IDENTIFICATION OF MOLECULAR MARKER FOR GENUS *ETLINGERA* BASED ON INTERNAL TRANSCRIBED SPACER (ITS) REGION

by

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A report submitted in fulfillment of the requirements for the degree of Bachelor of Applied Science (Natural Resources Science) with Honours

FACULTY OF EARTH SCIENCE
UNIVERSITI MALAYSIA KELANTAN

2019

DECLARATION

I declare that this thesis entitled "Identification of Molecular Marker for Genus *Etlingera* Based on Internal Transcribed Spacer (ITS) Region" is the result of my own research except as cited in the references. This thesis has not been accepted for any degree and is not concurrently submitted candidature of any other degree.

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APPROVAL

"I/ We hereby declare that I/ we have read this thesis and in our 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 : .....

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ACKNOWLEDGEMENT

The greatest thanks to Allah the Almighty upon the completion of this final year report entitled Identification of Molecular Marker for genus *Etlingera* based on Internal Transcribed Spacer (ITS) Region. It is a great pleasure to address people who helped me throughout this project to enhance my knowledge and practical skills in research area especially my supervisor, Dr. Suganthi A/P Appalasamy. She has made available their support in a number of ways including guidance and encouragement from the beginning till the end enabled me to develop and complete my final year research.

My gratitude also been to Faculty of Earth Science 's Dean, Universiti Malaysia Kelantan; Prof. Madya Dr. Aweng A/L Eh Rak, for his support throughout my years at Universiti Malaysia Kelantan by ensuring a fine study environment at all time for me to complete my 4 years studies successfully.

My fellow undergraduate students should also be recognized for their support such as Noor Azni Naziera Binti Mohamed, Rahayu Binti Mohamad Yusoff and Fatien Nur Syazwanie Binti Zabri. I also indebted to my research mate Nurhidayah Binti Saharizan for her support and help me throughout this project. I would like to extend my gratitude to the laboratory staffs of Universiti Malaysia Kelantan for assisting me to get all laboratory equipment and requirement on time. Lastly, I offer my regards and blessings to all of those who supported me in my any respect during the completion of my thesis especially my family members.

Identification of Molecular Marker for Genus *Etlingera* Based on Internal Transcribed Spacer (ITS) Region

ABSTRACT

This research aimed to identify the specific molecular marker for ginger (Zingiberaceae) as there is very limited molecular study on ginger especially for genus *Etlingera*. Three selected species from genus *Etlingera* was selected which are *E. elatior, E. megalocheilos* and *E. littoralis*. These three species were chosen from the twelve *Etlingera* species found in Peninsular of Malaysia. The DNA from these three species were isolated by using Cetyl Trimethylammonium Bromide (CTAB) extraction method and the results from the extraction were then used to conduct Polymerase Chain Reaction (PCR) with the Internal Transcribed Spacer region as the target sequence region. The Internal Transcribed Spacer specific primer was chosen and amplified via PCR. Results from two of the species were sent for sequencing and the sequences obtained were used for species identification through Basic Local Alignment Search Tool (BLAST). The results show these two species correctly belong to genus *Etlingera* (Zingiberaceae) by comparing with the closest match from Nucleotide BLAST. Three phylogenetic trees were constructed by using MEGA7 Software to show the phylogeny relationship among selected species in Zingiberaceae. Lastly specific primers for two species of *Etlingera* were designed by using Primer3 Plus. In a nutshell, the designed specific primers for *E. elatior* and *E. megalocheilos* identification were developed.

Identifikasi Penanda Molekular Bagi Genus *Etlingera* Berdasarkan Ruang Dalaman Salinan Rantau (ITS)

ABSTRAK

Kajian ini bertujuan untuk mengenal pasti penanda molekular khusus untuk halia (Zingiberaceae) kerana begitu terhad kajian molekular ke atas halia terutama untuk genus *Etlingera*. Tiga spesies *Etlingera* iaitu spesis *E. elatior*, *E. megalocheilos* dan *E. littoralis* telah terpilih. Ketiga-tiga spesis ini adalah daripada dua-belas spesis yang dijumpai di semenanjung Malaysia. Spesies ini telah diekstrak dengan menggunakan kaedah Cetyl Trimethylammonium Bromide (CTAB) dan hasil pengekstrakan ini kemudiannya diteruskan kepada kaedah Tindakan Balas Rantaian Polimerase dengan ruang dalaman salinan rantau sebagai kawasan yang terpilh. Primer khusus untuk ruang ini telah dipilih dan di uji di dalam kaedah Tindakan Balas Rantaian Polimerase. Keputusan dari kaedah Tindakan Balas Rantaian Polimerase tersebut mendapati dari dua spesies tersebut telah dihantar untuk proses urutan. Keputusan yang diperolehi daripada proses urutan tersebut digunakan untuk pengenalan spesies melalui perisian Basic Local Alignment Search Tool (BLAST). Hasil daripada pengenal pastian identiti species atau sampel, kedua-dua sampel tersebut berada di dalam genus dan spesis yang tepat iaitu *Etlingera*. Mengenal pasti identiti spesis tersebut dilakukan melalui pembandingan dengan nukleotid yang hampir sama diperolehi daripada Nukleotid BLAST. Selepas itu, tiga pokok phylogeni telah dibina dengan menggunakan perisian MEGA7 untuk memperlihatkan hubungan phylogeni antara beberapa spesies yang terpilih di dalam keluarga Zingiberaceae. Akhirnya, primer khusus telah direka untuk dua spesis *Etlingera* dan dicipta dengan mengunakan perisian Primer3 Plus. Secara keseluruhannya, primer untuk proses identifikasi bagi *E. elatior* dan *E. megalocheilos* telah direka dan di hasilkan.

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LIST OF ABBREVIATIONS

µl	Microliter
µM	Micromolar
BLAST	Basic Local Alignment Search Tool
Bp	Base Pair
CTAB	Cetyltrimethylammonium Bromide
DNA	Deoxyribonucleic Acid
dNTPs	Deoxyribonucleotide Triphosphate
EDTA	Ethylenediaminetetraacetic Acid
ITS	Internal Transcribed Spacer
$MgCl_2$	Magnesium Chloride
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
NCBI	National Centre for Biotechnology Information
O.D.	Optical Density
PCR	Polymerase Chain Reaction
PVP	Polyvinylpyrrolidone
Taq	*Thermus aquaticus*
TE	Tris-HCL-EDTA
Tris	Trisamine

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Approximately there are 369,000 species (94%) of vascular plants in the world (Dasgupta, 2016). However, most of the world's species is undiscovered or improperly identified. In identifying or characterizing species, genetic marker is widely used and known among the researchers or scientists. It is to identify species or to study any inherited disease within the gene that happened due to genetic linkage issues and possibly because of uncharacterised or unidentified genes such as single nucleotide polymorphisms (SNPs). Genetic marker includes the morphological markers, biochemical markers (alloenzymes, monoterpenes and other protein marker) and molecular markers (RAPD, AFLP, ISSR) (White, Adams, & Neale, 2007).

A molecular marker is a term to describe molecules that are taken from a sample such as plants and animals. It is used to reveal a particular organism's characteristics while differentiating it from the others. Molecular marker such as DNA contains information about genetic disorders, genealogy and the evolutionary of life. A molecular marker is a recognizable deoxyribonucleic acid (DNA) sequences associated at specific locations within the genome and transmitted by law of inheritance from generation to next generation (Singhi, Gyan, Mishra, Khan, &

Singhi, 2008). Hence, their inheritance is easily detected. The advancement and utilization of molecular markers in DNA polymorphism exploitation and detection is one of the most significant developments in the field of molecular genetic (Semagn, Bjørnstad, & Ndjiondjop, 2006).

1.2 Statement of Problem

There are limited studies on molecular maker for ginger especially for genus *Etlingera* and mostly did not use the Internal Transcribed Spacer as the target region. The previous study of molecular marker of *Etlingera* spp. only focused on *Etlingera elatior* which was conducted by Yunus, Aziz, Kadir, Daud, and Rashid (2013) by applying Random Amplified Polymorphic DNA (RAPD). However, the research done by Yunus and his team is a mutation breeding research that involved tissue culture and molecular marker. Thus, there is no definite research on identifying or developing molecular marker for *Etlingera* spp. Moreover, RAPD method that had been used in the previous study was reported to have low reproducibility, require high molecular weight DNA, need high precaution step to avoid contamination and the experimental procedure is not robust. Molecular markers are important especially for species identification in Zingiberaceae which are morphologically identical that will be hard for non-botanist to identify.

1.3 Significant of Study

The importance of this study is to identify and develop specific molecular marker for *Etlingera* species. At the end of the study, specified molecular marker for

Etlingera species were identified and developed that based on Internal Transcribed Spacer (ITS) region.

1.4 Objectives

1. To extract genomic DNA from three selected species of *Etlingera*
2. To identify specific molecular marker for *Etlingera* spp. identification.

1.5 Scope of Study

This study was focusing on sampling of selected *Etlingera* spp. around Kelantan and the samples were used to test the most suitable molecular marker of the selected *Etlingera* spp.

CHAPTER 2

LITERATURE REVIEW

2.1 Zingiberaceae

The Zingiberaceae is a ginger family that consist of perennial herbs and spreads horizontally by their tuberous rhizomes that enable them to cover the large area in short time period. Zingiberaceae which is one of the major group in Angiosperms and in order of Zingiberales consist approximately 50 genera and 1500 species known in the world that distributed in Americas, Tropical Africa and Asia (Krings, 2009). Currently, 20 out of 50 genera which is approximately 300 species are found in Malaysia (Khaw, 2001).

Zingiberaceae comprised genus of *Etlingera, Curcuma, Zingiber, Siamanthus* and others. *Etlingera* consist of 105 species and currently 12 species are found in Peninsular Malaysia including *E. elatior, E. maingayi, E. fulgens, E. venusta, E. pauciflora, E. punicea, E. subterranean, E. metriocheilos, E. triorgyalis, E. littoralis, E. corneri* and *E. pieace* as shown in Figure 2.1. Each of this species has significant antimicrobial activity and economically important.

Figure 2.1 shows the representative species of *Etlingera*. Sect. *Etlingera*: **A** *Etlingera maingayi* (Zaharil Dzulafly, 2014); **B** *Etlingera pauciflora* (David Monniaux, 2005); **C** *Etlingera venusta* (Amazon.com, 2018); **D** *Etlingera elatior* (World of Flowering Plant.Com, 2017); **E** *Etlingera fulgens* (Ron, 2007); **F** *Etlingera littoralis* (Ahmad Fuad Morad, 2012); **G** *Etlingera corneri* (Nhu Nguyen, 2011); **H** *Etlingera megalocheilos* (Fatien Nur Syazwani, 2018)

The inflorescence shoots of the *Etlingera* spp. are so short that do not emerge from the ground and all that can be seen is a circlet of flowers with prominent bright red petal-like structures (labella) radiating outward, the flower tubes and ovaries being below ground level. Fruits ripen below ground, and the seeds are usually dispersed by wild pigs or other animals. The leafy shoots are around 3 to 5 metres which is 10–16 feet tall making the leaves grows high in the air while the flowers are partially buried in the ground. Figure 2.2 shows the height leafy shoot of one of *Etlingera* spp. The species under this genus classified as perennial plants and have forked fleshy rhizomes (underground stems). By having aerial roots exposed to the humid atmosphere, some of these species are epiphytic which is growing upon or supported by another plant. However, they are not parasites that could harm the host plant. The Zingiberaceae flowers resemble to an orchids and commonly having green sepals but differ in texture and colour of the petals.

Figure 2.2: The mature leafy shoot may reach a height of 3-5 metres (Hairulafiz, 2003)

2.2 Economical and Medicinal Values of *Etlingera* spp.

Etlingera spp. has different purpose in traditional or commercial uses. In tropical region such as in Costa Rica and Australia, *E. elatior* were cultivated because of their high potential to colonize new habitat to be commercialize as ornamental (Larsen, 1999; Hammel, Grayum, Herrera, & Zamora, 2003). The flowers of *E. elatior* were used as an ingredient in making soap, shampoo and perfume. It also combined with other aromatic plants then used as deodorizers. Besides, in Peninsular Malaysia *E. elatior* or known as *bunga kantan* by Malay people are also used in local dishes as a flavouring such as in *laksa*, *nasi kerabu* and *nasi ulam*. Meanwhile, indigenous people in Borneo consumed *E. littoralis*, *E. rubrolutea* and *E. elatior* raw or cooked or as an added flavoured in a dishes. Whereas, local communities in Thailand preferred to consumed it as a vegetables (Sirirugsa, 1999). Furthermore, *E. elatior* were also applied to treat earache, wound and used in aromatic herbal bath as remedies to remove body odour by post-partum women.

2.3 Antimicrobial Activity and Phytochemical Screening of *Etlingera* spp.

An antimicrobial is an agent that can constraint and execute the production of microorganism. The plant extraction and phytochemicals analysis were evaluated by antibiotic susceptible and resistant microorganism to know its antimicrobial activity (Nascimento, 2000). *E. elatior* for instance, has the maximum inhibitory activity against microorganism against *Staphylococcus aureus* followed by *Bacillus thuringiensis*, *Bacillus subtilis* and lastly *Salmonella* sp. However, *Etlingera elatior* exhibits weak inhibition towards *E. coli* and *Micrococcus* sp.

Meanwhile, the phytochemicals screening of *E. elatior* extraction showed the presence of the tannins; a bitter taste present in plant, carbohydrates, and saponins; a glycoside found in root which is historically used in soap. Besides, terpenoids an natural chemical that give the distinctive aroma also present in the *E. elatior*. Furthermore, this species also absent of alkaloids which it does not contain nitrogen-containing bases that have diverse and physiological effects on human and animals (Lachumy, Sasidharan, Sumathy, & Zuraini, 2010).

2.4 Polymerase Chain reaction (PCR)

2.4.1 History of PCR

The Polymerase Chain Reaction technique was invented by American biochemist; Kary Banks Mullis in the year 1985 who been awarded Nobel prize in the year 1993 in recognition for his invention of this techniques. PCR is a rapid, in vitro deoxyribonucleic acid (DNA) synthesis process, which of a given nucleic acid target (Caskey & Metzker, 2009).

It is a laboratory technique used to copy the particular region of DNA ups to millions of copies of DNA sample. These copies of DNA are done to in order to make enough DNA regions for the further experiment such as for visualized by gel electrophoresis, cloned or DNA sequencing. PCR has been widely used in the identification process. For instance, in the identification of yeast strain done by Fujita, Senda, Nakaguchi, and Hashimoto (2001). Other than that, the criminologist also used PCR to link specific person to samples like blood or hair by DNA comparison. This can be seen where PCR is used in DNA profiling which is DNA

typing, genetic fingerprint and DNA testing (Huggett, Dorai-Raj & Falinska, 2014). Meanwhile, the qPCR is used for detection of nucleic acid in the forensic application such as microbiology, biomedical research and biotechnology (Sun, Park, Oh, & Hong, 2013).

