



**Faculty of Resource Science and Technology**

**Determination of Genetic Stability of Kelampayan plantlets regenerated from tissue culture using SSR markers**

**Natalie anak Gali  
(24267)**

**Bachelor of Science with Honours  
(Resource Biotechnology)  
2012**

**Determination of Genetic Stability of Kelampayan plantlets regenerated from tissue culture using SSR markers**

**Natalie anak Gali**

This project is submitted in partial fulfillment of the requirements for the degree of Bachelor of Science with Honours in Resource Biotechnology

Resource Biotechnology  
Department of Molecular Biology  
Faculty of Resource Science and Technology  
Universiti Malaysia Sarawak (UNIMAS)  
2012

## **ACKNOWLEDGEMENT**

Praise the Lord for His endless blessings and wisdoms in completing this project. My sincere appreciation goes to my supervisor, Dr. Ho Wei Seng for giving me the opportunity to conduct this study and also for his supervision, guidance, patience, knowledge and skills during this entire project. A special thank would be given to Dr. Pang Shek Ling and Miss Linna Chieng, FRC research officers, whom also assign as my co-supervisors for their guidance, suggestions and all the valuable informations given to me. Furthermore, I would like to thank Sarawak Forestry Research Centre (FRC) for providing the project samples. My appreciation also given to Madam Zeti Akhtar Binti Hj. Abdul Manan; science officer and other staffs from Faculty of Resource Science and Technology (FRST) as well for their cooperation. My deepest thanks and appreciation to Miss Kamalia and all the post-graduate students from the Forest Genomics and Informatics Laboratory (fGil), for their assistance on technical skills, advice and support from the beginning until this project done. Last but not least, my special thanks to my family members especially my parents for their continuous moral and financial support and also to all my friends for their advices, assistances, and patience during the critical moment in completing this project.

## Table of contents

<b>TITLE &amp; FRONT COVERS</b> .....	i
<b>ACKNOWLEDGEMENT</b> .....	ii
<b>TABLE OF CONTENTS</b> .....	iii - iv
<b>LIST OF ABBREVIATIONS</b> .....	v
<b>LIST OF TABLES</b> .....	vi
<b>LIST OF FIGURES</b> .....	vii
<b>ABSTRACT</b> .....	viii-ix
<b>1.0 INTRODUCTION</b> .....	1 - 3
<b>2.0 LITERATURE REVIEW</b>	
2.1 <i>Neolamarckia cadamba</i> (Roxb.) Bosser .....	4 - 6
2.2 Simple Sequence Repeats (SSRs) .....	6 - 7
2.3 Genetic stability .....	8 - 9
2.4 Polymerase Chain Reaction .....	9 -10
<b>3.0 MATERIALS AND METHODS</b>	
3.1 Materials .....	11
3.2 Sample collection .....	11
3.3 Sub-culturing of samples .....	11
3.4 DNA isolation .....	12
3.5 Polymerase Chain Reaction	
3.5.1 PCR condition .....	12 -13
3.5.2 PCR optimization .....	13

3.5.3 SSR-PCR analysis .....	13-14
3.5.4 SSR-Data analysis .....	14
<b>4.0 RESULTS AND DISCUSSION</b>	
4.1 Tissue culture .....	15
4.2 DNA extraction .....	16
4.3 SSR-PCR amplification	
4.3.1 PCR optimization .....	17-19
4.3.2 PCR amplification .....	19-22
4.4 SSR-Data analysis	
4.4.1 Data scoring .....	22- 23
4.4.2 Genetic Variation .....	23-29
<b>5.0 CONCLUSIONS AND RECOMMENDATIONS</b> .....	30
<b>6.0 REFERENCES</b> .....	31 – 34
<b>APPENDIX A</b> .....	35
<b>APPENDIX B</b> .....	36
<b>APPENDIX C</b> .....	37
<b>APPENDIX D</b> .....	38

## List of Abbreviations

SSRs	Simple Sequence Repeats
dNTPs	Deoxynucleotide–triphosphates
DNA	Deoxyribonucleotide Acid
PCR	Polymerase Chain Reaction
<i>Taq</i> polymerase	<i>Thermus aquaticus</i> polymerase
TAE	Tris–Acetate-EDTA
TBE	Tris-Borate-EDTA
MgCl <sub>2</sub>	Magnesium Chloride
°C	Degree Celsius
cm	Centimeter
m	Meter
ml	Milliliter
ng	nanogram
μl	microliter
bp	base pair
Pmol	picomole
Rpm	revolutions per minutes
Mother tree	A plant which the cells, organ or tissue been used to regenerate new plant.
Plantlets	a regenerated plant derived from the mother plant

