



Faculty of Resource Science and Technology

**DETERMINATION OF GENETIC STABILITY IN *KELAMPAYAN* PLANTLETS
REGENERATED FROM TISSUE CULTURE USING ISSR MARKERS**

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**Bachelor of Science with Honours
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List of Abbreviations

AFLP	Amplified Fragment Length Polymorphism
bp	basepair
cm	centimeter
CTAB	Cetyl Trimethyl Ammonium Bromide
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide-triphosphates
D _{SA}	shared allele distance
<i>fasTip-X</i>	Fast Incubated PCR-Extraction
kb	kilo basepair
log ₁₀	log to the power of 10
m	meter
ISSR	Inter Simple Sequence Repeats
ISSR-PCR	Inter Simple Sequence Repeats – Polymerase Chain Reaction
MgCl ₂	magnesium chloride
min	minutes
ml	milliliter
mm	millimeter
mM	milimolar
MP	mother plant
μl	microliter
NC	plantlets
<i>N. cadamba</i>	<i>Neolamarckia cadamba</i>

NTYSYS	Numerical Taxonomy System
PCR	Polymerase Chain Reaction
pH	potential of Hydrogen
pmol/ μ l	picomolar per microliter
POPGENE	Population genetics
RAPD	Random Amplified Polymorphic DNA
SSR	Simple Sequence Repeats
TAE	Tris-Acetate-EDTA
U	unit
UPGMA	unweighted pair group mean average
UV	ultraviolet
V	volt
%	percent
$^{\circ}$ C	celcius

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Determination of Genetic Stability in *Kelampayan* Plantlets Regenerated from Tissue Culture using ISSR Markers

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ABSTRACT

Kelampayan species from the family of Rubiaceae was studied in terms of its ability to conserve genetic stability throughout plantlets regeneration from tissue culture specifically by organogenesis, via the culturing of nodal segments and shoot tips. The assessment of genetic stability had been carried out through the employment of Inter-Simple Sequence Repeats (ISSR) Marker. Two ISSR primers had been selected, namely (ACC)₆G and (AG)₉C as these primers were capable of producing informative and reproducible outcome. Based on the ISSR band data, a total of 37 loci were generated. The binary data matrix based on the Jaccard's similarity coefficient was calculated, resulting to a similarity indices ranged from 0.14 to 0.95 while the Shannon's information index had indicated a value of 0.3431. 100% polymorphic loci had also been revealed. Additionally, the neighbor-joining tree constructed based on D_{SA} shared allele had implied similar result as UPGMA dendrogram, signifying a relatively distant relationship among *kelampayan* plantlets and its maternal genotype. The high level of genetic variation detected at the early stage of culturing had shown to be as a result of mislabeling of samples during subculturing process. These had ultimately proven the effectiveness of ISSR markers as reliable tools for genetic variation studies, and thus indicated that *kelampayan* plantlets regenerated from tissue culture in this study is genetically unstable.

Keywords: *Kelampayan*, Inter-Simple Sequence Repeats Marker, tissue culture, genetic stability, polymorphism

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ABSTRAK

Spesis Kelampayan yang berasal daripada keluarga Rubiaceae telah dikaji dari segi keupayaannya untuk memulihara kestabilan genetik melalui regenerasi anak pokok menerusi teknik kultur tisu secara spesifiknya melalui kaedah organogenesis, berlandaskan pengkulturan segmen nod dan pangkal pucuk. Kajian berkenaan kestabilan genetik tersebut telah dijalankan dengan mengaplikasikan penggunaan penanda Inter-Simple Sequence Repeats (ISSR). Sebanyak dua primer telah dipilih, iaitu (ACC)₆G dan (AG)₉C, memandangkan primer-primer ini berkeupayaan untuk menghasilkan keputusan yang berinformasi serta menghasilkan maklumat yang konsisten tanpa had. Berdasarkan data band ISSR, sejumlah 37 lokus telah dijanakan. Matriks data binari berdasarkan pekali keserupaan Jaccard telah dikira, menghasilkan indeks keserupaan dalam julat 0.14 hingga 0.95, manakala Kepelbagaian Shannon telah mengindikasikan nilai 0.3432. 100% lokus polimorfik juga telah dikenal pasti. Di samping itu, neighbor-joining tree yang dihasilkan berdasarkan D_{SA} shared allele juga telah memberikan keputusan yang seiras dengan UPGMA dendrogram, menunjukkan hubungan antara anak-anak kelampayan dengan genotip pohon induknya adalah saling kurang berkaitan. Pengenalpastian tahap variasi genetik yang tinggi pada peringkat awal pengkulturan telah menunjukkan berlakunya kesilapan pada pelabelan sampel semasa proses subkultur. Kajian ini telah membuktikan pengaplikasian penanda ISSR sebagai satu kaedah yang sah dan efektif untuk kajian berkenaan kepelbagaian genetik, justeru membuktikan bahawa pertumbuhan anak-anak pokok kelampayan melalui kaedah kultur tisu dalam kajian ini adalah menjurus kepada ketidakstabilan genetik.

