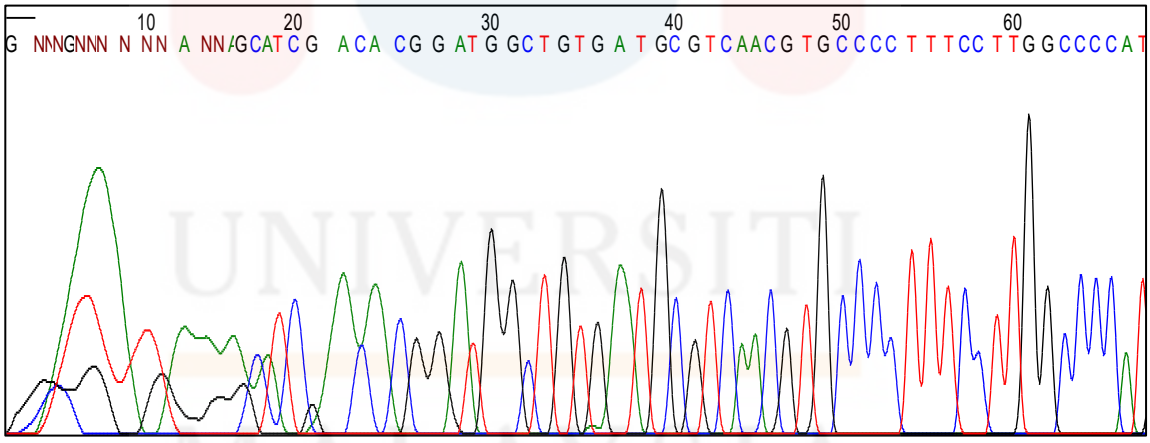
Zingiber officinale Forward



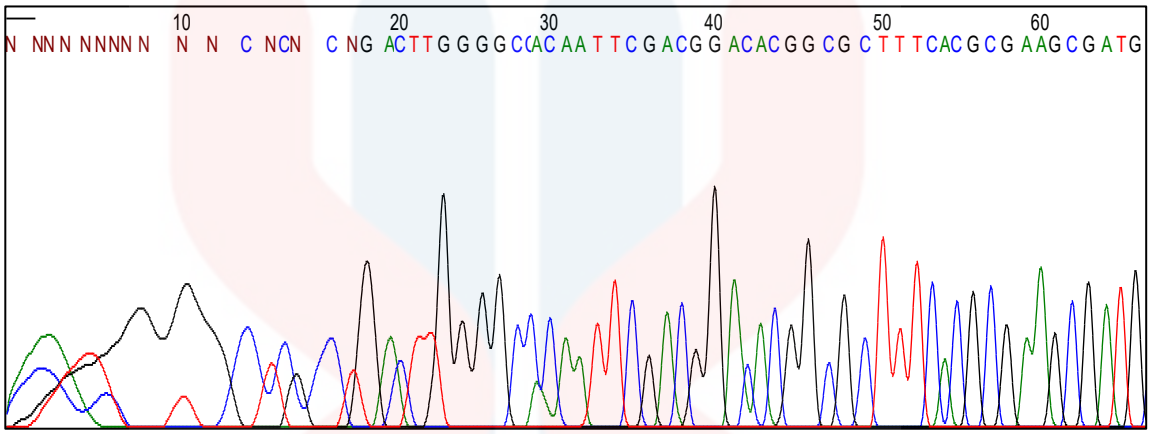
Zingiber officinale Reverse



Etlingera elatior Forward



Etlingera elatior Reverse



APPENDIX E

Internal Transcribed Spacer (ITS) gene sequence of *Zingiber officinale* and *Etlingera elatior*

Zingiber officinale Forward Sequences

GGGCGTACTGAAGAGCATATAGATGGACGGAGGGTTGGCGAACGGCTTGAT
TGTGTCCTTCTCTTCCCCCCACCCCGTTCGTCGGTGTGGGATTGGCCGAAACT
CTGTGCGATCGGGACTAAAGAAAAACGAAATCTGAAACGGAGGGGCCCCCT
CGCCGGGCGCGGGGGACCCATGCGTCTGAAATTTCCCGGAAACTATCTATCA
ATCAAACCTCTCGCCAATGTGGATATCTCCTCTCTTGCCTCTAAGAAAAATG
TAGAGAAGTGATATTTGTGGTGAGTATTGAAAAATCTCGAGCATTTTTGTCT
TTTTGAACACTTGTTGTGCCAGACGTTTTGTGGAAGAGGGCACCCCTGGGTG
GGTGTGGTGGCCTCCTCCCCTCTGCTCCTCCTCGCCTCGGCGGGTGGAAGGC
GCAAAGTGCGCAAGTTGGCCCCCTGTGCCCTCCGGCCCAGGCGGGTGAAAA
ACGGGTAAAACGCGGCGCTCGCGGGGCACGGCTGGTGTTGGTCGCCATGAG
AAAGAACACAACGTCGTCTTCGTAGTGAATTGACTGAGATCCTCAAAACGAT
CTGGGTGATTGCGGCGGCACGAGACAAAGAAAGAAAGCTGCGCGTCGTGCT
CTTCGTCATCTATTTGTGGCGACGCCACAGGCGACTTTCACTATATAATTAAT
CATATGAATAAGAAGAGG