2.4.2 Components of PCR

PCR requires the DNA template, primers, nucleotides and *Taq* polymerase. DNA template is obtained from the gene of interest through the separation of DNA strands under high temperature. *Taq* polymerase is chosen enzyme that replaced the DNA polymerase which is getting from the *E. coli*. *Taq* Polymerase (*Thermus aquaticus*) is a heat-tolerant bacterium that isolated from hot springs and hydrothermal vents. This enzyme is selected because of its ability to withstand the high temperature during protein-denaturing process compared to the *E. coli*, usually not function under high temperature. This enzyme will link the nucleotides blocks that are complementary to the first DNA strands. Nucleotides or deoxynucleotides triphoshate (dNTPs) include the four bases; Adenine (A), Thymine (T), Cytosine (C) And Guanine (G) (Garibyan & Avashia, 2013).

Oligonucleotide primers are the main component in order for the *Taq* polymerase to build on. Primers are short pieces of single-stranded DNA fragment about 20-30 nucleotides that provide a starting point for DNA synthesis. This fragment will define the sequence complementary of the target DNA and amplified.

2.4.3 Principles of PCR

PCR involved three stages which are denaturation of double stranded DNA template, annealing of primers and extension of double stranded DNA molecules. In denaturation process, the DNA strand is heated in 95ºC to separate the double stranded to single stranded DNA by breaking the hydrogen bond between the bases. During the process, maintained temperature is crucial to ensure the DNA strand separated completely. Now, this single stranded DNA strand serves as the template for production of new strand of DNA (Kadiyam, 2015). Next stage is annealing where the temperature of the reaction is lowered to 54^{o}C-60^{o}C to allow the primers to bind at the specific location on the single stranded template DNA and it indicates the starting point for DNA synthesis. After that, an extension process where the heat is increased to 72ºC enable the *Taq* polymerase enzyme adds nucleotides that are complementary to the first strand at 3' each primer and extending the DNA sequence in 5' to 3' direction (NCBI, 2017).

2.4.4 Advantages and Disadvantages of PCR

There are many advantages of polymerase chain reaction (PCR). Firstly, PCR very sensitive where only small amount starting materials required to amplify the gene of interest. Kary Mullis stated that " lets you pick the piece of DNA you're interested in and have as much of it as you want'' (Mullis, 1990; Garibyan & Avashia, 2013). According to Al-Attas, Al-Khalifa, Al-Qurashi, Badawy, and Al-Gualy (2000) in their research on evaluating PCR for Acute Human Brucellosis Diagnosis, to diagnose brucellosis disease frequently hard to be established due to its clinical sensitivity and ability to mimic any infectious and non-infectious disease.

Thus this team evaluate the capability of PCR to diagnose the disease and the outcome shown that PCR techniques could show the result very specificity, real sensitivity, quick, speedy and can be considered laboratory diagnosis of brucellosis.

Furthermore, the matured DNA also could yield adequate starting materials in amplify the interest DNA. PCR primer also targets a single loci with a single primer or primer set compared to the RAPD methods which are not locus-specific (Edwards & Gibbs, 1994). However, there are some limitations on this technology. Since PCR is very sensitive, it becomes the major disadvantages where small contaminating DNA from different sample could also be amplified. Furthermore, the *Taq* Polymerase (component of PCR) is quite expensive and false or cross reaction during the process may occur.

2.5 Advantages of Molecular Marker in Species Identification

Genetic marker can be divided into three markers which are biochemical marker, morphological marker and molecular marker (White *et al.*, 2007). Biochemical marker and morphological marker can vary in the different environment. Compared to molecular marker, the genes are free from the environmental and pleiotropic effect as they do not display phenotypic plasticity (environmental modification) (Kalia, Rai, Kalia, Singh, & Dhawan, 2011). Most morphological or biochemical marker are affected by polygenic control, influenced by epistatic control and limited number of independent markers available. The DNA based on molecular marker easily score as discrete states of alleles or DNA base pair unlike the morphology and biochemical marker. Thus, utilization of molecular markers is important for species identification research as it does not affect by any environmental modification.

Ghosh, Majumder & Mandi (2011) did a research on species identification for genus *Zingiber*. The Z. *officinale, Z. monatanum* and *Z. zerumbet* (Zingiberaceae) and species-specific Amplified Fragment Length Polymorphism (AFLP) marker was selected for the species identification due to morphologically similar but highly differs in therapeutic and pharmacological properties. Besides, isozyme marker was used by Apavatjrut, Anuntalabhochai, Sirirugsa, & Alisi (1999) for early flowering of *Curcuma Longa* that are taxonomically confused and this marker was utilized in order to confirm and distinguish the taxa that analysed.

2.6 Internal Transcribed Spacer (ITS) Region as Target Region in Polymerase Chain Reaction (PCR).

Internal Transcribed Spacer (ITS) sequences were used in first phylogenetic analysis of *Rubus* species (Alice & Campbell, 1999) that involved 57 taxa and 20 species of subgenus *Rubus* (Blackberries). ITS sequences were used as it is the most informative and has low variability between closely related species (Cheng, Xiao, Gu, & Xiao, 2015). ITS region currently used in the molecular target in species identification, phylogenetic research, epidemiological investigation and others.

This ITS coding region play fundamental role for molecular assays although not translated into protein (Iwen, Hinrichs, & Rupp, 2002) The ITS region is located in nuclear ribosomal DNA gene complex between 18S-5.8S-26S that have relatively high conserved nucleotide sequence. For an instance, ITS 1 situated between 18S and 5.8S rDNA genes includes the Intergenic Spacer Region (IGS) which consist the External Transcribed Spacer (ETS 1) region on 5' and External Transcribed Spacer (ETS 2) on 3' end (from 5' to 3' orientation). This region are used to identify the

taxonomic relationship between major groups and to separate genera and species based on sequence polymorphism (Einsele *et al*., 1997). Furthermore, this conserved sequence is really advantageous as a binding site for universal primers to amplify flanking spacer regions (White, Bruns, Lee, & Taylor, 1990).

2.7 Molecular Marker Study for Genetic Diversity of *Etlingera* spp.

Generally, lots of people know the importance of ginger in daily life. From using it in food additives until utilizes as medicinal properties. Due to this economically importance, many researched being done on biochemical aspects but there is very limited study in molecular aspects. Thus Ismail, Rafii, Mahmud, Hanafi, and Miah (2016) put an effort to review and accumulate the available molecular marker information and its application of ginger for usability in future study. Through this research paper, some of molecular markers were such as isozyme, RAPD, AFLP, SSR, ISSR were compared. The application of these markers is shown in Table 2.1 below.

Table 2.1: Application of Some Molecular Markers in Genetic Study

Marker	Application	References
Inter-Simple Sequence repeat (ISSR)	Asessing genetic diversity among micropropagated and clone, cultivar identification and relationship differentiation	(Syamkumar & Sasikumar, 2007; Jaleel, & Sasikumar, 2010)
Isozyme	Detecting variations and realtionship among taxa group.	(Paisooksantivatana, Kako & Seko, 2001; Jatoi, Kikuchi, Mimura, Yi, & Watanabe, 2008)

Random Amplified Polymorphic DNA (RAPD)	Genetic diversity evaluation, species identification, species relatinship and cultivar.	(Syamkumar & Sasikumar, 2007; Das, Kesari, Satyanarayana *et al.*, 2011)
Simple Sequence Repeat (SSR)	Genetic diversity within germplasm	(Harith, Retno, & Ishak, 2013)
Amplified Fragment Length Polymorphism (AFLP)	Determination of genetic relationship among species or genus and species-specification	(Kaewsri, Paisooksantivatana, Veesommai, Eiadthong, & Vadrodaya, 2007)

Thus, based on the research, most researchers utilize Random Amplified Polymorphic DNA (RAPD) as their molecular markers in their ginger study because RAPD marker is well established in generating polymorphic band. Besides, RAPD markers can distinguish the clones, varieties, accessions or genotype in high resolution power value. For instance RAPD marker was selected by Yunus *et al*., (2013) to identified irradiated clones of *E. elatior*. However, there are some limitation on marker system where a particular marker does not necessary its applicability for other species or genus. This is because, marker selection is depends on the purpose of the study need to be conducted. Thus this study aim to develop new molecular marker specify for genus *Etlingera* especially for the three selected species; *E. elatior, E. littoralis, E. megalocheilos* that based on Internal Transcribed Spacer (ITS) region for the species identification.

CHAPTER 3

METHODOLOGY

3.1 Material

3.1.1 Apparatus

Bio-Rad Thermal Cycler, Microcentrifuge Tubes, Spatula, Dropper, PCR Tube, PCR Rack, Micropipette, Tips (White [0.5-10 µl], Blue [10-100 µl], Yellow [2-200 µl]), Collection of Tubes, Microwavable Flask, Gel Tray & Box, Electrodes, UV Analyzer (Alphamager HP), Tomy SX-500 Autoclave Machine, Measuring Cylinder, Conical Flask, Media Bottles, Beakers, Magnetic Stirrer and Rod.

3.1.2 Chemicals

The 20g of CTAB Powder (R&M Chemicals), 81.82g of NaCl (Sigma-Aldrich), 121.1g of Tris Powder (Vivantis Technologies Sdn. Bhd), 186.12g of EDTA Powder (R&M Chemicals), Distilled Water, 20g of NaOH, 1% of PVP, 2g of Agarose Powder, 204.12g of Sodium Acetate, 100 ml of Acetic Acid, 70% of Ethanol, Green GoTaq$^{@}$ Plexi Buffer, PCR Nucleotide Mix (dNTPs), Green GoTaq$^{@}$ DNA Polymerase, $MgCl_2$, 1 kb of Molecular Weight Ladder (Promega), Isopropanol, Isomyl Alcohol and Chloroform (R&M Chemicals).

3.2 Method

3.2.1 *Etlingera* spp. Samples Collection

Selected *Etlingera* species samples (*E. elatior*, *E. megalocheilos* and *E. littoralis*) were collected around Kelantan using coordinate data provided by Sarmila (2018). The collected plant leaves were kept in zip lock bag temporarily and then the leaf surface was wiped with the 70% ethanol to clean before storing in -10^{O}C freezer at the B.A.P Laboratory 1.1 of Universiti Malaysia Kelantan, Jeli Campus until further used.

3.2.2 Preparation of CTAB Buffer for DNA Extraction of Three Selected *Etlingera* spp.

The Cetyltrimethylammonium Bromide (CTAB) buffer, Tris buffer and Ethylenediaminetetraacetic (EDTA) buffer were prepared with methodology by Vinod (2007) meanwhile the TE buffer was prepared with the methodology by Liwanag (2012). The first extraction buffer is the preparation of 1 L of 2% CTAB buffer. The 20g of the CTAB powder were added into the distilled water. The solution produced was added with 81.82g of NaCl, 100 ml of 1M of Tris buffer and 40 ml of 0.5M of EDTA buffer. After that, the solution was sterilized by autoclaving in 121^{o}C at 15 Ibs pressure for 15 minutes. This CTAB Buffer was stored in a media bottle at 27^{o}C±2^{o}C of room temperature.

Second buffer was 0.5 M of 1 L of EDTA buffer. The 186.12 g of EDTA powder was added in 750 ml of distilled water. After that, 20 g of NaOH pallets was added. After the pH 8.0 was obtained, the buffer was sterilized by in 121ºC at 15 Ibs pressure for 15 minutes.

The third buffer is the preparation of 1 L of Tris buffer. The 186.12 g of Tris powder was added with 700 ml of distilled water. The solution was placed on the hot plate with the magnetic stirrer to aid the dilution process. Once the solution was dissolved, concentrated hydrochloric acid was added slowly until the solution reach pH 8. After that, the buffer solution was sterilized by autoclaving in 121^{o}C at 15 Ibs pressure for 15 minutes.

The fourth buffer is 1 L of TE buffer where the 10 ml of 1 M of Tris buffer was added into 2 ml of 0.5 M EDTA buffer. After that, about 988 ml of distilled water was added. Next, the concentrated of HCI was added to adjust the pH buffer until it reaches 8.0. Lastly, the buffer solution was sterilized by autoclaving in 121ºC at 15 Ibs pressure for 15 minutes.

3.2.3 Genomic DNA Isolation from Leaves Samples of Selected Three *Etlingera* Species Using CTAB Method

The step for genomic DNA isolation was followed Devi, Punyarani, Singh, and Devi (2013). All the samples were desiccated for few days in a desiccator to remove the moisture in the leaves sample. Moisture content was calculated by the initial weight minus with final weight. Zero moisture content was achieved when the weight is constant. The extraction buffer containing 500 µl of Tris-HCL, ETDA and CTAB was pre-heat in water bath at 60ºC for 15 minutes. Next, the tissue was

pulverized in presence of 1% Polyvinylpyrrolidone (PVP) and pre-warmed extraction buffer by using a pre-chilled mortar and pestle at room temperature (27ºC±2ºC). This ground leaf samples were transferred into 2 ml centrifuge tubes and was incubated in water bath at 60ºC for one hour. After that, the tubes containing the sample were centrifuged at 10,000 rpm for 10 minutes at 4ºC. The supernatant that produced was collected in 1.5 ml centrifuge tube by using wide bored tip. In the supernatant, an equal volume of chloroform and isoamyl alcohol (24:1) was added and mixed by inversion for 15 minutes.

Next, tube was centrifuged at 10,000 rpm for 10 minutes at 4ºC and the supernatant was collected in 1.5 ml centrifuged tube. An equal volume of chloroform and isoamyl alcohol (24:1) was added and was mixed again by inversion for 15 minutes. Then, the tube was centrifuged at 10.000 rpm for 10 minutes at 4ºC and the supernatant was collected in 1 ml centrifuged tube. To the supernatant, 540 µl of ice cold isopropanol were added to precipitate the DNA and incubated it at -20ºC for 30 minutes. The tubes were centrifuged again at 10,000 rpm for 10 minutes at 4ºC and the pellets produced were collected. The pellets were washed with the 500 µl 70% ethanol twice and air dried the pellets in room temperature (27ºC±2ºC). Lastly, 50 µl of TE buffer was added to dissolve the DNA pellets and was stored at -20ºC till further use.

