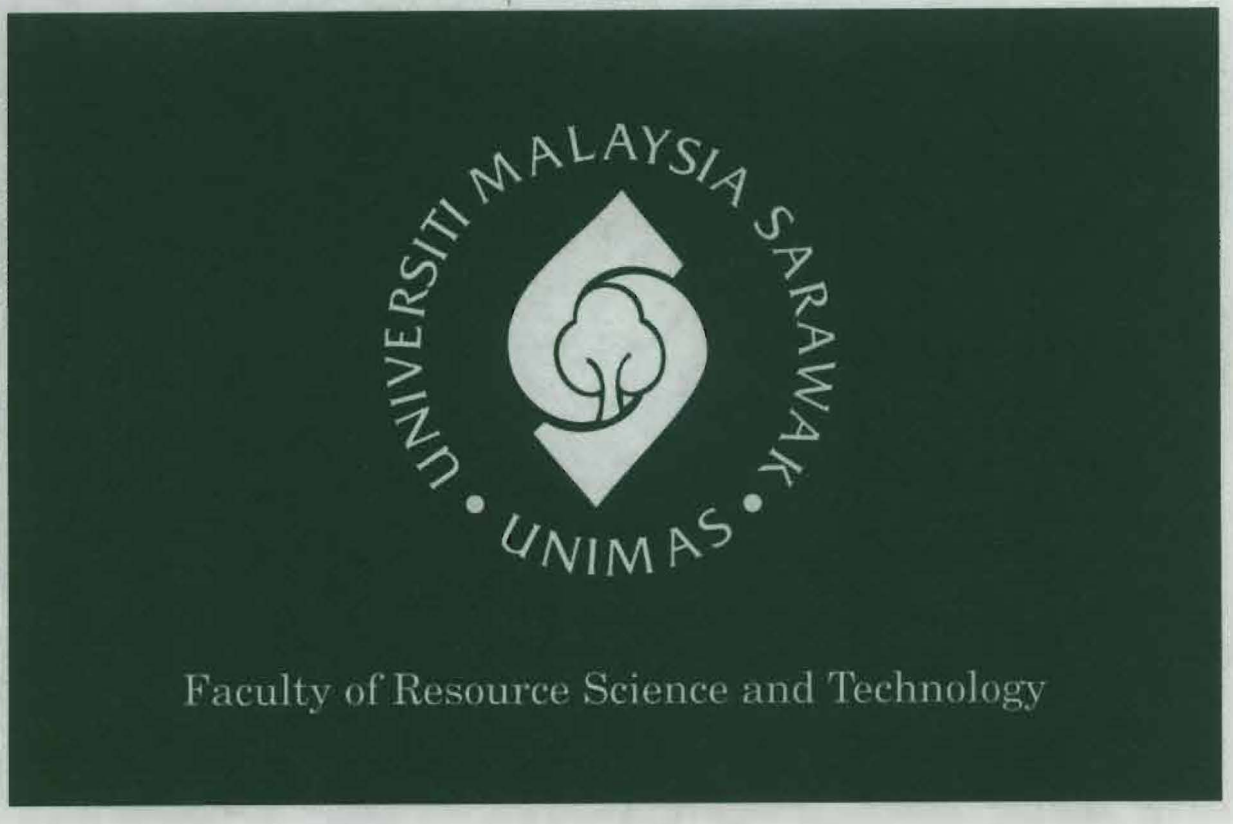

ESTABLISHMENT OF RAPD ANALYSIS PROTOCOL FOR Elephantopus scaber L.

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Bachelor of Science with Honours
(Resource Biotechnology)
2006

ACKNOWLEDGEMENT

First of all, I would like to thank the god for his grace and blessing upon the completion of this project. My sincere gratitude to my supervisor, Ms. Rosmawati Saat for her guidance, comments, suggestion and encouragement. Beside that, I would like to express my sincere appreciation to master students for sharing experiences and knowledge as well as giving valuable advice to me throughout my project. I also would like to thank lab assistant for their assistances especially providing technical support. Lastly, I would like to dedicate my special to friends and family for their moral support and understanding. .