## List of Tables

Table 3.1	Thermal cycling profile for SSR-PCR amplification .....	13
Table 4.1	The mother trees and its plantlets .....	15
Table 4.2	Thermal cycling profile for primer NCAC11 and NCAC14 .....	18
Table 4.3	The PCR ingredients for 25 µl of reaction mixture for primer NCAC11 and NCAC14 .....	19
Table 4.4	DNA fragment size of each alleles for primer NCAC11 and NCAC14 .....	23
Table 4.5	Level of polymorphism and information in alleles of Kelampayan samples using SSR markers .....	24

## Lists of Figures

Figure 2.1	Morphological characteristics of <i>N. cadamba</i> .....	6
Figure 4.1	Electrophoresis of PCR products for primer NCAC14 on 2 % agarose gel .....	17
Figure 4.2	Electrophoresis of PCR products for primer NCAC11 on 2 % agarose gel .....	18
Figure 4.3	Electrophoresis PCR production 3.5 % Metaphore® agarose gel using primer NCAC11 .....	20
Figure 4.4	Electrophoresis PCR production 3.5 % Metaphore® agarose gel using primer NCAC14 .....	20
Figure 4.5	Electrophoresis PCR production 3.5 % Metaphore® agarose gel using primer NCAC11 and NCAC14 .....	21
Figure 4.6	UPGMA of relationship among Kelampayan mother trees and plantlets using primer NCAC11 and NCAC14 based on genetic distance .....	25
Figure 4.7	Neighbor-joining tree of genetic similarity among Kelampayan mother trees and plantlets using primer NCAC11 and NCAC14 based on shared allele .....	27

# Determination of genetic stability in Kelampayan plantlets regenerated from tissue culture using SSR markers

**Natalie anak Gali**

Resource Biotechnology  
Department of Molecular Biology  
Faculty of Resource Science and Technology  
Universiti Malaysia Sarawak (UNIMAS)

## ABSTRACT

*Neolamarckia cadamba* (Roxb.) Bosser or locally known as Kelampayan is recognized as one of the fast growing forest tree and considered as an industrial species due to its multipurpose and great commercial values as well as possess various medicinal uses. The ability to retain its genetic traits is crucial before micropropagation for large-scale commercial production. The objective of this study is to access the genetic stability of Kelampayan plantlets regenerated from tissue culture using SSR markers. In this study, two primers namely NCAC11 and NCAC14 were selected. As a result, the mean number of observed alleles was 2.5 per locus together with 2.247 for the mean effective number of alleles per locus. Besides, the mean for PIC value (Polymorphism information content) was 0.451 and the mean expected heterozygosity was 0.561. The primers NCAC11 and NCAC14 were reported 100 % polymorphic. Moreover, a dendrogram constructed using unweighted pair group mean average (UPGMA) and a neighbor-joining tree constructed based on  $D_{SA}$  shared allele showed genetic relatedness among the Kelampayan samples. The Kelampayan plantlets regenerated via tissue culture are genetically unstable, due to mislabeling during samples collection or may be during sub-culturing process in the tissue culture laboratory, Sarawak Forest Corporation (SFC), Kuching.

**Keywords:** *Neolamarckia cadamba* (Roxb.) Bosser, Simple Sequence Repeats (SSRs) markers, Tissue culture, Genetic stability.