3.2.4 Agarose Gel Electrophoresis Analysis for DNA Isolated For Three Selected *Etlingera* spp.

The step of agarose gel electrophoresis was followed the methodology by Appalasamy (2018). The first step was preparing 1% of agarose gel. The 0.5 g of

agarose powder was weighted and mixed with 50 ml of 1 x TAE buffer in a microwavable flask. The mixture was weighted again to know the weight before microwave. The agarose solution was microwave for 1-5 min until the agarose was completely dissolved. After that, the microwavable agarose solution was weighted again. The volume of 1x TAE buffer was added equivalent to the volume of loss during microwave process. The 5 µl of Red Safe was added in the solution. Next, the agarose solution was poured into a gel casting tray with the well comb placed inside. This poured gel was placed at 27±2ºC of room temperature for 30-40 minutes until it completely solidified.

The second step was the methodologies of loading sample, running an agarose gel and analysing gel. Once solidification of the agarose gel was achieved, the agarose gel was placed in the gel box. The gel box was filled by 1 x TAE until the gel was covered. Next, the molecular weight ladder of 2.5 µl of 1 kb ladder of Promega were mixed with 1.0 µl of loading dye on a piece of plastic paraffin film. The molecular weight ladder was added into the first lane of the gel.

Then, 5 µl of samples that mix together with 1.0 µl of loading dye were loaded into the additional wells of the gel. The gel was run at 90 V until the dye line was approximately 75-80% of the way down the gel. Next, the power sources were shut off and the electrodes were disconnected so that the gel can be removed from the gel box. Lastly, the gel was analysed by using UV Analyzer (Alphamager HP).

3.2.5 Quantification and Qualification of Extracted Genomic DNA of Selected *Etlingera* Species

The extracted genomic DNA of all the three samples of *Etlingera* was quantified by measuring optical density (O.D.) at A260 and A280 with a Nanodrop Spectrophotometer (ND2000). Procedure of DNA quantification was done as described by Desjardins & Conklin (2010). Before starting, the upper and lower optical surfaces of the micro volume of spectrophotometer sample retention system were cleaned by wiped it using dry, lint-free lab wipe (Kim Tech). About 2 µl of TE buffer were pipetting onto the lower optical surface. Next, the lever arm were closed and ensured that the upper pedestal comes in contact with the TE buffer and blank measurement was done. Once the blank measurement were achieved, the upper and lower optical surfaces were cleaned again by using dry, lint-free lab wipe. Next, 1 µl of extracted genomic DNA of all the three samples of *Etlingera* sample was pipette onto the lower optical surface. The lever arm was closed and the optical density was measured.

3.2.6 Primer Sequences for Polymerase Chain Reaction (PCR)

The details of selected forward and reverse primers sequence for Polymerase Chain Reaction (PCR) for the three selected *Etlingera* spp. are listed in Table 3.1 below.

Table 3.1 Details of selected primers sequences for PCR

Primer	Code	Sequence	Reference
ITS	ITS1 ITS4	5′-TCCGTAGGTGAACCTGCG-3′ 5′-TCCTCCGCTTATTGATATGC-3′	(Fujita *et al*., 2001)

3.2.7 Polymerase Chain Reaction (PCR) with Internal Transcribed Spacer Region Specific Primers for Selected *Etlingera* spp. Identification

The component and process of Polymerase Chain Reaction (PCR) amplification for *Etlingera* spp. with the specific primer was conducted by following the method that described by Theerakulpisut *et al*. (2012). The PCR component and process are listed in Table 3.2 and 3.3 below.

Table 3.2 Component for PCR in 0.5 ml centrifuge tube

Component	25 µl Reaction (µl)	Final concentration
ddH2O	16.0	-
5 x of Green GoTaq @ plexi buffer	2.5	1.0 X
10 mM of PCR nucleotide mix (dNTP)	0.5	0.2 mM each
10 µM of Forward primer	0.5	0.5 µM
10 µM of Reverse primer	0.5	0.5 µM
5U/µl of Green GoTaq @ DNA Polymerase	0.5	1.5 units
Template DNA	2.0	25 ng
25 mM of $MgCl_2$ Solution	2.5	2.5 mM

Table 3.3 Process, Temperature, Cycles and Conditions of PCR

No	Process	Condition(s)	Cycles
I	Initial denaturation	94ºC for 4 min	1
II	Denaturation	94ºC for 1 min	45
III	Annealing	60ºC for 1 min	45
IV	Initial extension	72ºC for 2 min	45
V	Final extension	72ºC for 4 min	1

3.2.8 Agarose Gel Electrophoresis Analysis for PCR Product of Three Selected *Etlingera* spp.

Agarose gel electrophoresis were followed the method as described in section 3.2.4 for the products of PCR obtained in 3.2.7

3.2.9 DNA Sequencing and BLAST

The sequencing was accomplished by First Base Sdn. Bhd. by applying the same sequences of forward and reverse primer that were used in PCR. The sequences for PCR products from the section 3.2.7 were obtained in Fast Alignment Sequence Test for Application (FASTA) format and were be used to BLAST in Basic Local Alignment Search Tool (BLAST) again the sequences from National Center for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov/). Phylogenetic tree was constructed using Mega7 Software.

3.2.10 Designing Three Selected *Etlingera* spp. Specific Primer

The DNA sequence of PCR product that obtained in FASTA format was copied and the specific primer design was constructed by using Primer3 Plus Program at http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Quantification and Qualification of Extracted Genomic DNA That Isolated From Three Selected *Etlingera* Species.

The genomic DNA that isolated was quantified by measuring the absorbance (Optical Density, O.D.) at 260 nm wavelength by using ThermoFisher NanoDrop™ 2000c Spectrophotometer. This machine identified the concentration of samples, quality and the purity of extracted DNA and determine this variables is important before proceed to Polymerase Chain Reaction (PCR) process. Table 4.1 shows the optical density of three extracted genomic DNA species. From the table, it shows that the DNA concentration for *E. megalocheilos* was the highest meanwhile for *E. elatior* was the lowest.

Table 4.1: Optical density of genomic DNA extraction of three selected *Etlingera* species

DNA Sample	Conc.(ng/µl)	A260	Purity (A_{260}/A_{280})
E. elatior	54.222	1.0844	1.56
E. littoralis	69.345	0.8457	1.71
E. megalocheilos	162.481	3.2496	1.52

Low DNA concentration of *E. elatior* could be due to the incomplete crushing of sample leaves in CTAB buffer. The causes the plant cell walls were not completely lysed. Besides, the purity (A_{260}/A_{280}) of all three samples was low compared to expected ratio of ~1.8 that is generally accepted as "pure" for DNA while a ratio of ~2.0 is generally accepted as "pure" for RNA. The purity of these samples was in range 1.52-1.71, which indicates that low contamination with RNA. DNA samples could absorbs wavelength at near 280nm because the ratio is appreciably lower than that (Hercuvan, 2017). Thus, the reading at 280nm indicated presence of protein, phenol or other contaminants in the isolated DNA samples. Moreover, lower purity also results from changes in pH solution of the buffer extraction during sample isolation. Even though there are only small changes in pH, it will cause the A_{260}/A_{280} to vary. Since the extraction buffer was prepared two months before the isolation of DNA were conducted, change in pH might have occurred (Wilfinger, Mackey, & Chomczynski, 1997).

4.2 Agarose Gel Electrophoresis of Extracted Genomic DNA for the Three Selected *Etlingera* Species

Agarose is a polysaccharide from the seaweed of genus *Gelidium* and *Gracilaria* and it will form a matrix once it has been melted and re-solidified. Function of this agarose gel electrophoresis is to separate the varying size of DNA fragments ranging from 100 base pair up to 25 kilo base pair (Lee, Costumbrado, Hsu, & Kim, 2012). Agarose gel electrophoresis revolutionized DNA fragment separation. Previously, sucrose density gradient centrifugation was used and it provides an approximation DNA size only. Agarose gel (AGE) was chosen compared to polyacrylamide (PAGE) because polyacrylamide has higher resolution

and works best on small nucleic acid analysis such as tRNAs, miRNA, oligonucleotides and proteins. Moreover, preparation polyacrylamide is difficult and time consuming compared to agarose even though comparatively lower in resolution (Stellwagen, 2009).

An amount of 5 µl from the three extracted genomic DNA and 2.5 µl of 1 kb DNA marker was pipetted into the well of the gel, and tested on the electrophoresis set apparatus. After running in 45 minutes at 90 V, the gel was analyzed under UV analyzer. If a clear band was observed on top of the gel, it indicates the successful extraction of DNA. Figure 4.1 and Figure 4.2 show the genomic DNA extraction of three species of *Etlingera*, viewing under UV analyzer. Figure 4.1, in lane 1 is *E. megalocheilos*, and lane 2 is *E. littoralis* whereas Figure 4.2 is the DNA isolated from *E. elatior* leaves.

E. elatior and *E. megalocheilos* shows no visible band and produced a smear along the agarose gel when observed under UV. The observed smear indicate DNA degradation have taken place during the extraction. DNA degradation could be cause by frequent freezing and thawing or handling of DNA samples at room temperature (Lab Tech, 2016). Working on ice and cold DNA extraction reagents such as chilled isopropanol, chilled chloroform:isoamyl alcohol and chilled ethanol is crucial as lower temperature can protect the DNA by slowing the DNAse enzyme activities that could fragmentized the DNA. Chilled ethanol helps the DNA to precipitate more quickly. By exposing the DNA in room temperature eventually results in DNA samples exposed to heat or physical shearing (Bitesize Bio, 2009).

Besides, smearing could also resulted from poor quality sample. The DNA could be contaminated by the protein or contain too much of salts (Mayer, 2018). Contamination of protein could happen during phase separation. An aqueous phase

that collected during the DNA extraction steps could be mistakenly mixed with the organic phase that contains proteins (Singh, 2012). Meanwhile, contamination of soluble salts is due to improperly wash of DNA with isopropanol or ethanol. However, DNA smear is a norm in DNA extraction process and still can be used for DNA region amplification (Lorenz, 2012). For *E. littoralis* (Figure 4.2), a visible band was observed on the top of the Agarose gel. It is high molecular weight band hence indicating good DNA preparation was achieved. Successful DNA extraction for *E. littoralis* could be due to little changes had been made on the protocol by following method done by Keb-Lianes, Gonzalez, & Chi (2012). Liquid nitrogen was used to crush the young fresh leaves samples rather than desiccate the leaves samples in the desiccator. Liquid nitrogen can yield good quality of DNA by reducing the large quantities of secondary metabolites, poly-saccharides and protein (Sika *et al*., 2015).

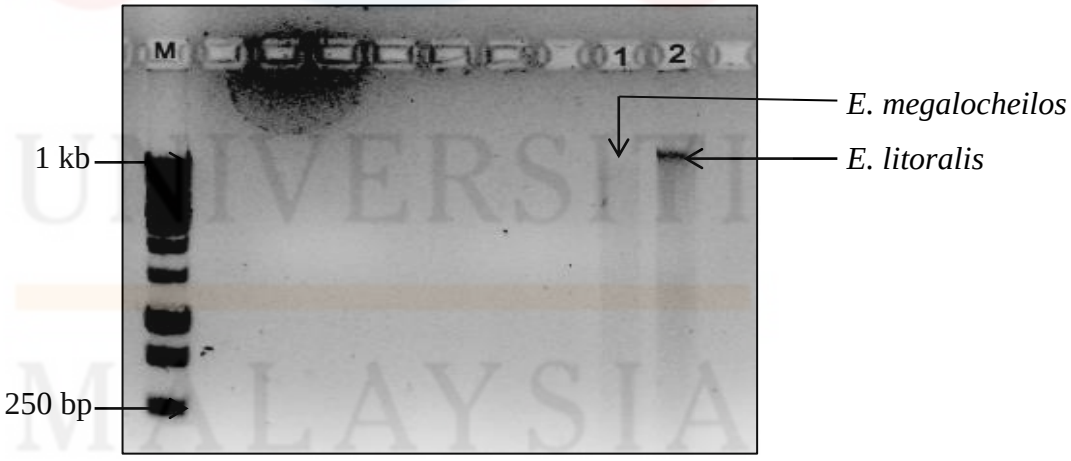

Figure 4.1: Agarose gel electrophoresis of DNA extraction for selected species of *Etlingera*. Lane M is the 1 kb DNA Ladder (Promega, United States), lane 1 is the *E. megalocheilos* and lane 2 is the *E. liitoralis*. Concentration of the agarose gel is 1% and 5 µl DNA was loaded in the well per sample.

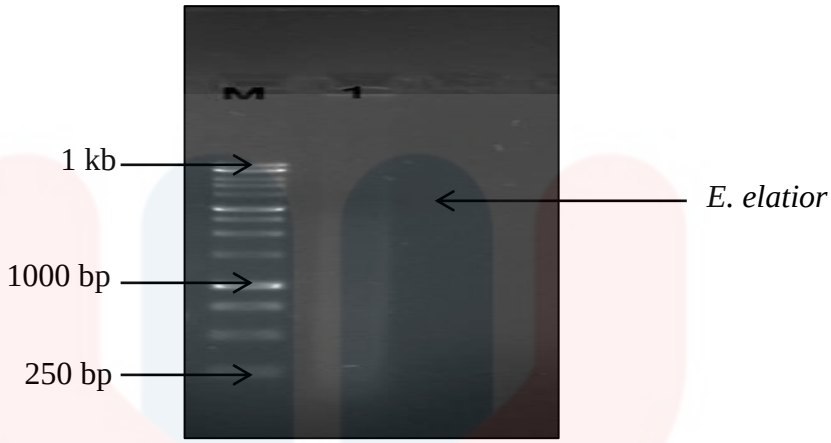

Figure 4.2: Agarose gel electrophoresis of DNA extraction *E.* elatior. Lane M is the 1 kb DNA Ladder (Promega, United States), lane 1 is the DNA of *E. elatior*. Concentration of the agarose gel is 1% and 5 µl DNA was loaded in the well per sample.

4.3 Polymerase Chain Reaction (PCR) of Extracted Genomic DNA for Three Selected *Etlingera* Species

4.3.1 Optimization of PCR Amplification

In general, there are three categories of PCR which are standard PCR, long PCR and multiplex PCR. Unfortunately, there are no single set of conditions that is optimal for all PCR. Therefore, each PCR is required specific optimization for the primer pairs chosen (Grunenwald, 2003). Failure to amplify under optimum conditions can lead to the generation of multiple, undefined and unwanted products, even to the exclusion of the desired product. At the other extreme situation, no product may be produced (Roux, 2003). Grunenwald (2003) also point out several problems that will arise if lack of optimization in PCR such as no detectable PCR product, formation of primer dimer, presence of non-specific bands and smeary

background. Therefore, gradient PCR was performed to find out the optimum temperature for annealing stage in PCR (Prezioso, 2000).