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LIST OF ABBREVIATIONS

AP-PCR	Arbitrary primed PCR
CIA	chloroform-isoamyl alcohol
CTAB	cetyl triethylammonium bromide
DNA	dioxyribonucleotide acid
ddH_2O	double distilled water
EDTA	ethyenediaminetetra-acetate
EtOH	ethanol
$MgCl_2$	magnesium chloride
NaCl	sodium chloride
NaOH	sodium hydroxide
PCI	phenol: chloroform: isoamylalcohol
PCR	polymerase chain reaction
RAPD	Random Amplified Polymorphic DNA
RNA	ribonucleotide acid
SAHN	Sequential agglomerative hierarchical nested cluster analysis magnesium chloride
SDS	sodium dodecyl sulfate
UV	ultra violet

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Establishment of RAPD Analysis Protocol for *Elephantopus scaber* L.

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ABSTRACT

Elephantopus scaber L. is a pantropical weed, which is originally from America and can be found locally through out Sarawak. The objective of this project is to establish a Random Amplified Polymorphic DNA (RAPD) protocol for *E.scaber*. In this study, SDS and CTAB genomic DNA extraction methods were used to extract DNA. Among the two extraction methods SDS extraction method produced better quality and sufficient amount of DNA compared to CTAB method. Good quality of bands of DNA recovery was observed on 1% agarose gel. Sets of primers OPA, OPB, and OPC were used in the RAPD analysis and only OPA05 produced satisfactory result. Optimization was done on the concentration of magnesium chloride and annealing temperature of primers. The optimal annealing temperature and magnesium chloride concentration for OPA05 were 33^0C and 3mM respectively. This condition produced good and consistent RAPD profiles. NTSYSpc version 2.0 software (Rohlf, 1998) was used to analyze the profile in order to construct dendogram. Similarity matrix was generated based on Jaccard coefficient (Godwin *et al.*, 2001). This analysis revealed that the 10 individuals of *E.scaber* were clearly divided into clusters and there were genetic variations within these 10 individuals.

Keywords: CTAB, SDS, RAPD-PCR, primer.

ABTSRAK

Elephantopus scaber L. adalah rumpai pantropikal yang berasal dari Amerikayang boleh dijumpai di Sarawak. Objektif kajian ini ialah untuk menghasilkan satu protokol RAPD bagi E.scaber. Ini kerana tumbuhan tersebut mempunyai kebaikan dari segi perubatan.kepekatan magnesium klorida dan suhu yang optimal telah ditentukan untuk RAPD. Teknik SDS dan CTAB telah digunakkan untuk mengekstrak DNA. Teknik SDS didapati menghasilkan DNA yang lebih berkualiti dan dalam kuantiti yang mencukupi berbanding dengan teknik CTAB. Jalur DNA yang jelas telah dilihat setelah elektroforesis gel agarose 1 % dijalankan. Bagi analisis RAPD, primer OPA, OPB, dan OPC telah digunakan, antara primer-primer yang digunakan hanya primer OPA05 telah menghasilkan keputusan yang memuaskan. Suhu setinggi 33^0C dan kepekatan magnesium klorida sebanyak 3.0mM berjaya menghasilkan produk yang optimum bagi primer OPA05. NTSYSpc versi 2.0 (Rohlf, 1998) telah digunakan untuk menganalisis profail RAPD untuk menghasilkan dedogram. Daripada analisis ini didapati bahawa kesemua individu E.scaber mempunyai perbezaan dari segi genetic

Kata kunci: CTAB, SDS, RAPD-PCR, primer.