# Determination of genetic stability in Kelampayan plantlets regenerated from tissue culture using SSR markers

Natalie anak Gali

Resource Biotechnology  
Department of Molecular Biology  
Faculty of Resource Science and Technology  
Universiti Malaysia Sarawak (UNIMAS)

## ABSTRAK

*Neolamarckia cadamba* (Roxb.) Bosser atau dikenali sebagai Kelampayan telah diiktiraf sebagai salah satu daripada pokok hutan yang membesar dengan cepat dan dianggap sebagai satu spesies perindustrian oleh kerana ia mempunyai pelbagai fungsi dan nilai komersial yang tinggi serta memiliki pelbagai kegunaan dalam perubatan. Keupayaan untuk mengekalkan ciri-ciri genetik adalah penting sebelum mikropropagasi untuk pengeluaran komersil berskala besar. Objektif kajian ini adalah untuk mengakses kestabilan genetik anak pokok Kelampayan yang dijana semula dari kultur tisu menggunakan penanda SSR. Dalam kajian ini, dua primer iaitu NCAC11 dan NCAC14 telah dipilih. Hasilnya, bilangan min alel diperhatikan adalah 2.5 bagi setiap lokus bersama-sama dengan 2.247 untuk bilangan keberkesanan min alel pada setiap lokus. Selain itu, min bagi nilai PIC (polimorfismo kandungan maklumat) adalah 0.451 dan heterozigositi min yang dianggarkan adalah 0.561. Primer NCAC11 dan NCAC14 dilaporkan 100 % polimorfik. Tambahan pula, dendrogram yang dibina menggunakan unweighted pair group mean average (UPGMA) dan neighbor-joining tree yang dibina berdasarkan perkongsian alel  $D_{SA}$  menunjukkan bahawa anak pokok Kelampayan saling berkaitan dengan pokok induk. Anak pokok Kelampayan yang digenerasikan semula melalui kultur tisu mempunyai genetik yang tidak stabil disebabkan oleh kesilapan melabel sampel ketika proses pengumpulan sampel atau ketika proses sub-kultur di makmal kultur tisu, Sarawak Forest Corporation (SFC), Kuching.

**Kata kunci:** *Neolamarckia cadamba* (Roxb.) Bosser, Simple Sequence Repeats (SSRs) markers, Kultur tisu, Kestabilan genetik.

## 1.0 INTRODUCTION

Malaysia is a very fortunate country which covers wide areas of valuable natural tropical forest. This blessed country had been recognized as one of the top twelve of mega-diversity countries in the world. This land of species richness is associated with diverse stages of natural biodiversity from ecosystems to species, population, individuals and genes. In Malaysia, the natural biodiversity indeed is a very important resource for Malaysian's development especially in tourism as well as in research and development purposes. In addition, the demand on the ecosystems especially for woods is increasing from time to time where the World Resource Institute has reported that the demand for wood is expected to be double by the year 2020 (Taylor, 2004). Therefore, the process of conserving and developing the forest plantation is significant in order to achieve the objectives of yielding the greatest sustainable benefits for the present generations and at the same time maintaining their potential to meet the needs of future generation (FAO, 1993). There are eight selected species which had been targeted for forest plantation program in Malaysia; *Hevea brasiliensis* (Malaysian Rubberwood), *Acacia hybrid* (Akasia), *Khaya ivorensis* (African Mahogany), *Tectona grandis* (Teak), *Azadirachta excels* (Sentang), *Paraserianthes falcataria* (Batai), *Octomelea sumatrana* (Binuang) and *Neolamarckia cadamba* (Kelampayan).

*Neolamarckia cadamba* (Roxb.) Bosser or locally known as Kelampayan under the family Rubiaceae is recognized as one of the fast growing forest tree and had become the most frequently planted trees in the tropics. Kelampayan is considered as an industrial species due to its multipurpose and great commercial value as one of the best raw material especially in plywood industry, light construction and pulping industry (Lim *et al.*, 2005) as well as possess

various medicinal uses (Patel and Kumar, 2007). Therefore, prior to discover and determine its genetic stability; a study was conducted in order to achieve the goal which is essential for the efficiency of plant breeding and conservation for forest trees. At the same time, promoting and further employed for large-scale commercial production both locally and internationally.

The maintenance of clonal fidelity is an important issue to be focus on especially in developing a secure and stable *in vitro* clonal repository of elite Kelampayan germplasm. According to Marum *et al.*, (2009) and Xing *et al.*, (2010), the basic fundamental for plant regeneration is the maintenance of the genetic integrity with respect to the mother tree in which the production of clonal individuals should be genetically identical. This is done in order to make sure the precious characteristics which contain in the genetic of the mother tree will be present too in the clonal individual that produced later on.