Kata kunci: Kelampayan, penanda Inter-Simple Sequence Repeats, kultur tisu, kestabilan genetik, polimorfisme

INTRODUCTION

Neolamarckia cadamba (Roxb.) Bosser or locally known as *kelampayan* is a favored plantation species having wide range crop-growing area, where it grows naturally in Australia, China, India, Indonesia, Papua New Guinea, Philippines, Singapore, Vietnam including Malaysia (Krisnawati *et al.*, 2011). Distribution of *N. cadamba* is wide, and can be found abundantly in the lowland area, mountain forests below 1000 meter altitude, often by streams and rivers, and mainly in open sites in the forest (Lim *et al.*, 2005). Due to the characteristics of *N. cadamba* which provides essential benefits in biodiversity (Joker, 2000), economical (Krisnawati *et al.*, 2011) and also medicinal values (Patel *et al.*, 2011), these specialties thus makes it a potential crop for commercialization.

Through the vast advance method of cultivating plant established globally, the adoption of new technologies on plant improvement had come to an exponential improved technique, such as genetic engineering and also plant tissue culture (Jain, 2001). In this study, typical conventional breeding of *N. cadamba* trees which basically utilizes the presence of soil, water, fertilizer and sunlight had been substituted by an alternative method introduced as plant tissue culture technique.

Plant tissue culture technique is defined as a bio-technique based on the premise that the plant organs, tissues, protoplasts or cells can be *in-vitro* manipulated to grow back into a complete or functional plant. This technique has long been recognized as an efficient tool for rapid clonal multiplication and conservation of desirable genotypes (Nayak *et al.*, 2010). It involves the preparation of media with specific basic supplement composition, and can be micropropagated into large scale of product in shorter period of time.

Compared to the conventionally breeding practice, labor work is needed and it requires a lengthy time of years to be able to obtain the yield (Panda *et al.*, 2007). However, in this proficient technique, there is some questioning in terms of its reliability level for generating true-to-type cloning; whether it has the ability to retain and preserve well the inheritable genotype encoded in the donor plant to its plantlets.

Microsatellites are widely represented in the genome of eukaryotic species. However, the usage of microsatellites has not been widely used to study lesser-known species (Dje *et al.*, 2006). Among various microsatellites, inter-simple sequence repeats (ISSRs) use repeat-anchored primers to amplify sequences between two inverted SSRs (Zietwiecki *et al.*, 1994). This marker also has features of high annealing temperature and longer sequence of ISSR primers, which yields reliable and reproducible bands at relatively low cost (Guo *et al.*, 2009).

According to Xu *et al.* (2010), ISSRs had been widely used to reveal the genetic variation of medicinal plants. Additionally, ISSR had also amassed the advantages of AFLP markers and SSR with the convenience of RAPD in molecular analyses (Santos *et al.*, 2011). These features thus suggest ISSR markers as a valuable tool in the study of genetic variability in plant species.

To date, there are no studies undertaken on the assessment of genetic stability of *N. cadamba* species from tissue culture by using ISSR markers. Therefore, in order to establish effective conservation strategies of *N. cadamba*, assessment pertaining genetic stability of this genuine species is urgent.

The objective of this study was:

1. To determine the genetic stability of tissue culture derived plantlets of *kelampayan* using ISSR markers.

LITERATURE REVIEW

2.1 *Neolamarckia cadamba*

Sarawak is a well-known state in Malaysia with astonishingly diverse constituent of flora element within its ecosystem. To date, there are several studies have been carried out concerning numerous plantation species as the potential source of wide-range economical production. One of the examples is *kelampayan* or botanically known as *Neolamarckia cadamba*.