1.0.INTRODUCTION

Elephantopus scaber L. is a pantropical weed which is originally from America found by Linn. It is also known as Elephant's foot and in Malay it is called Tutup bumi (Barnes and Chan, 1990). *E.scaber* commonly can be found in places where grass grow, and exists as troublesome weed (Barnes and Chan, 1990). In this study a Random Amplified Polymorphic DNA (RAPD) protocol for *E.scaber* was established. It is an excellent technique to embark upon first for studying genetic resources about this species, as not much information is known especially in molecular and genetic aspect (Waugh, 1997). RAPD is ideal for screening polymorphisms in most species and particularly attractive because its primers are readily available (Waugh, 1997). RAPD analysis for *E.scaber* which possesses medicinal values has been done to distinguishing this particular plant with others. The variable stretches of DNA will bring a big difference among species and more informative when genomic DNA is used in order to differentiate individuals from one-another. RAPD analysis can be used for species identification using its genome as well as to reveal the genetic variation present in the sample that will be collected (Draper *et al.*, 1988). Other than that, RAPD analysis also used to screen genetic polymorphism among the *E.scaber* samples (Draper *et al.*, 1988). This information can be used to identify the species and detect genetic mutation in the future studies. This is because some stretches of DNA within a genome tend to vary among individuals.

2.0.OBJECTIVES

The objectives of this project are to establish a rapid and simple procedure as well efficient method of isolating high-quality genomic DNA for RAPD amplification as to establish simple and efficient protocols for RAPD analysis for *E.scaber* through optimization. Optimization is achieved by setting optimal annealing temperature and magnesium chloride concentration for different sets of primers.

3.0.LITERATURE REVIEW

3.1.*E.scaber* Morphology

E.scaber stem normally can grow until an average tall of 30 cm. The leaves of *E.scaber* generally grow at the base of the plant with white hairy like structure on top of the leaves. Crowded leaves at the base of stem form rosset close the ground and the size of the leaves vary as well as the shape of the leaves. *E.scaber* leaves are oblong in shape and its tip is blunt. The flowers normally grow at the end of white wooly brunch which grows from leaves. The flowers are normally white in color and with corolla. The flowers located on top of a white tube. *E.scaber* also produces fruit. Its fruit has hairy like structure or bristle surrounding its surface (Barnes and Chan, 1990).

3.2.RAPD Analysis

In every individual of plants the genetic information or features are based on two main things. they are morphological and agronomical traits. Sometimes the biochemical features also used to determine the genetic characteristics (Waugh, 1997). However, these methods are subject to environmental influences and their overall effectiveness in estimating genetic relationship has been subjected to some debates (Waugh, 1997). Therefore, the polymorphic information obtained from DNA gives the best estimation of genetically diversity.

In Random Amplified Polymorphic DNA (RAPD) analysis the DNA samples has been amplified using PCR. RAPD-PCR normally uses arbitrary sort primers, but in certain cases there were longer and more than one arbitrary primer was used (Williams *et al.*, 1990). Nucleotide sequence of the primer used and the source of the template DNA can determine the sequence of amplified fragments in different organisms to get a genome specific "DNA fingerprint". RAPD data will be obtained by staining agarose electrophoresis gel containing fragments that synthesized using the automated technology of PCR (Stiles *et al.*, 1993).

The genetic differences or polymorphisms between individuals result from sequence differences in primers biding site can be identified by directly looking at band patterns produced in a particular RAPD profiles (Rafalski *et al.*, 1994). To date, there were few studies have been done using RAPD method, they are genetic fingerprinting for dipterocarps (Rath *et al.*, 1998), olive (Germas *et al.*, 2000), cashew (Dhanaraj *et al.*, 2002), guava (Prakash *et al.*, 2002) and mango (Karihaloo *et al.*, 2003).

Basically, RAPD is a technique that involves PCR reaction to determine genetic diversity present in any population of species. The difference of PCR in RAPD analysis compared to other PCR based technique is the usage of single arbitrary primers. These short arbitrary primes produce different length of DNA fragment trough the amplification process. The technique was developed independently by two different laboratories (Williams *et al.*, 1990; Welsh and McClelland. 1990) and called as RAPD and AP-PCR (Arbitrary primed PCR) respectively. The differences or similarities in genetic information can be determined by analyzing the DNA fragments that was amplified. According to Williams *et al.* (1990), various sizes of DNA segments can be amplified in order to identify polymorphism, and the sizes of amplified fragments can be affected by certain incidents such as such as deletions of a priming site and insertions that render priming sites.