Zingiber officinale Reverse Sequences

CAACTGCGGCGCTCGCTGACTTGGGGCCACAAGTTGATGACGGGCAGCACG
GCGCGCTTTCTTTCATTCGCTGCCCGGTGCCGCCATCACCCAGGGTCTCTTGA
GGAATTCTTTCCTAAAACAACGGGGACGACGTCCTGTTGCCGCTCACGGCGA
CCACCACCCGTCGTGCCGCCCGACTGCCGACTTTTATTAGCTCTTCTGCCGAC
TGTGGCCGAGGACAGACGGGGGCAATTTTTTCGCTCCTCACCTGCCAACCAA
CGACGAAGCAAGGAGGACAAGAGACTATCACCCCATGACCCCCGACCCGGG
CCGCCCGCCCCCAGGCCGCCTCGGGCACTTGTTGCTCAAAAATACTCGATGT
TCTACAATTTTCAGCTATTACCACATATCATATCTTTCTACTTCGTTCTTCAAC
CCAAGAAAAGCCATGTCAATTGTTGACAATTGTTTTTTTGTTGAATGATTGCC
AACGAACAACCCACCACTGCGCCCGCCCCGCGGCGCACGGCGAGCCACCTC
CTTCTTCCTTGTTGCTTATTGCTTACCGCCCCTCGCAACCACCTTCCGACCAC
CCACGAGCGGCGGGAGGGGGGGGGGAAAAAAAAAACCTCCTCCATCCCTCCT
CCACCATCCTTCGTTCTTTTATCTGCTCTCACTATGCTGCTTCCTCCCCATAAA
CAAAAAAAGAAATGTTCTCAAGAGAAGGGTTTGGTCGAGAAGGATGGATCG
CATGTTCTGATACCCACCGCCCTCGCCTGCGTGATGAAGTGGATGGAAAAAG
TATCAATTGTTCTG

Etlingera elatior Forward Sequences

GNNNGNNNNNNANNAGCATCGACACGGATGGCTGTGATGCGTCAACGTGCC
CCTTTCCTTGGCCCCATGTCGGAGGCCAACTGACCGTAGCTCGGTGCGATCG
GCACCAAGGAACAACGAACTCAGAAGCAGCGGGCCCTCGGCGTGCACGAG
GAGCCCACTGCATAAGAGATGCTTGGAATCGAATGACTCTCGGCAATGGAT
ATCTCGGCTCTTGCATCGATGAAGAACGTAGTGAAATGCGATACTTGGTGTG
AATTGCAGAATCTCGTGAACCATTGAGTCTTTGAACGCAAGTTGTGCCCAAG
GCTTTGTGGCCGAGGGCACGTCTGCTTGGGCGTCATGGCATCACCGCCTTTG
CTCCTTGCTCTGCTGGTGCCAAGCGCGGAAATTGACCTCGTGTGCCCTCGGA
CACAGTCGGTCAAAGAGCGGGCAGTCGCCAGTCGTCGAGCGCGATGGGTGC
TGGTAAACCCCGCGCGCGAATAGAACGTCGCCCTCGACGTGTCTGGACGAG
TCCTCGAGAGACCCTGCGGGACTGCGGCATCGCTTCGCGTGAAAGCGCCGT
GTCCGTCGAATTGTGGCCCCAAGTCAGGCGAGGCCCACCCGCCGAGTTTAA
GCATATCATAAGCCCGGGAGGAAA

Etlingera elatior Reverse Sequences

NNNNNNNNNNNCNCNCNGACTTGGGGCCACAATTCGACGGACACGGCGCTT
TCACGCGAAGCGATGCCGCAGTCCCGCAGGGTCTCTCGAGGACTCGTCCAG
ACACGTCGAGGGCGACGTTCTATTCGCGCGCGGGGTTTACCAGCACCCATCG
CGCTCGACGACTGGCGACTGCCCGCTCTTTGACCGACTGTGTCCGAGGGCAC
ACGAGGTCAATTTCCGCGCTTGGCACCAGCAGAGCAAGGAGCAAAGGCGGT
GATGCCATGACGCCCAAGCAGACGTGCCCTCGGCCACAAAGCCTTGGGCAC
AACTTGCGTTCAAAGACTCAATGGTTCACGAGATTCTGCAATTCACACCAAG
TATCGCATTTCACTACGTTCTTCATCGATGCAAGAGCCGAGATATCCATTGC
CGAGAGTCATTCGATTCCAAGCATCTCTTATGCAGTGGGCTCCTCGTGCACG
CCGAGGGCCCGCTGCTTCTGAGTTCGTTGTTCCTTGGTGCCGATCGCACCGA
GCTACGGTCAGTTGGCCTCCGACATGGGGCCAAGGAAAGGGGCACGTTGAC
GCATTCACAGCCATCCGTTGTTCGATGCTCTCTCAACAATGATCCTTCCGCAG
TCCCCCCCATTCGAANA