Gradient PCR is a technique that will determine the optimum annealing temperature (T_a). By using Eppendorf Mastercyler Gradient (Appendix A), the gradient PCR can function in a one single run and can evaluate up to 12 different annealing temperatures. Furthermore, in the same run, different concentration of parameters could be tested too. The different gradient temperatures were determined by taking the starting temperature is at 5ºC below of the primer melting point (T_m) (Prezioso & Jahns, 2000). Beside PCR conditions were also can be optimized by changing the volume of PCR reagents which are the Magnesium ($MgCl_2$), DNA template, dNTPs, primer and *Taq* Polymerase.

Figure 4.3 is the optimization of PCR conditions for *E. megalocheilos*. Figure 4.4 and Figure 4.5 is the optimization of PCR conditions for *E. littoralis* whereas Figure 4.6 and Figure 4.7 is optimization of PCR conditions for *E. elatior*. All optimization of PCR conditions is under temperature gradient between 52ºC-62ºC by referring the melting temperature (T_m) of the universal primer ITS 1 (F) and ITS 4 (R). The detail of this pair of primer was shown in Table 4.2 below.

Table 4.2: Detail of primers; ITS 1 (F) and ITS 4 (R)

Primer	Melting Temperature	Sequences
ITS 1 (F)	60.1 ºC	5′-TCCGTAGGTGAACCTGCG-3′
ITS 4 (R)	52.1 ºC	5′-TCCTCCGCTTATTGATATGC-3′

E. megalocheilos (Figure 4.3) showed no result in amplification. After analyzed the failure, few reasons can point out to explain the failure in PCR amplification optimization of *E. megalocheilos*. High concentration of extracted DNA used in the PCR preparation could be one of the reasons. Based on the optical density (O.D.) reading of *E. megalocheilos*, the DNA concentration was ~ 160 ng/µl which is too high for PCR amplification. This failure also occurred in *E. littoralis* (Figure 4.4) where no bands appeared on the gel. This is because, based on Figure 4.1, *E. littoralis* produces a thick band which means the concentration of the DNA ~60 ng/ul considered high. High DNA concentration will make the extra DNA binds with the magnesium ions to stabilize its own structure and eventually hindered the *Taq* polymerase to function (Santalucia, 2015).

To overcome this problem, few protocols being adjusted by reducing volume of DNA template and dilution of extracted genomic DNA samples. Firstly the volume for DNA template for *E. megalocheilos* was reduced from 0.8 µl to 0.6 µl in PCR process but the results still negative. Second trial was done on *E. littoralis*, by diluting first the concentrated extracted genomic DNA in TE buffer to approximate 25 ng/µl and the result is positive as shown in Figure 4.5. However, only *E. littoralis* was proceed for gene amplification as there is no more stock for *E. megalocheilos* for dilution.

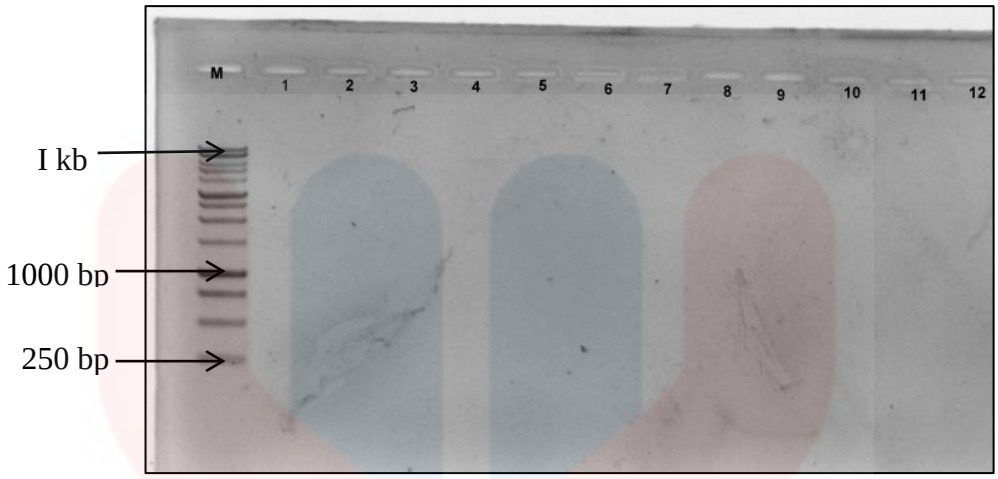

Figure 4.3: Optimization of PCR condition for *E. megalocheilos* by applying 45 cycles in the denaturation, annealing and extension process respectively. Lane M was 1 kb DNA ladder (Promega, United States). Lane 1 to lane 12 contained 5 µl of PCR products that does not visible any band

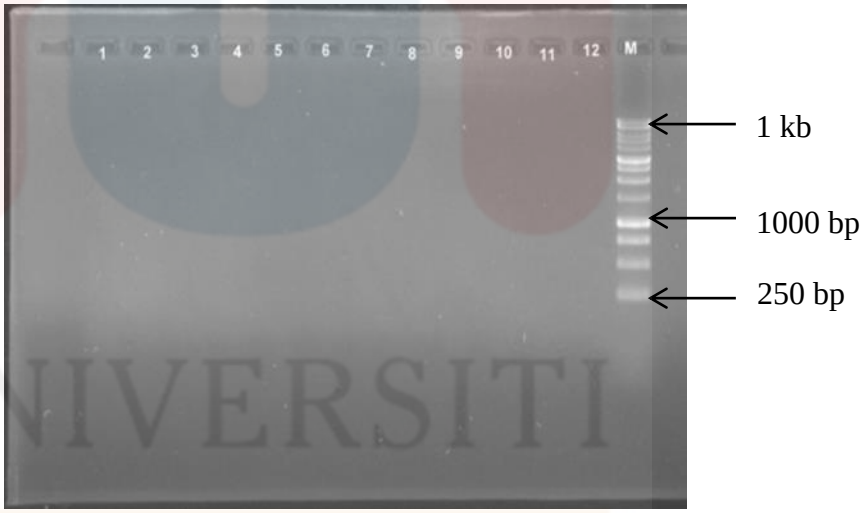

Figure 4.4: Optimization of PCR condition for *E. littoralis* by applying 45 cycles in the denaturation, annealing and extension process respectively. Lane M was 1 kb DNA ladder (Promega, United States). Lane 1 to lane 12 contained 5 µl of PCR products that does not produce a visible band

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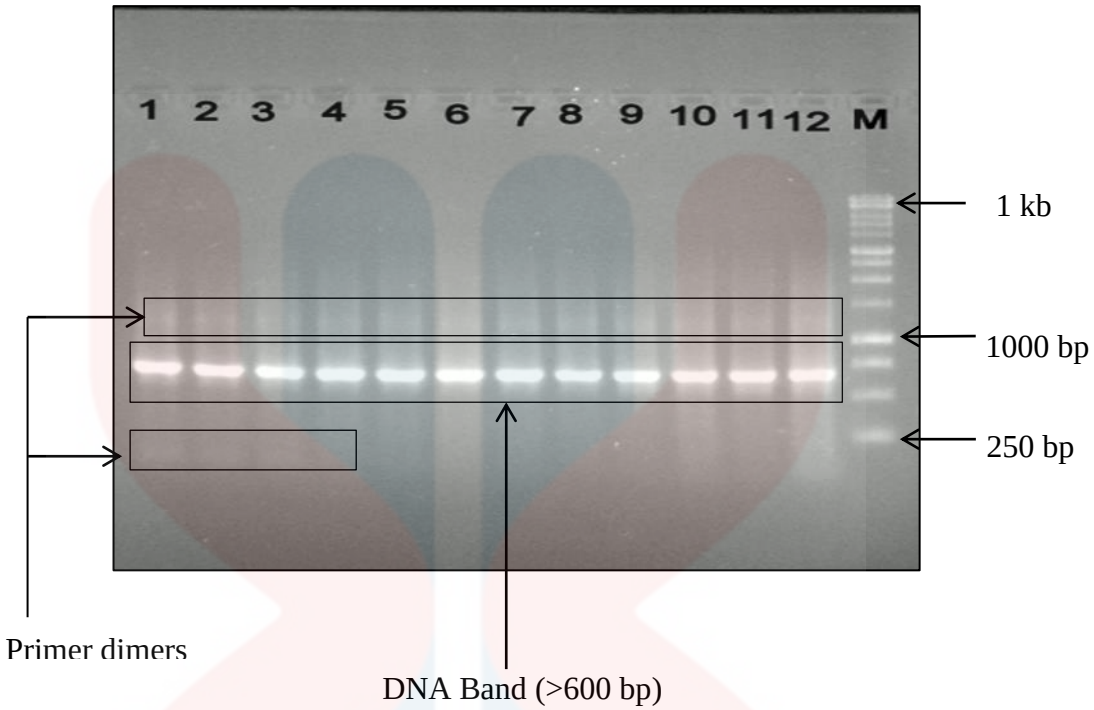


Figure 4.5: Optimization of PCR conditions for diluted *E. littoralis* by applying 45 cycles in the denaturation, annealing and extension process respectively. Lane M was 1 kb DNA ladder (Promega, United States). Lane 1 to lane 12 contained 5 µl of PCR products.

For *E. elatior*, the first trial optimization of PCR condition failed due to different reason. The negative results were shown in Figure 4.6. Based on that figure, only smeary background observed in lane 2 and 5 and very low size of band were produced in lane 11 and 12. The low size DNA band (<250 bp) was very small compared to the expected base pair (562-630 bp) (Theerakulpisut *et al*., 2012). Thus second PCR amplification trial was conducted by using different extracted genomic DNA of *E. elatior*. The volume for DNA template was re-calculated again by using $M_1V_1=M_2V_2$ formula by considering the concentration of extracted genomic DNA (O.D.) in order to find out the best volume for DNA template in PCR. The second trial of PCR for this species was re-do by using the same PCR profile and the positive results were achieved as shown in Figure 4.7.

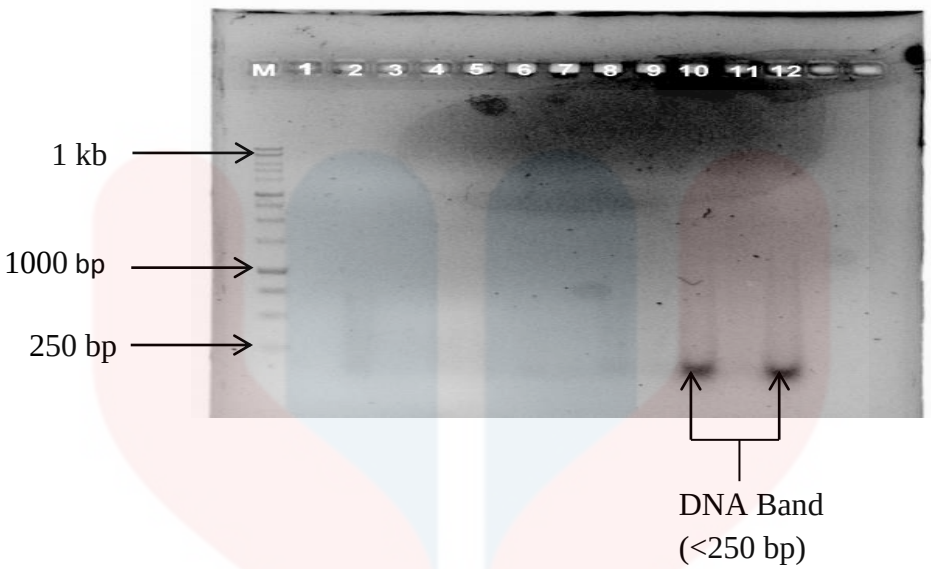


Figure 4.6: Optimization of PCR condition for *E. elatior* by applying 45 cycles in the denaturation, annealing and extension process respectively. Lane M was 1 kb DNA ladder (Promega, United States). Lane 1 to lane 12 contained 5 µl of PCR products.

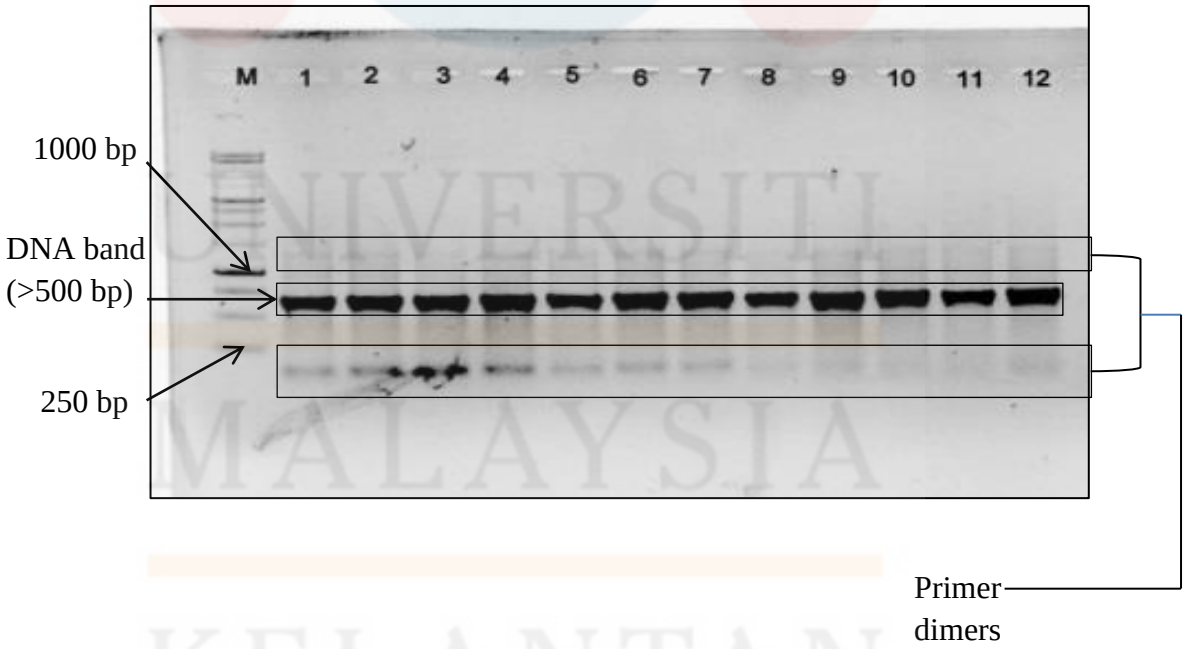


Figure 4.7: Optimization of PCR condition for *E. elatior* (different extracted genomic DNA) by applying 45 cycles in the denaturation, annealing and extension process respectively. Lane M was 1 kb DNA ladder (Promega, United States). Lane 1 to lane 12 contained 5 µl of PCR products.