RAPD differs from standard PCR reaction in terms of numbers of primers used. RAPD requires only one primer that would bind to template DNA but standard PCR needs two primers to amplify target DNA (Bova and Micheli, 1997). In RAPD, the primers will bind to several locations in template DNA and amplify the fragments that they bound in order to produce a discrete DNA product through thermocyclic amplification. The polymorphisms between individuals result from sequence differences in one or both of the primer binding sites, and are visible as the presence or absence of a particular RAPD band (Rafalski *et al.*, 1994).

3.3.RAPD primers

RAPD primers that were used (OPA05), normally shot and single stranded. The number of base in these primers will be around 10 bases of maybe more. The primers are constructed randomly or in another word the primer's bases are sequenced with a random selection of bases. Thus, information on neither the sequence of amplified fragment nor the sequence of template DNA is unknown. Another feature of these primers are, these primers do not contain any palindromic sequences and have a minimum of 40% G+C content (generally 50%-80%) will be used in this analysis (William *et al.*, 1990).

3.4.RAPD-PCR reaction

PCR is a method where isolated DNA will be amplified by synthesizing new ideal DNA fragments with the isolated one. This process is done in vitro. In a cycle of PCR, there are few steps will be done repetitively until the interested amount of DNA is produced. In RAPD analysis only a certain parts of the template DNA is amplified. These amplified fragments based on the location where the primers attach to the template DNA. The main factors that influence the primer bind to a template DNA are dNTPs, DNA polymerase, magnesium chloride concentration and reaction buffer. The PCR reaction in RAPD involves three steps, they are denaturation, annealing and extension. These three steps are repeated many times in order to multiply the number of fragment that produced by the primers. The temperature settings for each of these steps vary from 94°C (denaturation) to 30°C (annealing). High temperature is required to denature the DNA which is double stranded. Lower temperature is required for the primers to

ind to the template. The setting of temperature considered as a crucial part in PCR. improper emperature setting will cause the PCR reaction unable to produce any products. The proper emperature settings will produce good quality product which can be separated by gel lectrophoresis and clearly visualized under ultra violet light after ethidium bromide staining.

3.5.RAPD reproducibility

In every analysis, the main concern goes to the reproducibility. The RAPD capability of producing reliable products based on two main factors. The two factors are the quality and quantity of DNA templates. These two factors have to be the main concern in RAPD analysis in order to obtain reproducible results. Thus both parameters should be controlled using proper techniques to produce results that can be reproduced in future. Therefore, the best DNA extraction method that gives high quality and quantity yield must be appropriately selected. The best extraction method should be able to produce DNA templates with minimal level of contamination to prevent interruption in amplification process. In another hand, the amount of DNA also must be precisely quantified as it is a crucial part to obtain reproducible results. The patters of RAPD profiles can vary drastically if different concentrations of DNA template are used (Waugh, 1997).

3.6.RAPD Application

RAPD is a commonly used in many fields of studies, among these fields of studies, only population genetic studies uses RAPD analysis more frequently. This field has grown wide with

he RAPD analysis method. Population genetic studies use RAPD markers to distinguish ndividuals in a population. DNA fingerprints were created for any organism with DNA make up or the purpose of individual identification and for the taxonomical study. Other than that, RAPD lso used to detect polymorphism in closely related organisms such as different population of ingle species or individuals within a population. Therefore, this technique provides a powerful ool for gene mapping, marker-assisted selection in breeding programs, population and pedigree nalysis, phylogenetic studies, and individual and strain identification (Bova and Micheli, 1997).

3.7.Problems in RAPD analysis

The contamination of DNA template has been the main problem in RAPD analyses. There are few factors contribute to the contamination of DNA samples. Firstly, due to the improper disruption of plant cell wall. The incompletely disrupted plant cells produce shredded DNA molecules and damage the integrity of DNA. This problem can be solved if the plant tissues are freeze-dried prior to cell disruption (Draper *et al.*, 1988).