The emergence of molecular markers in today's molecular world had assisted in the process of screening the tissue culture derived plants for the purpose of restoring true-to-type plants. One of the most commonly used DNA markers for the study done on determination of the genetic stability of regenerated plants is Simple Sequence Repeats (SSR) markers. SSRs had become the genetic markers of choice in this study because they are capable of revealing multiples alleles at a single locus and gave a very highly reproducible outcome (Powell *et al.*, 1996; Zhebentyayeva *et al.*, 2003). At the same time, SSRs marker is one of the PCR-based marker that is co-dominant, polymorphic and highly available in most of the plant genomes (Powell *et al.*, 1996; Zhebentyayeva *et al.*, 2003). This had been successfully proven by a few studies conducted previously on the genetic stability using SSR markers such as in almond, cherry and apricot germplasm (Cantini *et al.*, 2001; Martinez-Gomez *et al.*, 2001; Hormaza, 2002; Zhebentyayeva *et al.*, 2003) where these plants gave a similar result that concludes about

the genetic stability where the present of somaclonal variation had been successfully eliminated among the plantlets and the mother trees.

Since the existence of Kelampayan is realized only by a small group of people, therefore only very little is known about its genetic stability especially in regenerated plants. Thus, this present study report for the first time the results of molecular analysis, based on two SSR primers, namely NCAC11 and NCAC14 were selected to determine the genetic stability of the mother trees and plantlets regenerated from tissue culture of Kelampayan via SSR-PCR method. The objective of this study was to access the genetic stability of Kelampayan plantlets regenerated from tissue culture using SSR markers; NCAC11 and NCAC14.

## 2.0 LITERATURE REVIEW

### 2.1 *Neolamarckia cadamba* (Roxb.) Bosser

*Neolamarckia cadamba* under the family Rubiaceae is commonly known as ‘Kelampayan’ (Asia), ‘kadam’ (India), ‘kaatoan bangkal’ (Philippines), ‘mal sa kho’ (Laos), ‘jabon’ (Indonesian) and also ‘bur-flower’ (English name). It is well distributed in Sri Lanka, India, E. Himalayas, Bangladesh, Nicobar IsIs, Thailand, Laos, Cambodia, Vietnam, Sumatra, Java, Lesser Sunda IsIs, Borneo, Philipphines (introduced), Sulawesi and New Guinea. The taxonomical classification of *N. cadamba* is shown as below:

Kingdom	: Plantae
Subkingdom	: Tracheobionta
Division	: Magnoliophyta
Class	: Magnoliopsida
Sub-class	: Asteridae
Order	: Rubiales
Family	: Rubiaceae
Genus	: <i>Neolamarckia</i>
Species	: <i>Cadamba</i>
Scientific name	: <i>Neolamarckia cadamba</i>

Kelampayan is a tropical forest tree that has a physical appearance of creamy white to reddish brown tree with a canopy shape like an umbrella. The mature medium-sized to large tree can grow up to 40 m to 45 m tall and without branches over 25 m tall (Lim *et al.*, 2005). It has a

diameter around 100 cm to 160 cm and comprises thicker leaves with a measurement of 13 cm to 32 cm long. Similar to other types of tropical trees, Kelampayan also produce flower which is smaller in size and give an attractive solid and hairy orange balls appearance whereas the fruits are best described as a small capsule that packed closely to produce a fleshy, yellow colored infructescence containing approximately 8000 seeds. This fast growing tropical tree can be easily grown in areas with a maximum altitude of 1300 m above sea level and mostly found at logged-over lowland dipterocarp forest to mountain forest at about 1000 m and can thrives well in freshwater swamps, in open sites and also deep moist alluvial soils as a pioneer species (Nair, 2007).

Kelampayan is considered as an industrial species as it possesses a great economic value. The light and soft properties of this indigenous plant species had become crucial in plywood industry, light construction and pulping industry as one of the best raw materials with production such as picture frame, packing case moulding, skiring, wooden sandals, disposable chopstick, toys, general utility furniture, vaneer and also plywood (Lim *et al.*, 2005). Besides that, Kelampayan is not restricted to commercial value alone but also medicinal value as it possesses various medicinal uses. The dried bark and leaves are useful to relieve fever, as mouth washer and can also serve as a tonic. At the same time, serve as an astringent anti-hepatotoxic, antidiuretic and antiseptic (Patel and Kumar, 2007).