Based on those two results, optimization of PCR conditions for two species; *E. littoralis* (Figure 4.5) and *E. elatior* (Figure 4.7) and were succeed. However, there is production of primers dimer in optimization of PCR product for both species. Based on the Figure 4.5 and 4.7, primer dimers were seen above and below the DNA band for both species. Primer dimers are products of non-specific annealing and primer elongation events. It forms when the PCR reagents are mixed together especially if the procedure carried out in room temperature. Later this primer dimer will competes with the formation of specific PCR product leading to less successful PCR process (Roche, 1999). For *E. littoralis*, primer dimers were seen below the DNA band at approximate 250 bp which is from lane 1 to lane 4 and above the DNA band in between 1000 bp-1500 bp from lane 1 to 12. Meanwhile for *E. elatior*, primer dimers were seen in all lanes which are in lane 1 to 12 above the DNA band in between 1000 bp-1500 bp and below the DNA band at approximate 250 bp. Despite that, there are several lane that produce less visible primer dimers comparatively such as in *E. littoralis*, where no primer dimers below DNA band from lane 6 to 8. In contrast, in *E. elatior*, all primer dimers were visible in all lanes but in lane 8 until 12 produce less visible primer dimers.

Based on the analyzed gel, two annealing temperatures were chosen for *E. littoralis* and *E. elatior*. For *E. littoralis*, the optimum temperature for annealing that been chosen are 56.4ºC which are the temperature got from lane 6. Differs for *E.*

elatior, where optimum temperature for annealing is 58.9ºC got from lane 8. Several reasons were considered in choosing this optimum temperature. Firstly, both of the bands produced are high in intensity. Second, because of less visible primer dimers and lastly, less background smear.

4.3.2 Amplification of Internal Transcribed Spacer Region of the Genomic DNA of Selected *Etlingera* Species.

There are two internal transcribed spacer (ITS) region of plant which are ITS 1 located between 18S and 5.8S rRNA genes and ITS 2 located between 5.8S and 26S rRNA genes. These target gene were amplified by using universal primers (ITS 1 and ITS 4) and the estimated size or base pair of the PCR product for the rDNA sequence amplification was approximately between 562-630 bp (Theerakulpisut *et al*., 2012). Internal trasncribed spacer region for *E. littoralis* and *E. elatior* were amplified with the oprimum temperature of 56.4ºC and 58.9ºC by using PCR machine (Eppendorf Mastercycler Nexus, United States). The results for the amplification of Internal Transcribed Spacer (ITS) region were shown in figure below.

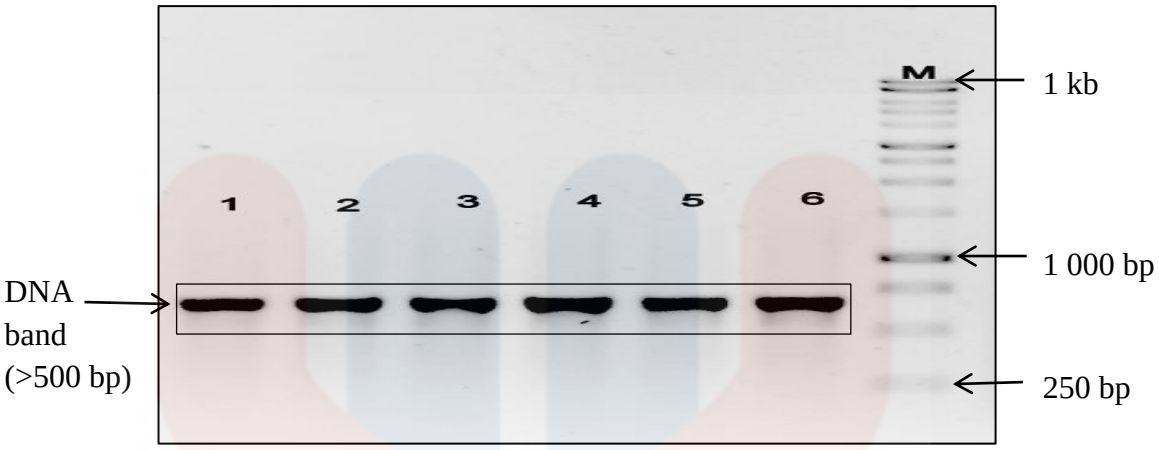


Figure 4.8: Amplification of Internal Transcribed Spacer (ITS) region for diluted *E. littoralis* by applying 45 cycles in the denaturation, annealing and extension process respectively in optimum temperature of 56.4ºC for annealing (T_a). Lane M was 1 kb DNA ladder (Promega, United States). Lane 1 to lane 6 contained 5 µl of PCR products.

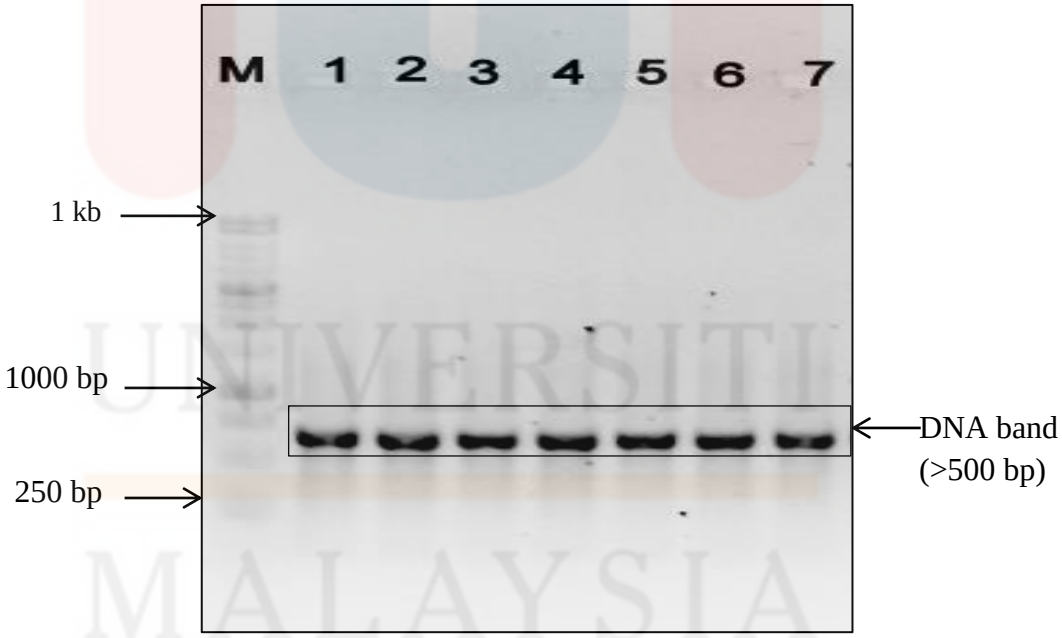


Figure 4.9: Amplification of Internal Transcribed Spacer (ITS) region for *E. elatior* by applying 45 cycles in the denaturation, annealing and extension process respectively in optimum temperature of 58.9ºC for annealing (T_a). Lane M was 1 kb DNA ladder (Promega, United States). Lane 1 to lane 6 contained 5 µl of PCR products.

4.4 DNA Sequencing and BLAST of *Etlingera* Species

PCR products of *E. elatior* and *E. littoralis* were sent for sequencing. The chromatogram for both forward and reverse sequences of these two samples contained high levels of background noise on the front and at the back of the sequences. But in the middle part of the chromatogram for both forward and reverse sequences of these two samples produced fairly good sequencing result indicates low noise background and less N sign. The chromatograms for forward and reverse sequences of the two species were shown in APPENDIX B.

By blasting in the Basic Local Alignment Search Tool (BLAST) against the sequences from National Center for Biotechnology Information (NCBI) database, these two species were successfully identified. These samples were correctly belongs to genus of *Etlingera* by comparing the similarities of closest match from Nucleotide BLAST.

Tables 4.3: Identification of DNA Samples of *Etlingera* by comparing the percentage of similarities with the closest match from nucleotide BLAST. (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch)

DNA samples	Closest match from Nucleotide BLAST (Sequence ID)	% Similarity
E. elatior	*E. elatior* (AF414465.1)	99%
E. littoralis	*E. littoralis* (AF414461.1)	98%

Next, phylogenetic tress was constructed by using MEGA7 software and the Clustal W was used for sequence alignment. Phylogenetic trees consists four types of construction which are Neighbour-Joining (NJ), Unweighted Pair Group Method with Arithmetic Mean (UPGMA), Maximum parsimony (MP) and Maximum Likelihood (ML). The Neighbour-Joining (NJ) was selected in this research. Two species which are *Zingiber wrayi* and *Zingiber officinale* were selected as the outgroup. *Zingiber* is one of genus in Zingiberaceae. The phylogenetic tree for *E. elatior* was shown in Figure 4.10 and phylogenetic tree of *E. littoralis* was shown in Figure 4.11. Meanwhile, Figure 4.12 shows comparison of phylogeny relationship between all species from phylogenetic tree of *E. elatior* and *E. littoralis*.

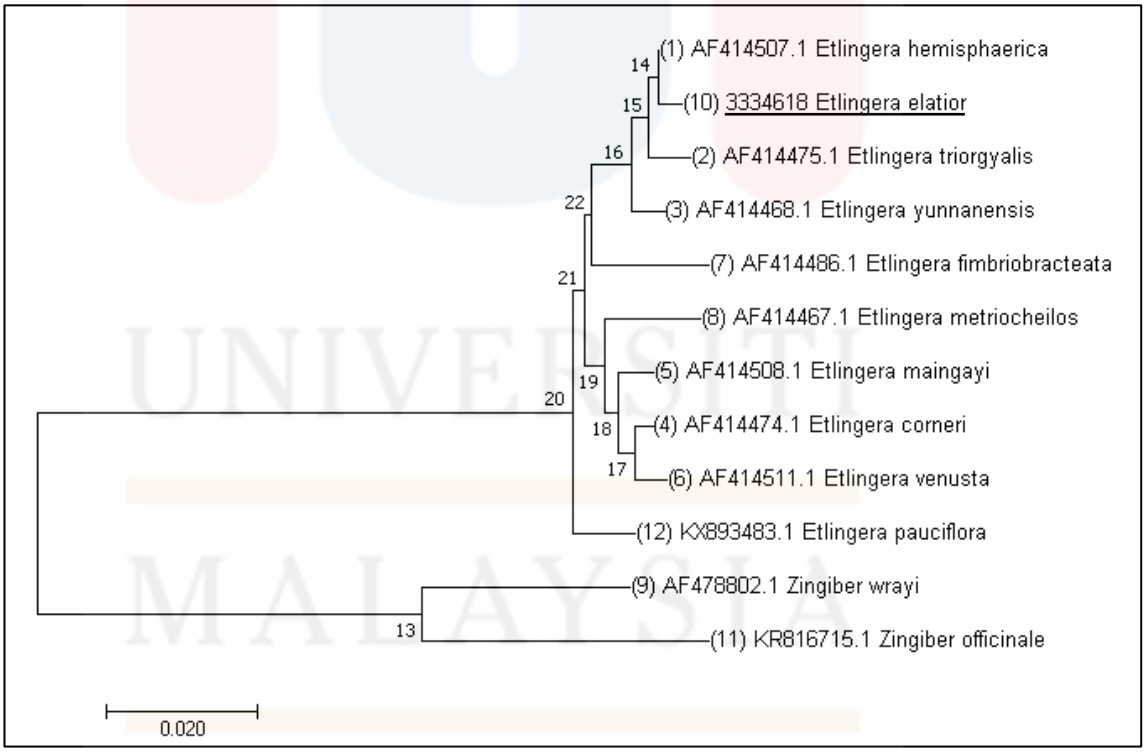


Figure 4.10: The phylogenetic relationship between *E. elatior* (underlined) with the closest sequences found in Nucleotide Blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch). The percentage of similarity that had taken for comparing with *E. elatior* were 99%, 98% and 97%. The phylogenetic relationship were constructed using Mega7.

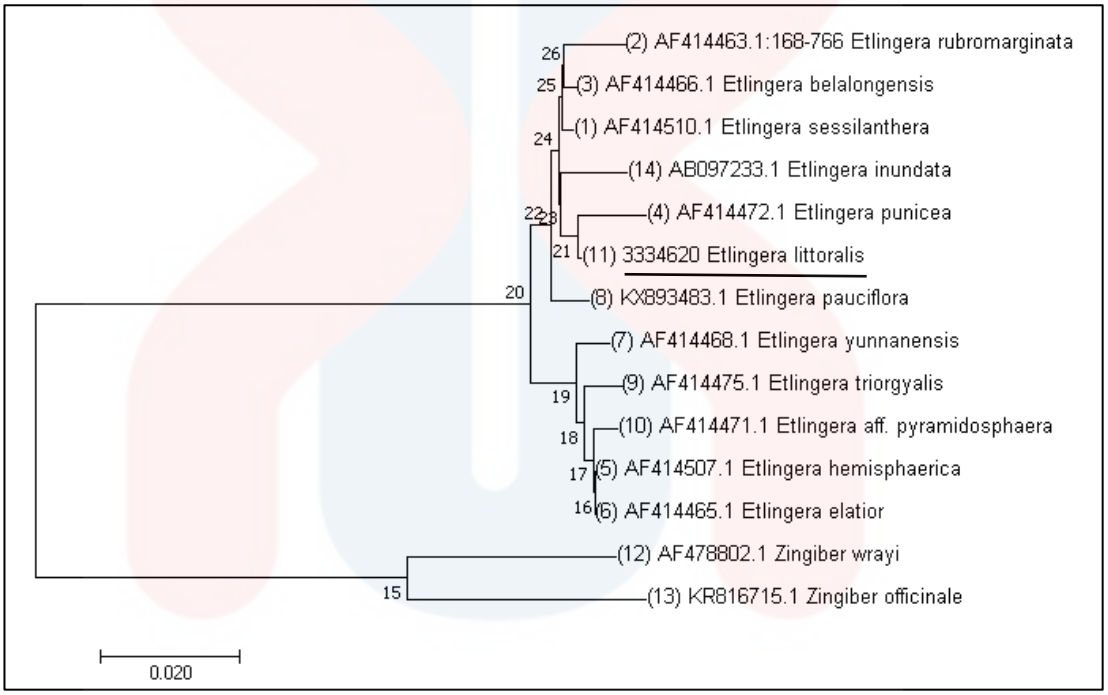


Figure 4.11: The phylogenetic relationship between *E. littoralis* (underlined) with the closest sequences found in Nucleotide Blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch). The percentage of similarity that had taken for comparing with *E. littoralis* were 99%, 98% and 97%. The phylogenetic relationship were constructed using Mega7.