Secondly, the presence of bimolecules such as polysaccharide and tannins also can increase the level of DNA sample contamination. These biomolecules makes the samples viscous and complicate the extraction processes. The high concentration of polysaccharide reduces the chances of obtaining pure DNA templates which an important factor for PCR. This is because the contaminants would inhibit the endonuclese and polymerase activities during PCR; therefore the PCR would fail to produce any products (Lodhi *et al.*, 1994).

astly, the DNA samples also can be contaminated by microorganisms that exist on the plant issue surfaces. The DNA from these microorganisms could be extracted and amplified along vith the plant DNA to produce a false result. Thus, the plant tissues should be surface cleaned vith ethanol to get rid of microorganisms (Dodds, 1991).

3.8.Cetyl triethylammonium bromide (CTAB) method

The CTAB method used in this study is a modified method by Doyle and Doyle (1990). It is most preferred method and commonly used by researchers. This is because CTAB method produces an excellent result in a short period of time. It is capable to produce results immediately comparing other techniques. This has been proved by Stewart and Via (1993), through the isolation of DNA samples from 5 plants and 1 fungus species. It is the most successful technique in plant DNA extraction. The CTAB-based DNA extraction procedure for grapevine species, hybrids and *Ampelopsis* (Vitaceae) has produced high amount of DNA. The DNA yielded from this procedure is high (up to 1 mg/g of leaf tissue) and the DNA is completely digestible with restriction endonucleases and amplifiable in the PCR. This method is concluded to be suitable not only to extract DNA from flowering plants but also suitable for fruit species DNA extraction such as apple, and cherry (Doyle and Doyle, 1990).

3.9.SDS (Sodium Dodecyl Sulfate) extraction method

SDS extraction method (Dellaporta *et al.*, 1983) is another plant genomic extraction method. With this method most proteins and polysaccharides were removed by a short centrifugation as a

omplex with the insoluble potassium dodecyl sulfate precipitate. Thus, this method needed only short centrifugation to separate DNA from all other contaminants. It took approximately one minute from grinding a leaf piece to the final DNA sample without counting the time for sampling and labeling. although it varied depending on ability of the centrifuge used. Thus. this method is named "One-minute DNA extraction" method.

4.0.MATERIALS AND METHODS

4.1.Sampling of Plant Materials

Fresh young leaves from 10 different individuals of *E.scaber* plant were collected from Samarahan using plastic bags and labeled properly with location and date of collection. The young leaves were isolated then sterilized using 70% alcohol and rinsed with distilled water and dried using paper towel. Finally the leaf tissues were stored at -80^0C (Gawal and Jarret, 1991).

4.2.Methods

4.2.1.Reagents Preparation

The stock solutions and reagents were prepared according to the concentrations and amount acquired using Dellaporta *et al* (1983) and Doyle and Doyle (1990) methods for SDS and CTAB extraction method respectively. The preparations of solutions are as follows.

M Tris HCl pH 8.0

21.1 g of Tris-base (biotechnology grad) was measured and dissolved in 800ml of distilled eionized water. The pH was adjusted using approximately 42ml of concentrated HCl. Then, istilled water was added to bring the volume till 1L.

.5M EDTA pH 8.0

86.1g of disodium ethyenediaminetetra-acetate dihidrate ($Na_2EDTA.2H_2O$) (molecular weight = 72.2g) was added into 800ml of distilled deionized water. The solution was stirred vigorously sing a magnetic stirrer. Then the pH was adjusted using approximately 20g of NaOH to until it eached pH 8.0. The solution turned into a clear solution when it reached the pH of 8.0. Finally istilled deionized water was added to bring the solution level to 1litre.

CTAB extraction buffer

100ml of 100mM Tris buffer pH 8.0 and 40ml of 20mM EDTA pH 8.0 was mixed and 600ml of distilled deionizer. At last 81.82g of 1.4M NaCl was added to the solution and the volume level was set at 1litre.

CIA (Chloroform-isoamyl alcohol) (24:1)

240ml of Chloroform and 10ml of isoamyl alcohol was mixed and stored at room temperature.

ash buffer

0ml of distilled deionizer water and 760 ml of 100% ethanol was mixed. Then 0.77g of mmonium acetate was added and stirred vigorously on a magnetic stirrer.