Since Kelampayan is a fast and easily growing tree, it can gives early economic returns within 8 years to 10 years. Due to its multipurpose function and utility, Kelampayan species is favoured in plantation programmes in some of the country such as India, Sri Lanka, Myanmar, Indonesia, Malaysia and Philippines (Nair, 2007).



**Figure 2.1:** Morphological characteristics of *N. cadamba*. (a) The mature tree; (b) twig with inflorescence and (c) the fruit.

## 2.2 Simple Sequence Repeats (SSRs)

The development and application of molecular markers in revealing polymorphism at the DNA level has become an important segment in the field of plant biotechnology and their genetics studies. The development of molecular techniques had assist in assessing the genetic variation more precisely, quickly and cheaply ways. There are various types of molecular markers can be found and each of them with different principles, methodologies and applications. According to Kumar *et al.* (2009), there are some desirable properties upon selection for an ideal molecular marker such as highly polymorphic nature, co-dominant, frequent occurrence in genome, selective neutral behaviors, fast and easy assay, highly reproducibility and ease of data exchange between laboratories. However, it is very difficult to find the exact molecular markers which would encounter such criteria. Thus, the most applicable way to choose the molecular marker is highly depending on the type of research to be undertaken (Weising *et al.*, 1996).

Simple sequence repeats (SSRs) or known as microsatellite is a type of molecular marker that consist of one to six nucleotide units repeated in tandem and randomly spread in eukaryotic genomes. According to Palombi and Damiano (2002), SSR is highly polymorphic due to the high mutation rate affecting the number of repeat units. SSR provides co-dominant Mendelian markers which are much better and more powerful compare to dominant markers (Perumal *et al.*, 2008). In addition, SSR offers several advantages of being more simpler to use, less expensive and less time consuming as well as having special features such as PCR-based, highly reproducible, polymorphic and also abundant in plant genomes which made them more convenient to be used in evaluating the somaclonal variability in micropropagated plants compared to other available molecular markers (Palombi and Damiano, 2002). Due to such properties, SSR is favoured in the study of genetic stability of various plant species.

The application of SSR marker in genetic stability had been successfully tested in the previous study done by Xing *et al.* (2010) on regenerated *Solanum melongena*. In the study, 13 SSR DNA loci were used to examine the somaclonal variation for 40 plants which had been selected randomly among the regenerated plants. As a result, no SSR DNA variation was observed among the regenerants. Other than that, there are few more study had been carried out on genetic stability using SSR markers such as in almond, cherry and apricot germplasm (Cantini *et al.*, 2001; Martinez-Gomez *et al.*, 2001; Hormaza, 2002; Zhebentyayeva *et al.*, 2003) as well as in Actinidia (Palombi and Damiano, 2002), Norway spruce (Helmersson *et al.*, 2004), cork oak (Lopes *et al.*, 2006), cotton (Jin *et al.*, 2008), potato (Sarkar *et al.*, 2010) and olive species (Brito *et al.*, 2010).

### 2.3 Genetic Stability

The first successful plant tissue and cell culture was created by Gottlieb Haberlandt near the turn of the 20<sup>th</sup> century (Caponetti *et al.*, 2005). Plant tissue culture or also known as micropropagation is defined as a technique used to grow large numbers of plants where small pieces of tissues or organs are removed from the mother tree (donor plant) to be cultured aseptically on a nutrient medium under controlled environment in order to protect these tissues and cells from microorganisms (Bonga and Durzan, 1982).

The development of tissue culture techniques and molecular genetics had produce a huge impact in the world of plant multiplication as it allowed the exploration of novel pathways for plant development especially for conservation, breeding and commercial purposes. Through tissue culture techniques, some of the limitations of conventional breeding can be overcome and facilitated large scale production of plants especially woody plants. The tissue culture techniques not only offer large-scale production but also capable to retain the genetic information among the species of individual plant and its progenies. According to Xing *et al.* (2010), the most crucial fundamental in plant regeneration is to retain the genetic integrity with respect to the mother trees.