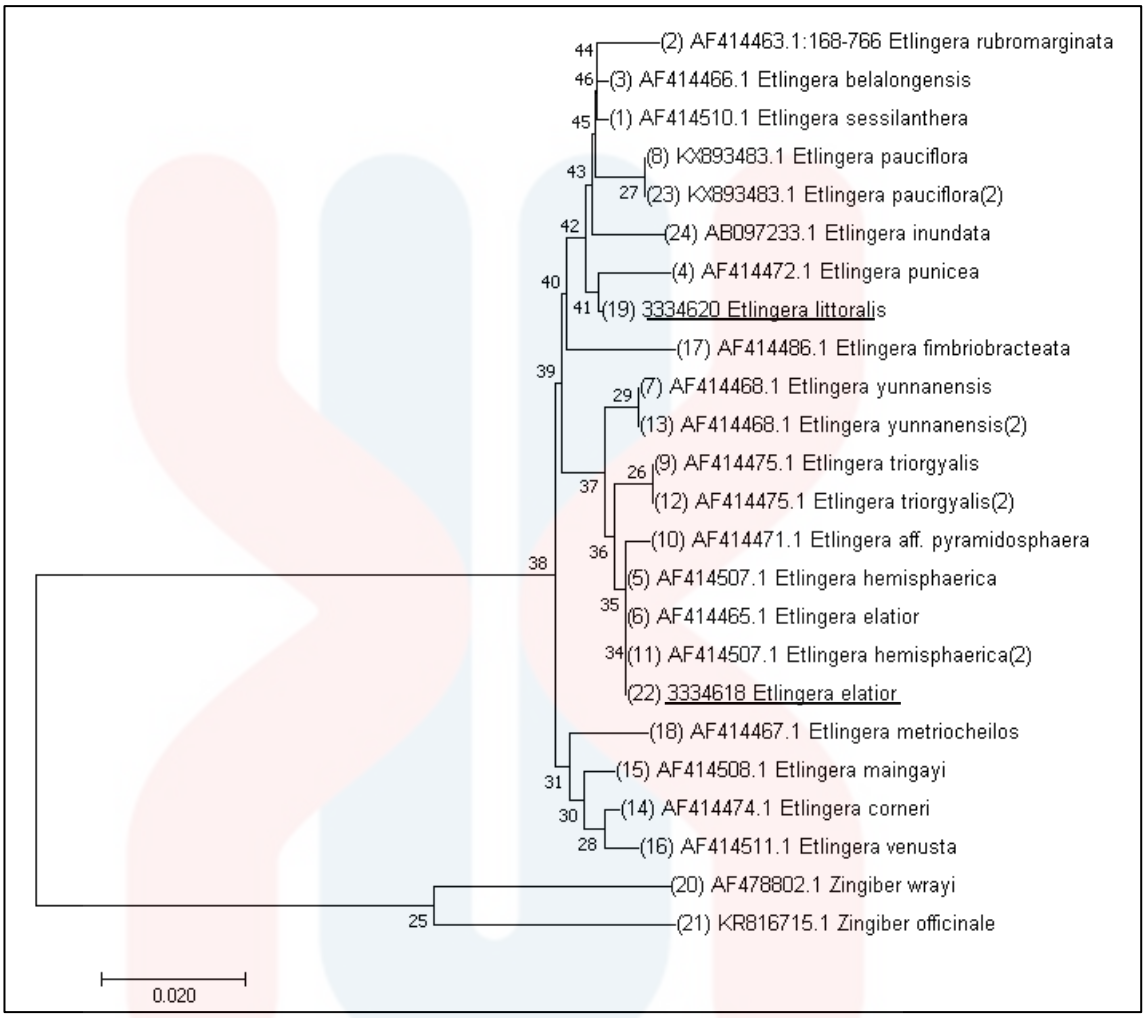


Figure 4.12: The comparison of phylogeny relationship between *E. littoralis* (underlined) and *E. elatior* (underlined) with the closest sequences found in Nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch). The phylogenetic tree was constructed using MEGA7.

Based on the comparison of phylogeny relationship between *E. littoralis* and *E. elatior* above (Figure 4.12), three major cluster were produced where the *E. littoralis* and *E. megalocheilos* located in first and second cluster. In the first cluster, eight species including *E. littoralis* clustered together with the *E. rubromarginata*, *E. belalongensis, E. sessilanthera, E. pauciflora, E. inundata, E. punicea* and *E. fimbriobracteate*. Its indicate these species are more related in recent common ancestor than the second and third cluster.

Meanwhile, *E. yunnanensis, E. triorgyalis, E. aff. pyramidosphaera, E. hemisphaerica* were clustered together with *E. elatior*. Whereas, *E. metriocheilos. E. maingayi, E. corneri* and *E. venusta* were in the third major cluster and share the most less related recent common ancestor. *Z. wrayi* and *Z. officinale* remain as the outgroup.

4.5 Designization of *E. elatior* and *E. littoralis* Specific Primer

The FASTA format for *E. elatior* and *E. littoralis* were copied and used in designing primer by using Primer3 Plus (http://www.bioinformatics.nl/cgi bin/primer3plus/primer3plus.cgi). The target sequences were choosed and copied in Primer3 Plus software. From the FASTA format of these two spesies, the primer were designed. The details of designed primer for *E. elatior* and *E. littoralis* were shown in Table 4.3.

Table 4.4: Details of designed primer for *E. elatior* and *E. littoralis*

Species	Primer	Length	Melting temperature (T_m)	GC Content
E. elatior	Forward: GCACCAAGGAACAACGAACT	20 bp	60.2ºC	50.0%
	Reverse: AAAGCCTTGGGCACAACTT	19 bp	59.7ºC	47.4%
E. littoralis	Forward: TCTTTGAACGCAAGTTGTGC	20 bp	60.0ºC	45.0%
	Reverse: GAGGGCGACGTTCTATTCAC	20 bp	59.7ºC	55.0%

Next, the sequences of forward and reverse primer were blast against the nucleotide sequence database from NCBI to check or testing its specificity. Based on the blasting result, the primer sequences for *E. elatior* sample were closely matched by 100% with the *E. elatior*, 18S ribosomal RNA gene, partial sequence; and internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence (Sequence ID: AY769845.1). However, for *E. littoralis* sample, there is no perfectly match with any *E. littoralis* nucleotides sequences database from NCBI. But, the forward and reverse primer sequences at least match with many species in the genus of *Etlingera* which are *E. venusta* (Sequence ID: AF414511.1), *E. sessilanthera* (Sequence ID: AF414510.1), *E. punicea* (Sequence ID: AF414472.1), *E. maingayi* (Sequence ID: AF414508.1), *E. fimbriobractaeta* (Sequence ID: AF414506.1).

The primers that had been designed were a species specific primer for *E. elatior* and *E. littoralis*. The designed primers also can be tested in *in silico* PCR primer. *In silico* PCR is a computational tools used to calculate PCR results theoritically using a given set of primers sequences. This *in silico* PCR will analyses the individual primers or pair of primers. Moreover, it will calculate the melting temperature for standard and degenerate oligonucleotides, analyses primers properties, dilution and resuspension calculator (Kalendar, Lee & Schulman, 2011). But due to time restriction in this research, *in silico* PCR was not able to perform.

Compared to the universal primer; ITS 1 and ITS 4, the species specific primer can be purposely designed to amplify the particular gene which gives a greater chance of a successful PCR outcome. However, species specific primer must be tested first and optimization of PCR reactions is a very crucial. This developed PCR-assay must be evaluated for the specificity and sensitivity. Moreover, this

develop primer should be tested on various field samples (Wallinger *et al*., 2012). But there are some limitation in developed PCR assay where to get the reliable primers were challenging. For an example, Wallinger *et al*., (2012) stated in their research of plant identification using species specific primers targeting chloroplast DNA, the selected chloroplast sequences has highly ambiguous alignment caused by high rated of indels – the insertion, deletions and inversion in the chloroplast genome (Ingvarsson, Ribstein & Taylor, 2003). Thus, selecting either universal or specific primers is depends on the purpose or the variables of the research. For an example, two universal primer based on 12S and 16S rRNA loci region were chosen by Kitano, Umetsu, Tian & Osawa (2007) because they were identifying species level among the vertebrates which are cow, human, horse, sheep, pig, dog and mouse.

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

The genomic DNA of three species of *Etlingera* was successfully isolated by using CTAB method with some modification for *E. littoralis*. The optimization of PCR conditions were successfully done on two species which are *E. elatior* and *E. littoralis*. The PCR condition for *E. megalocheilos* was unsuccessful due to lack of extracted genomic DNA stock. The amplification of Internal Transcribed Spacer (ITS) region as the target region with the specific primer was successfully amplified. The nucleotide sequences of two species were blast in the National Center for Biotechnology Information (NCBI) and the species were correctly belongs to genus of *Etlingera*. Phylogenetic trees were constructed by using MEGA7 software to show the phylogeny relationship among the selected species and *E. littoralis* shared the most common recent ancestor traits. Lastly, the specific forward and reverse primers were designed in Primer3 Plus software. The designed primer for *E. elatior* was 5'GCACCAAGGAACAACGAACT'3 (forward) and 5'AAAGCCTTGGGCACAACTT'3 (reverse). Meanwhile, for *E. littoralis* was 5'TCTTTGAACGCAAGTTGTGC'3 (forward) and 5'GAGGGCGACGTTCTATTCAC'3 (reverse). In a nutshell, the species identification for *E. elatior* and *E. littoralis* were successfully identified and the molecular marker specifically for this species is developed.

5.2 Recommendation

There is a need to further study in molecular marker for this genus as limited research was done on genus *Etlingera.* The designed primer should proceed to wet lab analysis after running in the *in silico* PCR. The designed primers should be tested on *E. littoralis* or *E. elatior* together with the other species of *Etlingera* to confirm its applicability as the species specific primer. Therefore the applicability can be more precise. Besides, gene such as MaturaseK gene (*MatK*), trnH-psbA and ribulose-bisphosphate carboxylase gene (*rbcL*) is the other three plant barcode marker that are widely used in species identification other than nuclear ribosomal Internal Transcribed Spacer (ITS) in the future study.

REFERENCES

Abdelmageed, A. H. A., Faridah, Q. Z., Norhana, F. M. A., Julia, A. A., & Kadir, M. A. (2011). Micropropagation of *Etlingera elatior* (Zingiberaceae) by using axillary bud explants. *Journal of Medicinal Plants Research, 5*(18), 4465-4469.

Agbagwa, I. O., Datta, S., Patil, P. G., Singh, P., & Nadarajan, N. (2012). A protocol for high-quality genomic DNA extraction from legumes. *Genetic and Molecular Research, 11*(4), 4632-2639.

Al-Attas, R. A., Al-Khalifa, M., Al-Qurashi, A. R., Badawy, M., & Al-Gualy, N. (2000). Evaluation of PCR, culture and serology for the diagnosis of acute human brucellosis. *Journal Article of Annals of Saudi Medicine, 20*(3), 3-4.

Alice, L. A., & Campbell, C. S. (1999). Phylogeny of *Rubus* (Rosaceae) based on nuclear ribosomal DNA internal transcribed spacer region sequences. *American Journal of Botany, 86*(1), 81-97.

Apavatjrut, P., Anuntalabhochai, S., Sirirugsa, P., & Alisi, C. (1999). Molecular markers in the identification of some early flowering *Curcuma Longa* (Zingiberaceae) species. *Annals of Botany, 84*(4), 529-534.

Appalasamy, S. (2018). *Genetics and molecular biology: students guide.* Universiti Malaysia Kelantan, Kelantan.

Bitesize Bio. (2009). DNA Precipitation: Ethanol vs. Isopropanol. Retrieved from https://bitesizebio.com/2839/dna-precipitation-ethanol-vs-isopropanol/

Caskey, C. T., & Metzker, M. L. (2009). Polymerase Chain Reaction (PCR). Retrieved from http://www.els.net/WileyCDA/ElsArticle/refId-a0000998.html

Chan, L. K., & Thong, W. H. (2004). In vitro propagation of Zingiberaceae species with medicinal properties. *Journal of Plant Biotechnology, 15*(2), 35-37.

Cheng, D., Xiao, H., Gu, J., & Xiao, P. G. (2015). *Potentilla* and *Rubus* medicinal plants: potential non Camellia tea resources. *Medicinal Plants, Chemistry Biology and Omics, 4*(3), 227-237.

Dagupta, S. (2016). How many plant species are there in the world? Scientists now have an answer. Retrieved from https://news.mongabay.com/2016/05/many-plants-world-scientists-may-now-answer/

Das, A., Kesari, V., Satyanarayana, V., & *et al.*, (2011). Genetic relationship of *Curcuma* species from Northeast India using PCR-based markers. *Molecular Biotechnology, 49*, 65-76. doi:10.1007/s12033-011-9379-5

Desjardins, P., & Conklin, D. (2010). NanoDrop microvolume quantitation of nucleic acids. *Journal of Visualized Experiments* : JoVE, (45), 2565. doi:10.3791/2565

Devi, K. D., Punyarani, K., Singh, N. S., & Devi, H. S. (2013). An efficient protocol for total DNA extraction from the members of order Zingiberales- suitable for diverse PCR based downstream applications. *SpringerPlus, 2*(1), 669. doi:10.1186/2193-1801-2-669

Edwards, M. C., & Gibbs, R. A. (1994). Multiplex PCR: advantages, development, and applications. *Genome Research, 3*(4), S65-S75.

Einsele, Hebart, Müller, Bowden, Burik, V., Engelhard & Schumacher. (1997). Detection and identification of fungal pathogens in blood by using molecular probes. *Journal of Clinical Microbiology 35*(6), 1353–1360.

Fatien, N. S. Z. (2018). *Initiation of In Vitro of Etlingera megalocheilos*. (Unpublished degree's thesis). University of Kelantan, Kelantan, Malaysia.

Fujita, S. I., Senda, Y., Nakaguchi, S., & Hashimoto, T. (2001). Multiplex PCR Using Internal Transcribed Spacer 1 and 2 Regions for Rapid Detection and Identification of Yeast Strains. *Journal of Clinical Microbiology, 30*(10), 3617-3622. doi:10.1128/JCM.39.10.3617-3622.2001

Garibyan, L. & Avashia, N. (2013). Research techniques made simple: polymerase chain reaction (PCR). *The Journal of Investigative Dermatology, 133*(3), 6.

Ghosh, S., Majumder, P. B., & Mandi, S. S. (2011). Species-specific AFLP markers for identification of Zingiber officinale, Z. montanumand Z. zerumbet (Zingiberaceae). *Genetics and Molecular Research, 10*(1), 218-229.