M potassium acetate stock solution

22.75g of potassium acetate (SIGMA) was dissolved in 180ml of distilled water. Then distilled ater added to make the solution up to 250ml.

M NaCl stock solution

.58g of NaCl was dissolved in 50ml of distilled deionized water. Then after all the NaCl issolved distilled deionized water was added till the volume reach 100ml.

SDS lysis buffer

1.08g PVP was dissolved in 1.055ml of distilled water. Then 1.6ml of 1M Tris-HCl, 480µl 0.5M EDTA (pH 8), 600µ of 1M NaCl, 267µl of 20% SDS and 10µl was mixed well and incubated at 60°C until the it turn into a clear solution.

SDS 2% stock solution

2g of SDS was dissolved in 20ml of distilled deionized water. The solution then stored under room temperature.

NAase A

)mg of RNAase A dissolved in 1000µL RNAase storage buffer and heated to 100^0C and boiled r 15minutes. The working solution is 1µg/µl: for 1000µl of RNAase solution. Then 50µl NAase stock solution was diluted with 950µl distilled deionized water.

.3.Method

.3.1.Genomic DNA Extraction

Method 1: CTAB (Cetyltrimethylammoniumbromide)

TAB extraction method Doyle and Doyle (1990) was used to extract genomic DNA from the *scaber* leaf tissues. At the beginning stage, with pestle and mortar, leaf tissue were ground into ine powder form under the treatment of liquid nitrogen which will be previously stored at -20°C. A little more liquid nitrogen will be added if necessary to keep the powder from thawing while grinding.

A ratio of 10 ml prewarmed DNA extraction buffer per gram of tissue was performed during the extraction. The content was mixed gently than incubate in a water bath at 65°C for an hour and after that the tube was removed from the water bath and left to cool down to room temperature approximately for 5minutes. Than, equal volume of chloroform: isoamylalcohol 24:1 (CIA) was added. The aqueous layer then was transferred to a new fresh 1.5ml tube and the DNA was precipitated by 2/3 volume of Isopropanol at -20°C for overnight. The next day the DNA was

covered by centrifuging the tubes at 4°C, 13.000 rpm for 10 minutes. Then 70% ethanol was ed in order to wash the DNA. After that the washed pallet was air-dried at room temperature d re-suspend in sterile distilled water. Finally the DNA was stored at -20°C.

lethod 2: SDS (Sodium Dodecyl Sulfate)

he SDS method used in this study is a modified version of the initial Kikuchi *et al.*, (1998) ethod. First, young fresh leaves were collected and washed with 70% ethanol. Then, the leaves ere rinsed with distill water. Approximately 0.1g of fresh leaves was ground in liquid nitrogen ntil fine powder is formed. The leaf powder was then transferred into an Eppendorf tube ontaining 600µl SDS lysis buffer. The tube was inverted several times to mix the solution. Then he tube was filled with 200µl of 5Mpotassium acetate and inverted gently several times. The ixture was incubated at 60°C for 20 minutes and the tube was gently inverted periodically.

After that, the mixture was placed on ice for 20 minutes. Then, 600µl of cold chloroform was added to the mixture. The mixture was inverted gently to mix and leave at room temperature for 10 minutes. After incubation, the mixture was centrifuged at 13,000rpm for 10 minutes at 4°C. The aqueous phase was transferred into a new tube. 600µl of isopropanol was added and inverted gently to mix. The tube was stored at -20°C overnight. On the next day, the tube was centrifuged at 13,000rpm for 20 minutes at 4°C and the supernatant was discarded. Pellet was washed twice with 70% ethanol by adding 600µl of 70% EtOH, and then centrifuged at 5000rpm for 5 minutes at room temperature. Supernatant was discarded and the washing repeated. The pellet was dried

ide laminar flow cabinet for 20-30 minutes. The pellet was dissolved in 50µl sterile ddH_2O d stored at -20°C.