Meristem culture is one of the approaches used in micropropagation techniques. It is commonly refers to a technique that involved very small shoot apices which being dissected from the terminal or lateral buds; the microscopic apical dome with only the smallest leaf primordial evident which usually less than 2 mm across (Surendran *et al.*, 2000). This culture technique offers an advantage over other types of culture in which it is widely used in obtaining pathogen-free clones and thus makes it as an essential method for breeding and conservation of forest trees as well as for commercial purposes (Babaoglu *et al.*, 2002). At the same time, this

technique was capable in producing a complete plantlet that has genetic similarity with the mother tree. Moreover, this technique was successfully proven in the study of *in vitro* propagation of *Maytenus canariensis* (Mederos, 2002) and micropropagation of *Gloriosa superba* (Hassan *et al.*, 2005). Although micropropagated plants may associated with the risk of somaclonal variation, but this variation only occurs at low level and the presence of variation is one of the disadvantages of using tissue culture especially when culturing the desired elite tree. Therefore, the study of genetic stability is crucial to maintain the advantages of the desires elite genotypes (Modgil *et al.*, 2004).

## **2.4 Polymerase Chain Reaction**

The first person whom developed and introduced polymerase chain reaction (PCR) was Kary Mullis in 1980s. PCR is a molecular technique which used for unrestricted amplification of targeted DNA and provides an extremely sensitive means of amplifying small quantities of DNA. This revolutionary technique has been widely applied in medical and biological research field such as DNA cloning for sequencing, functional analysis of genes, identification of genetic fingerprinting, detection and diagnosis of infectious diseases as well as diagnosis of hereditary diseases. A basic PCR set up is comprise of DNA template which contains DNA region to be amplified, buffer solution, dNTPs, magnesium chloride, primers which are complementary to the DNA regions at 5' or 3' ends of the DNA region, distilled water and *Taq* polymerase in a final volume of 25  $\mu$ l.

Generally, there are three major steps in PCR; denaturation, annealing and extension. These steps are usually repeated for 25 to 40 cycles in order to obtain the optimum yields of DNA fragments and done in thermocycler or automated cycler. First, the reaction is initiated by

an initial denaturation at 96°C for one minute in order to activate the DNA polymerase. Then, the cycles begin with the denaturation step in which DNA template is heated at temperature between 92°C to 95°C. During this step, the double stranded DNA is denatured to form two single strands of DNA and the enzymatic reaction is stop as the enzyme denatured at high temperature. Next is the annealing step which is preferable at a sufficiently high temperature such as 55°C. At this stage, the short single strands of DNA which also known as primers bind at a lower annealing temperature to the single stranded complementary templates at ends flanking the target sequences. However, the annealing temperature varies for different kinds of species and depending on the length and nucleotide sequence of the primers. Subsequently, the temperature is raised up to 72°C; the extension step. This is the final stage where the high temperature is raised in order for the DNA polymerase enzyme to catalyze the template-directed syntheses of new double-stranded DNA molecules which identical in sequence to the starting material. The newly synthesized double-stranded DNA target sequences are denatured at high temperature and the cycle is repeated (Semagn *et al.*, 2006).

## **3.0 MATERIALS & METHODS**

### **3.1 Materials**

Isolated DNA, agarose gel, MetaPhor® Agarose gel, 1x TAE buffer, 1x TBE buffer, ultra-pure water, 1x PCR buffer, dNTPs, MgCl<sub>2</sub>, SSR primers (NCAC11 and NCAC14), *Taq* polymerase (Promega, USA), *fasTiP-X* kit and ethanol 70%.

### **3.2 Samples Collection**

A total of 40 Kelampayan stock plants were collected from the tissue culture laboratory, Sarawak Forest Corporation (SFC), Kuching. The molecular works, except for the sub-culturing and DNA isolation of the stock plants were done in the Forest Genomics and Informatics Laboratory (*fGil*), UNIMAS.

### **3.3 Sub-culturing of Samples**

A total of 40 Kelampayan mother trees were obtained from the tissue culture laboratory with temperature of 24 °C. Each stock plants was subjected for their first sub-culture in the laminar flow hood cabinet and aseptic techniques were applied. The sub-culturing process begins with separating the stock plants from the adventitious root. Then, the leaves were removed using scalpel; exposing the terminal and lateral buds. The node was excised and the shoot was divided into two sections. After that, transferred to Gamborg's B5 medium (Gamborg *et al.*, 1968) in a petri dish and stored in culture room.