Grassi, F., Labra, M., & Minuto, L. (2009). Haplotype richness in refugial areas: Phylogeographical structure of *Saxifraga callosa*. *Journal of Plant Research, 122*(4), 377-367. doi:10.1007/s10265-009-0230-z

Grunenwald, H. (2003). Optimization of polymerase chain reactions. In *PCR protocols* (pp. 89-99). Humana Press.

Hammel, B. E., Grayum, M. H., Herrera, C., & Zamora, N. (2003). *Manual of Plants of Costa Rica, Volume III: Monocotyledons (Orchidaceae-Zingiberaceae)*. Missouri Botanical Garden Press.

Harith, J. M., Retno, A., & Ishak, A. (2013). Determination of phylogenetic and molecular characteristics of three Malaysian ginger cultivars (*Zingiber officinale*) using microsatellite DNA. *Genetic Molecular Research, 10*(1), 419-428.

Hercuvan. (2017). how to determine the concentration and purity of a DNA sample? Retrieved from https://www.hercuvan.com/how-to-determine-the-concentration-and-purity-of-a-dna-sample/

Huggett, J., Dorai-Raj, S., Falinska, A., & O'Grady, J. (2014). Molecular Diagnostics: An Introduction. *Molecular Diagnostics*.

Ingvarsson, P. K., Ribstein, S., & Taylor, D. R. (2003). Molecular evolution of insertions and deletion in the chloroplast genome of Silene. *Molecular Biology and Evolution, 20*(11), 1737-1740.

Ismail, N. A., Rafii, M. Y., Mahmud, T. M. M., Hanafi, M. M., & Miah, G. (2016). Molecular markers: a potential resource for ginger geneticdiversity studies. *Molecular Biology Report, 43*, 1347–1358. doi:10.1007/s11033-016-4070-3

Iwen, P. C., Hinrichs, S., & Rupp, M. (2002). Utilization of the internal transcribed spacer regions as molecular targets to detect and identify human fungal pathogens. *Medical Mycology, 40*(1), 87-109.

Jaleel, K., & Sasikumar, B. (2010). Genetic diversity analysis of ginger (*Zingiber officinale Rosc*.) germplasm based on RAPD and ISSR markers. *Scientia Horticulture, 125*, 73-76. doi:10.1016/j.scienta. 2010.02.024

Jatoi, S., Kikuchi, A., Mimura, M., Yi, S., & Watanabe, K. (2008). Relationships of *Zingiber* species, and genetic variability assessment in ginger (*Zingiber officinale*). *Breed Science, 58*, 261-270.

Kadiyam, R. R. (2015). Polymerase Chain Reaction (PCR) : Principle, Procedure, Components, Types and Applications. Retrieved from https://laboratoryinfo.com/polymerase-chain-reaction-pcr/

Kaewsri, W., Paisooksantivatana, Y., Veesommai, U., Eiadthong, W., & Vadrodaya, S. (2007). Phylogenetic analysis of Thai Amomum (Alpinioideae: Zingiberaceae) using AFLP markers. *Natural Science, 41*(213-226).

Kalendar, R., Lee, D., & Schulman, A. H. (2011). Java web tools for PCR, in silico PCR, and oligonucleotide assembly and analysis. *Genomics, 98*(2), 137-144.

Kalia, R. K., Rai, M. K., Kalia, S., Singh, R., & Dhawan, A. (2011). Microsatellite markers: an overview of the recent progress in plants. *Euphytica, 177*(3), 309-334.

Käss, E., & Wink, M. (1997). Phylogenetic relationships in the Papilionoideae (family Leguminosae) based on nucleotide sequences of cpDNA (rbcL) and ncDNA (ITS 1 and 2). *Molecular Phylogenetics and Evolution, 8*(1), 65-88.

Keb-Lianes, M., Gonzalez, G. & Chi, Bartolome. (2012) A rapid and simple method for small-scale DNA extraction in Agavaceae and other tropical plants. Plant Molecular Biology Reporter, Retrieved from https://www.researchgate.net/publication/257178589_

Khaw, S. (2001). The genus *Etlingera* (Zingiberaceae) in Peninsular Malaysia including a new species. *Gard Bull Singapore, 53*(1-2), 191-239.

Kitano, T., Umetsu, K., Tian, W., & Osawa, M. (2007). Two universal primer sets for species identification among vertebrates. *International Journal of Legal Medicine, 121*(5), 423-427.

Kress, W. J., Prince, L. M., & Williams, K. J. (2002). The phylogeny and a new classification of the gingers (Zingiberaceae): evidence from molecular data. *American Journal of Botany, 89*(10), 1682-1696.

Krings, T. N., (2009). *Flowering Plants The Biology and Evolution of Fossil Plants*. Academic Press.

Lab Tech. (2016). DNA storage: to freeze or not to freeze?. Retrieved from https://www.ttplabtech.com/whats-new/news/blog-dna-storage-to-freeze-or-not-to-freeze/

Lachumy, S. J. T., Sasidharan, S., Sumathy, V., & Zuraini, Z. (2010). Pharmacological activity, phytochemical analysis and toxicity of methanol extract of *Etlingera elatior* (torch ginger) flowers. *Asian Pacific Journal of Tropical Medicine, 3*(10), 769-774.

Larsen, K., Ibrahim, H., Khaw, S. H., & Saw, L. G. (1999). *Gingers of Peninsular Malaysia and Singapore*. Natural History Publications (Borneo).

Lee, P. Y., Costumbrado, J., Hsu, C.Y., & Kim, Y. H. (2012). Agarose gel electrophoresis for the separation of DNA fragments. *Journal of Visualized Experiments* (62), 3923. doi:10.3791/3923

Liwanag, H. (2012). Making TE Buffer Solution. Retrieved from https://sites.google.com/site/sciencebridgetechsites/sop-list/purification-sops/solutions/making-te-buffer-solution

Lorenz, T. C. (2012). Polymerase chain reaction: basic protocol plus troubleshooting and optimization strategies. *Journal of Visualized Experiments*: JoVE, (63).

Mayer, M. (2018). What Causes Smearing in Electrophoresis? Retrieved from https://sciencing.com/causes-smearing-electrophoresis-6404726.html

Mendez, A. M. V., Moctezuma, J., & Lao, J. (2004). Propagation of torch ginger (*Nicolaia elatior* (Jack.) Horan) through in vitro shoot tip culture. *Propagation Ornamental Plants, 4*(2), 48-52.

Möller, M., & Cronk, Q. C. (1997). Origin and relationships of *Saintpaulia* (Gesneriaceae) based on ribosomal DNA Internal Transcribed Spacer (ITS) sequences. *American Journal of Botany, 84*(7), 956-965.

Mullis, K. B. (1990). The unusual origin of the polymerase chain reaction. *Scientific American, 262*(4), 56-65.

Nascimento, J. L., Paulo C., Freitas, Giuliana L & Silva. (2000). Antibacterial Activity of Plant Extracts And Phytochemicals on Anti Biotic Resistant Bacteria. *Brazilian Journal of Microbiology, 31*, 247-256.

NCBI. (2017). Polymerase Chain Reaction (PCR). Retrieved from https://www.ncbi.nlm.nih.gov/probe/docs/techpcr/

Paisooksantivatana, Y., Kako, S., & Seko, H. (2001). Genetic diversity of *Curcuma alismatifolia* (Zingiberaceae) in Thailand as revealed by allozyme polymorphism. *Genetic Resources Crop Evolution, 00*, 1-7.

Prezioso, V. R., (2000, April 12) Using Gradient PCR to Determine the Optimum Annealing Temperature. Retrieved from https://www.drugdiscoveryonline.com/doc/using-gradient-pcr-to-determine-the-optimum-a-0002doi:10.1023/A:1012078728003

Roche. (1999). Optimization of Reactions to Reduce Formation of Primer Dimers. Retrieved from https://www.gene-quantification.de/roche-primer-dimer.pdf

Roux, K. H. (2003). Optimization and Troubleshooting in PCR. Retrieved from http://cshprotocols.cshlp.org/content/2009/4/pdb.ip66.full

Santalucia, J. (2015). At the End of PCR, Amplification Efficiency Is Not Exponential Because the Primers or NTPs Are Exhausted or the Polymerase Looses Activity. Retrieved from https://www.dnasoftware.com/myth-6-at-the-end-of-pcr-amplification-efficiency-is-not-exponential-because-the-primers-or-ntps-are-exhausted-or-the-polymerase-looses-activity/

Semagn, K., Bjørnstad, Å., & Ndjiondjop, M. (2006). An overview of molecular marker methods for plants. *African Journal of Biotechnology, 5*(25).

Sharmila, R. (2018). *Distribution and Micropropagation of Selected Species of Zingiberaceae At Lojing Highlands, Kelantan And Sungai Gadong, Tasik Temengor, Perak*. (Unpublished degree's thesis). University of Kelantan, Kelantan, Malaysia.

Sika, K. C., Kefela, T., Adoukonou-Sagbadja, H., Ahoton, L., Saidou, A., Baba-Moussa, L., ... & Gachomo, E. W. (2015). A simple and efficient genomic DNA extraction protocol for large scale genetic analyses of plant biological systems. *Plant Genetic*, *1*, 43-45.

Singh, G. (2012). DNA Extraction Methods & Estimation. Retrieved from https://www.slideshare.net/Geet_singh/dna-extraction-12082102

Singhi, Gyan, Mishra, Khan, A., & Singhi, S. B. (2008). Molecular Markers in Plants *Molecular Plant Breeding: Principle, Method and Application. Crop Protection, 77, 74-86.*

Sirirugsa, P. (1999). Thai Zingiberaceae: species diversity and their uses. Retrieved from http://www.iupac.org/symposia/proceedings/phuket97/sirirugsa.html

Stellwagen, N. C. (2009). Electrophoresis of DNA in agarose gels, polyacrylamide gels and in free solution. *Electrophoresis*, *30*(S1), S188-S195.

Sun, Y. L., Park, W. G., Oh, H. K., & Hong, S. K. (2013). Plant-specific primers for the amplification of the nrDNA ITS region in fungus-associated *Pulsatilla* species *Journal of Medicinal Plants Research, 7*(27), 1969-1978,

Syamkumar, S., & Sasikumar, B. (2007). Molecular marker based genetic diversity analysis of *Curcuma* species from India. *Scientia Horticulture, 112*, 35–24. doi:10.1270/jsbbs.56.107

Theerakulpisut, P., Triboun, P., Mahakham, W., Maensiri, D., Khampila, J., & Chantaranothai, P. (2012). Phylogeny of the genus Zingiber (Zingiberaceae) based on nuclear ITS sequence data. *Kew Bulletin, 67*(3), 389-395. doi:10.1007/s12225-012-9368-2

Vinod, K. K. (2007). DNA extraction- Cetyltrimethylammoniumbromide (CTAB) method. Retrieved from http://kkvinod.webs.com/prot1.htm

Wallinger, C., Juen, A., Staudacher, K., Schallhart, N., Mitterrutzner, E., Steiner, E. M., ... & Traugott, M. (2012). Rapid plant identification using species-and group-specific primers targeting chloroplast DNA. *Plos One, 7*(1), e29473.

White, T. L., Adams, W. T., & Neale, D. B. (Eds.). (2007). *Forest Genetics*. Cabi.

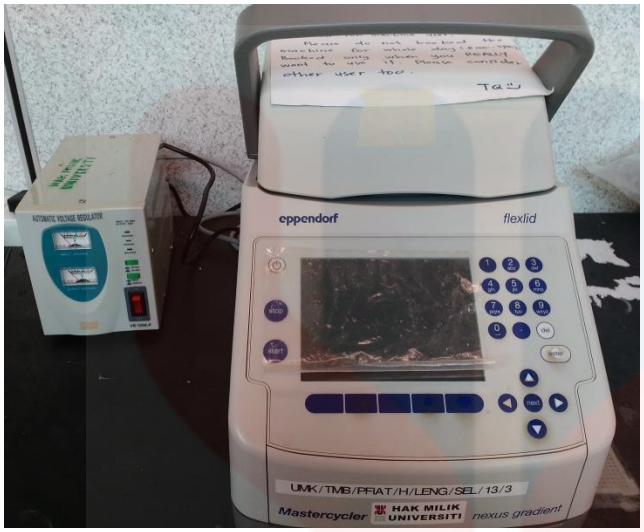
White, T. J., Bruns, T., Lee, S. J. W. T., & Taylor, J. L. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR protocols: a guide to methods and applications*, *18*(1), 315-322.

Wilfinger, W. W., Mackey, K., & Chomczynski, P. (1997). Effect of pH and ionic strength on the spectrophotometric assessment of nucleic acid purity. *Biotechniques*, *22*(3), 474-481.

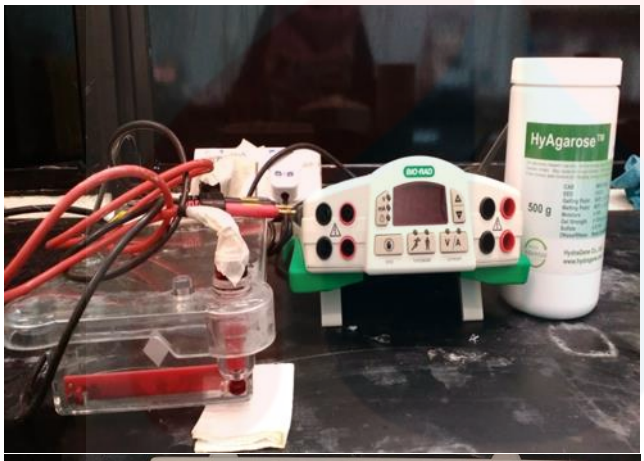
Yunus, M. F., Aziz, M. A., Kadir, M. A., Daud, S. K., & Rashid, A. A. (2013). In vitro mutagenesis of Etlingera elatior (Jack) and early detection of mutation using RAPD markers. *Urkish Journal of Biology*, *37*(6), 716-725.

APPENDIX A

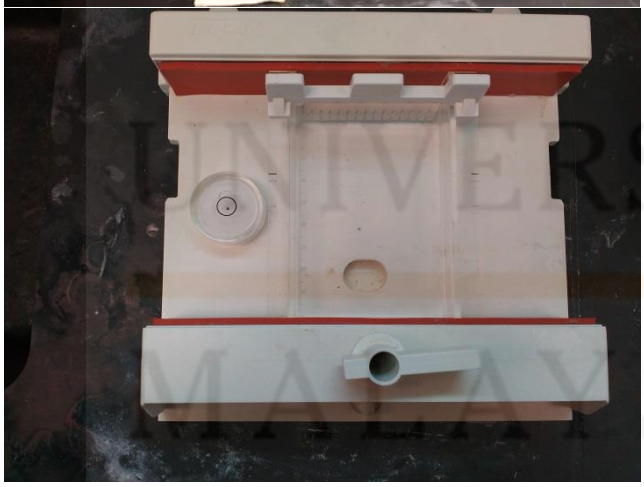
FIGURE	DESCRIPTION
	Process of shredding the young and fresh leaf sample for DNA extraction process. The mortar pestle were wrapped in aluminium foil for autoclaved and then chill in the freezer.
	The PCR reagent includes Go *Taq Polymerase*, dNTPs, $MgCl_2$, 5x PCR buffer. All of these reagents were stored in -10ºC to ensure these reagents safe.
	The PCR tube were placed align in the PCR machine. All the tubes were labelled the number and date of experiment.