4.DNA Purification

final concentration of 0.02µg/µL RNAase A was added to the DNA samples which were rived from SDS and CTAB methods. The mixtures then was incubated at 37°C for an hour and llowed by equal phenol:chloroform:isoamylalcohol 25:24:1 (PCI) purification. Then 2.5volume f cold absolute ethanol was used to precipitate DNA. The tubes were left at-20°C 20 minutes. hen the tubes were centrifugation at 13,000 rpm for 10minutes. Then DNA pellet that was rmed at the bottom of the tubes was washed with 75% ethanol and air-dried at room mperature, after that the pellet was re-suspended in appropriate amount of sterile distilled water nd stored at -20°C.

4.5.DNA Quantification

The DNA samples extracted from both CTAB and SDS method were quantified using spectrophotometer Ultrospec® 1100 pro. This machine uses UV absorbance to quantify the amount of DNA present in every sample. The amount of DNA was quantified using the absorbance at 260nm and the amount of protein contaminant was quantified using absorbance at 280nm. According to Asif (2000), the A_{260}/A_{230} absorbance ratio indicates polysaccharide or polyphenolic contamination and the A_{260}/A_{280} absorbance ratio indicates the protein

ntamination. These ratios will be estimated should be greater than 1.8 for DNA with high rity. Otherwise, the contamination with polysaccharide, polyphenolic and protein is suspected. s mentioned by Sambrook (1989), an O.D. at the wavelength 260nm is equivalent to 50µg of NA per ml. Therefore, the DNA concentration was determined according to the formula stated Brown (1990):

NA Concentration (µg/ml) = A_{260} x 50µg/mL x Dilution Factor

uring DNA quantification 5µl of DNA was diluted in 495µl of distilled water in a quartz uvette. The wavelength absorbance at the 260nm and 280nm (A_{260} and A_{280}) and the reading of $_{260}$: A_{280} ratio from the spectrophotometer was recorded.

.6.Agarose gel electrophoresis

% of agarose gel was used for the gel electrophoresis of DNA samples extracted from both TAB and SDS methods. The concentration of agarose gel used depends on the size of molecules that are to be separated. Thus, 1% gel was sufficient for visualize the genomic DNA. µl of 6x loading dye with 5µl DNA stock loaded in to the gel well together with 1kb ladder and the gel was ran at 100volt for 30 minutes. The RAPD-PCR products were separated using 1.6% w/v agarose gel at 80 volts for approximately 3.5 hours and stained with ethidium bromide. All the gels were visualized under UV light and Polaroid 667 films were used to photograph of the gels.

2.RAPD analysis

decamer primers: OPA1 to OPA6, OPB1 to OPB6, OPC2, OPC3, OPC5, OPC7, OPC11, and PC13 (1ST Base Inc.) were used for screening of DNA extracted from *E.scaber* using the PCR achine (eppendorf® PCR Mastercycler gradient). Primers that produced good and consistent CR products were selected for final screening. Different $MgCl_2$ concentrations (1.5 mM, 2.0 M, 2.5 mM, 3.0 mM and 3.5mM) and different settings of annealing temperature (30°C, 31°C, °C, 33°C and 34°C) were used to optimize the PCR. Concentration of $MgCl_2$ and annealing mperature that produces best profile was used for the final primers screening. The 20µl reaction ixtures containing: 1X PCR buffer (Yeastern Biotech Co.Ltd), with 2mM $MgCl_2$, 100mM of ch dNTPs (1ST Base Inc.), 10pmol primer (1ST Base Inc.), 1 Unit *Taq* DNA polymerase and 5ng of template DNA.

he PCR cycling for initial screening was as follows: 1 min initial denaturation at 94°C followed y 45 cycles of 1 min denaturation at 94°C, 1 min annealing at 29°C and 27°C and 2 min xtension at 72°C, with a final extension of 7 min.

rimers which produced clear and distinct band in the initial screening were selected to be ptimized. Optimization of primers that produced clear and distinct band in initial screening would produce good, descriptive and reliable band pattern. The $MgCl_2$ concentration and nnealing temperature were adjusted to determine the optimal condition for the primers that were elected from the initial screening. The optimal $MgCl_2$ concentration and annealing temperature