### **3.4 DNA Isolation**

The DNA for SSR marker was isolated from the leaves of the stock plants using the Fast Incubated PCR-Extraction (*fasTiP-X*) method developed by Lai (2009) from the Forest Genomics and Informatics Laboratory (*fGil*). Six leaf discs with diameter of 0.5 mm were punched from the leaf using the special tool. Then the leaf discs were placed in PCR tube followed by addition of 50  $\mu$ l of extraction buffer and undergone incubation process at 95 °C for 10 minutes. After incubation, the tube was intermittently mixed by inverting and tapping. Then, 120  $\mu$ l of dilution buffer was added and finally the extracted DNA was stored in -20 °C freezer.

### **3.5 Polymerase Chain Reaction**

#### **3.5.1 PCR condition**

The PCR reaction was carried out using Mastercycler Gradient PCR (Eppendorf, Germany) and a total of 35 cycles was carried out. A total of 25  $\mu$ l PCR reaction mixture was prepared before the PCR reaction was carried out; which consisted of 1x PCR buffer, 0.2 mM of dNTPs, 2.5 mM  $MgCl_2$ , 2.5 pmol/  $\mu$ l of reverse primer, 2.5 pmol/  $\mu$ l of forward primer, 1 unit *Taq* DNA polymerase, 1  $\mu$ l of template DNA and 8.75  $\mu$ l of distilled water. The PCR profile is shown in the Table 3.1 below.

**Table 3.1:** Thermal cycling profile for SSR-PCR amplification

Parameter	Temperature (°C)	Timing	Number of cycles
Initial denaturation	94.0	2 min	
Denaturation	94.0	1 min	} 35
Annealing	61.0	30 sec	
Extension	72.0	2 min	
Final extension	72.0	10 min	

### 3.5.2 PCR optimization

The PCR optimization was carried out using the selected primer (NCAC11 and NCAC14) to determine the annealing temperature ( $55^{\circ}\text{C} \pm 10^{\circ}\text{C}$ ),  $\text{MgCl}_2$  concentration (1.0 mM-3.0 mM) and optimal DNA template concentration (100x dilutions from the original stock – 4 ng/  $\mu\text{l}$ ). Then, PCR products were separated with agarose gel electrophoresis and the product was stained with Ethidium Bromide.

### 3.5.3 SSR-PCR analysis

The extracted DNA sample was subjected to optimized PCR conditions with the selected SSR primers. Then, the SSR-PCR product was analyzed firstly using agarose gel electrophoresis with 2.0 % agarose gel in 1x TAE buffer for two hours at 70 Volts. 100 bp DNA ladder (Invitrogen) was used in agarose gel electrophoresis. Then, post-staining was done using Ethidium Bromide (25  $\mu\text{l}$ / 100 ml) for 2 minutes and de-stained for 30 minutes in distilled deionized water. The presence of bands was visualized under ultra-violet light. The photograph of DNA banding gel was taken using DNA visualize machine.

After the presence of DNA samples been confirmed using the agarose gel, the SSR-PCR products were finally analyzed using 3.5 % Metaphor® agarose gel electrophoresis in 1x TBE

buffer for 15 hours at 30 Volts. 100 bp DNA ladder (Invitrogen) and 25 bp DNA ladder (Invitrogen) were used. The post-staining was done using GelStar stain for 2 hours and visualized under ultra-violet light.

#### **3.5.4 SSR data analysis**

The SSR-PCR results were used for data analysis. The size of PCR bands produced; indicated different allele was estimated in the range of 100 bp to 200 bp. The sizes of the alleles were estimated using the graph  $\log_{10}$  marker fragment size versus migrated distance of the band which was done using Microsoft Excel. The SSR allelic composition was determined for each accession. The alleles were indicated in alphabetical order (A from the biggest fragment); single band was labeled as AA and more than one band produced was labeled as AB and so on.

The POPGENE version 1.31 software (Yang and Boyle, 1999) was used to examine the genetic stability between the mother trees and its plantlets. The analysis carried out was including the allele frequency, allele number, effective allele number (Hartl and Clark, 1989), observed heterozygosity and expected heterozygosity (Nei's 1978). The software PowerMaker version 3.25 (Chakraborty and Jin, 1993) was used to determine the polymorphism informative content (PIC) and at the same time used to construct dendrogram using UPGMA method and neighbor-joining tree.