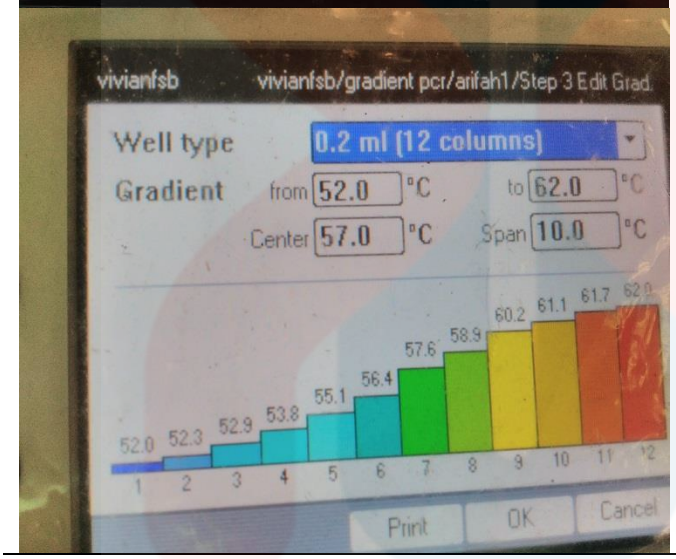
PCR machine (Eppendorf, United States). This PCR machine can load 96 PCR tube per reaction. (Abdelmageed, Faridah, Norhana, Julia, & Kadir, 2011; Chan & Thong, 2004; Mendez, Moctezuma, & Lao, 2004)



Components of electrophoresis: casting tray and comb. The agarose solution will be poured into the casting tray. Casting tray is vary in size depending the of number of wells and size of gel needed. The comb will provide the gel wells.



Components of electrophoresis-electrophoresis chamber and power supply.

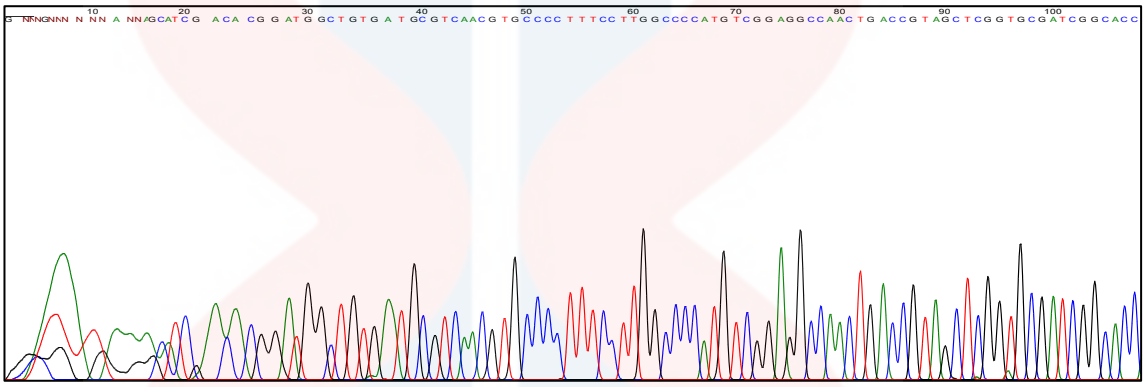
	UV Analyzer (AlphaImager HP): High-performance gel imaging for a wide range of UV fluorescent
	The gradient temperature in PCR

APPENDIX B

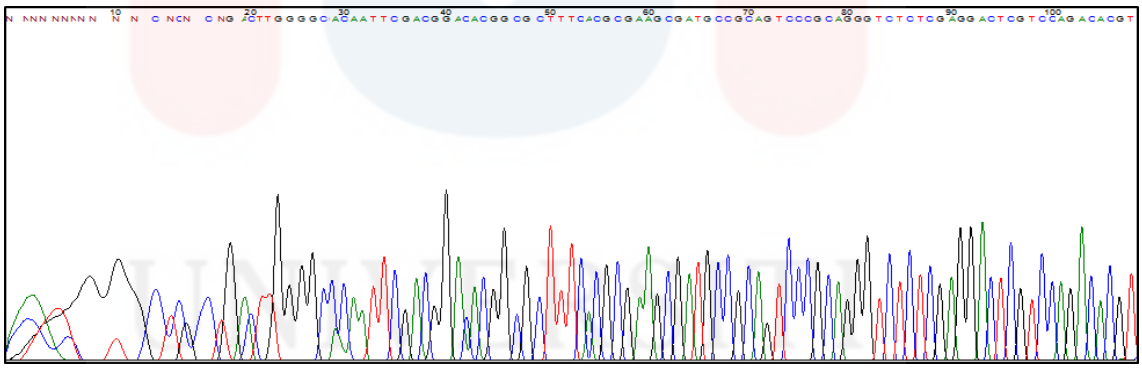
Chromatogram images of forward (ITS1) and reverse (ITS4) of Internal Transcribed Spacer region sequences for species *E. elatior* and *E. liitoralis*.

Note: APPENDIX only shows a portion of chromatogram for each ITS1 (FOW) and ITS4 (REV) sequences ranging from 1 to 100 nucleotides.

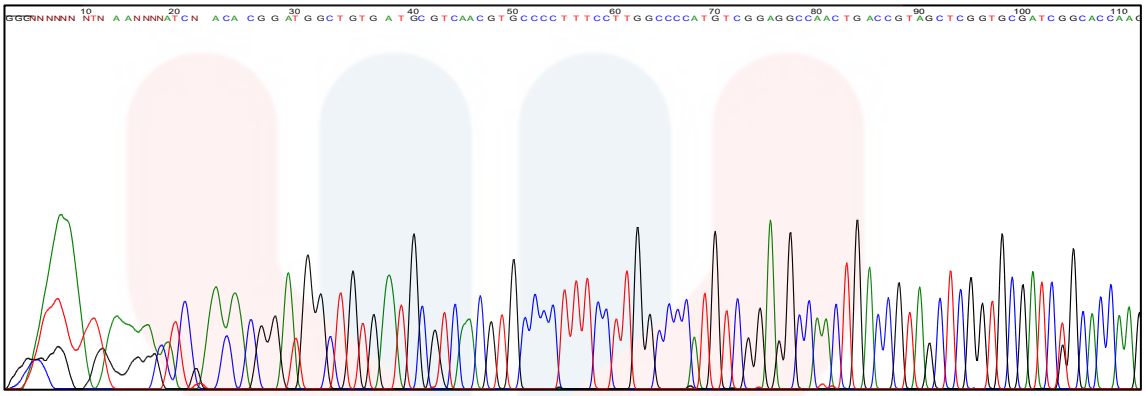
E. elatior (ITS1, Foward)



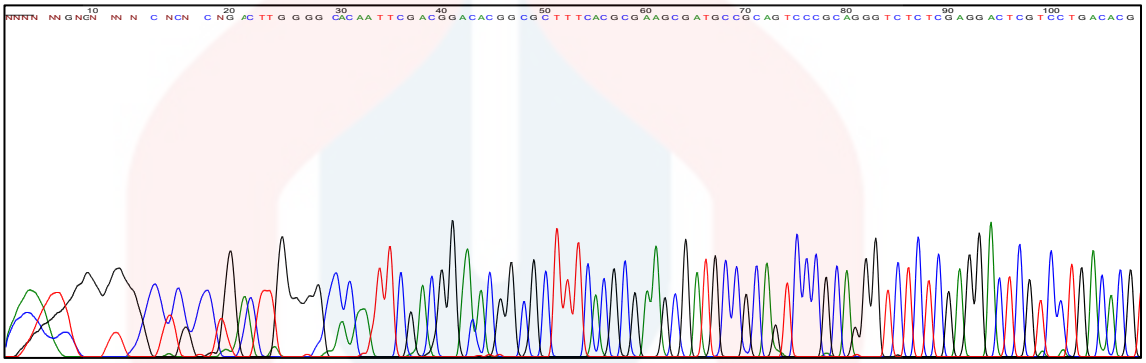
E. elatior (ITS4, Reverse)



E. littoralis (ITS1, Foward)



E. littoralis (ITS4, Reverse)



APPENDIX C

>3334618_*Etlingera elatior* _ITS1_Foward Sequences

GNNNGNNNNNNANNAGCATCGACACGGATGGCTGTGATGCGTCAACGTG
CCCCTTTCCTTGGCCCCATGTCGGAGGCCAACTGACCGTAGCTCGGTGCG
ATCGGCACCAAGGAACAACGAACTCAGAAGCAGCGGGCCCTCGGCGTGC
ACGAGGAGCCCACTGCATAAGAGATGCTTGGAATCGAATGACTCTCGGC
AATGGATATCTCGGCTCTTGCATCGATGAAGAACGTAGTGAAATGCGATA
CTTGGTGTGAATTGCAGAATCTCGTGAACCATTGAGTCTTTGAACGCAAG
TTGTGCCCAAGGCTTTGTGGCCGAGGGCACGTCTGCTTGGGCGTCATGGC
ATCACCGCCTTTGCTCCTTGCTCTGCTGGTGCCAAGCGCGGAAATTGACC
TCGTGTGCCCTCGGACACAGTCGGTCAAAGAGCGGGCAGTCGCCAGTCG
TCGAGCGCGATGGGTGCTGGTAAACCCCGCGCGCGAATAGAACGTCGCC
CTCGACGTGTCTGGACGAGTCCTCGAGAGACCCTGCGGGACTGCGGCATC
GCTTCGCGTGAAAGCGCCGTGTCCGTCGAATTGTGGCCCCAAGTCAGGCG
AGGCCCACCCGCCGAGTTTAAGCATATCATAAGCCCGGGAGGAAA

>3334619_*Etlingera elatior*_ITS4_Reverse Sequences

NNNNNNNNNNNCNCNCNGACTTGGGGCCACAATTCGACGGACACGGCGC
TTTCACGCGAAGCGATGCCGCAGTCCCGCAGGGTCTCTCGAGGACTCGTC
CAGACACGTCGAGGGCGACGTTCTATTCGCGCGCGGGGTTTACCAGCACC
CATCGCGCTCGACGACTGGCGACTGCCCGCTCTTTGACCGACTGTGTCCG
AGGGCACACGAGGTCAATTTCCGCGCTTGGCACCAGCAGAGCAAGGAGC
AAAGGCGGTGATGCCATGACGCCCAAGCAGACGTGCCCTCGGCCACAAA
GCCTTGGGCACAACTTGCGTTCAAAGACTCAATGGTTCACGAGATTCTGC
AATTCACACCAAGTATCGCATTTCACTACGTTCTTCATCGATGCAAGAGC
CGAGATATCCATTGCCGAGAGTCATTCGATTCCAAGCATCTCTTATGCAG
TGGGCTCCTCGTGCACGCCGAGGGCCCGCTGCTTCTGAGTTCGTTGTTCCT
TGGTGCCGATCGCACCGAGCTACGGTCAGTTGGCCTCCGACATGGGGCCA
AGGAAAGGGGCACGTTGACGCATTCACAGCCATCCGTTGTTCGATGCTCT
CTCAACAATGATCCTTCCGCAGTCCCCCCCATTCGAANA

>3334620_*Etlingera littoralis*_ITS1_Foward Sequences

GGGNNNNNNNTNAANNNNATCNACACGGATGGCTGTGATGCGTCAACGT
GCCCCTTTCCTTGGCCCCATGTCGGAGGCCAACTGACCGTAGCTCGGTGC
GATCGGCACCAAGGAACAACGAACTCAGAAGCAGCGGGCCCTCGGCGTG
CACGAGGAGCCCATTGCATAAGAGATGCTTGGAAACGAATGACTCTCGG
CAATGGATATCTCGGCTCTTGCATCGATGAAGAACGTAGTGAAATGCGAT
ACTTGGTGTGAATTGCAGAATCTCGTGAACCATTGAGTCTTTGAACGCAA
GTTGTGCCCAAGGCTTTGTGGCCGAGGGCACGTCTGCTTGGGCGTCATGG
CATCACCGCCTTTGCTCCTTGCTTTGCTGGTGCCAAGTGCGGAAATTGAC
CTCGTGTGCCCTCGGACACAGTCGGTCAAAGAGCGGGCAGTCGGCAGTC
GTCGAGCGCGATGGGTGCTGGTAACCCTGCGCGTGAATAGAACGTCGCC
CTCGACGTGTCAGGACGAGTCCTCGAGAGACCCTGCGGGACTGCGGCAT
CGCTTCGCGTGAAAGCGCCGTGTCCGTCGAATTGTGGCCCCAAGTCAGGC
GAGGCCCACCCGCCGAGTTTAAGCATATCATAAACCGGGGAAAGAAAAG

>3334621_*Etlinera littoralis*_ITS4_Reverse Sequences

NNNNNNGNGNNNNCNCNCNGACTTGGGGCACAATTCGACGGACACGGC
GCTTTCACGCGAAGCGATGCCGCAGTCCCGCAGGGTCTCTCGAGGACTCG
TCCTGACACGTCGAGGGCGACGTTCTATTCACGCGCAGGGTTACCAGCAC
CCATCGCGCTCGACGACTGCCGACTGCCCGCTCTTTGACCGACTGTGTCC
GAGGGCACACGAGGTCAATTTCCGCACTTGGCACCAGCAAAGCAAGGAG
CAAAGGCGGTGATGCCATGACGCCCAAGCAGACGTGCCCTCGGCCACAA
AGCCTTGGGCACAACTTGCGTTCAAAGACTCAATGGTTCACGAGATTCTG
CAATTCACACCAAGTATCGCATTTCACTACGTTCTTCATCGATGCAAGAG
CCGAGATATCCATTGCCGAGAGTCATTCGTTTCCAAGCATCTCTTATGCA
ATGGGCTCCTCGTGCACGCCGAGGGCCCGCTGCTTCTGAGTTCGTTGTTC
CTTGGTGCAGATCGCACCGAGCTACGGTCAGTTGGCCTCCGACATGGGGC
CAAGGAAAGGGGCACGTTGACGCATTCACAGCCATCCGTTGTTCGATGCT
CTCTCAACAATGATCCTTCCGCAGGTCACCCCCATTCGGAA