Kingdom: Plantae

Subkingdom: Tracheobionta

Superdivision: Spermatophyta

Division: Magnoliophyta

Class: Magnoliopsida

Subclass: Asteridae

Order: Rubiales

Family: Rubiaceae

Genus: *Neolamarckia* F. Bosser

Species: *Neolamarckia cadamba* (Roxb.) F. Bosser

Scientific name: *Neolamarckia cadamba*

Source: *United States Department of Agriculture (2012)*



Figure 2.1: Glossy green leaves of *N. cadamba*. Usually of 15-50 cm long by 8-25 cm wide long and elliptical in shape.



Figure 2.2: Tree characteristics of *N. cadamba*. Large tree with a broad umbrella-shaped crown. (Adapted from Krisnawati *et al.*, 2011)

Neolamarckia cadamba is described as a plantation species capable of growing up to 45 m tall, and has a diameter up to 100-160 cm. It has straight and columnar barks, sometimes having steep buttresses up to 2 m tall. It can grow on a variety of soils (Krisnawati *et al.*, 2011) and tolerates periodic flooding (Joker, 2000). Fundamentally, it is also a fast growing plantation species suitable for reforestation in watershed and eroded areas.

Having broad umbrella shaped crown as its specialty features, it can therefore serves as an excellent shade tree along roadsides and villages, ornamental use, as well as windbreaks in agroforestry systems (Krisnawati *et al.*, 2011). In addition, *N. cadamba* also has its medicinal value and known as an important tropical tree used for treatment of blood disease, cough and uterine complaints (Patel *et al.*, 2011).

Current predominant interest of this plantation species involves the values it offers in the commercialization sector, specifically in the demand of its woods. The wood is recognized as a lightweight hardwood with texture moderately fine and even (Lim *et al.*, 1999). This type of wood is commonly utilized for plywood manufacture, and also to produce variety of products such as packing case, wooden sandals, toys disposable, chopsticks, matches, ceiling boards and possibly as a pulp for production of medium quality paper (Soerianegara and Lemmans, 1993; Joker, 2000; Krisnawati *et al.*, 2011).



Figure 2.3: The grey and smooth stem bark of young *N. cadamba* trees.

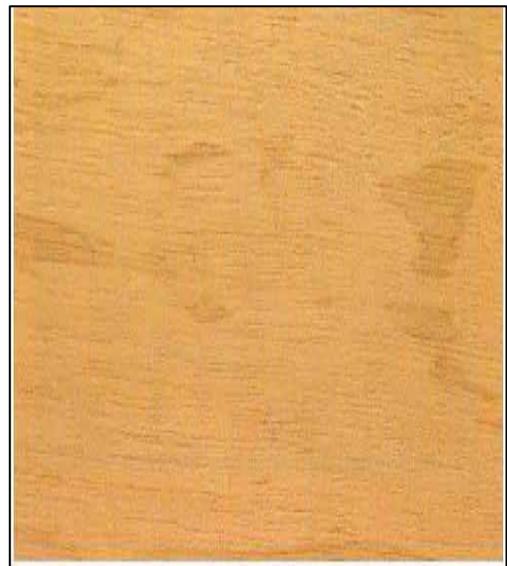


Figure 2.4: Wood characteristics of *N. cadamba*.

According to Krisnawati *et al.*, (2011), this plantation species is expected to become an increasingly important source for wood industries, particularly when supplies for plywood from natural forests declined. Revealing the potential of *N. cadamba*, series of research are conducted in Forest Genomics and Informatics Lab (UNIMAS) to develop strategies related to the genetic conservation of this trait.

2.2 Inter-Simple Sequence Repeats Marker

Molecular markers are sequence of protein or DNA that can be screened to show the variation of the organism at molecular level. The use of molecular marker has revolutionized the genetic study and is capable of giving direct, fast and accurate diagnostic. As stated by Weising *et al.* (2005), the employment of molecular markers has complemented the study on genetic variability, such as comparative anatomy, morphology, embryology and physiology. For ISSR markers specifically, it has been used in genomic fingerprinting, studies of genetic variation, phylogenetic analyses and evolutionary biology (Poczai, 2011).

Inter-simple sequence repeats marker (ISSR marker) – a DNA-based molecular marker, is an example of microsatellite which is often used due to its simplicity and wide distribution in genome of eukaryotic species. The technique is inexpensive, powerful, rapid, reproducible and simple to use (Abd-Alla, 2010; Santos *et al.*, 2011, Parashami and Thengane, 2012) as it provides generally reliable products and yield a large number of polymorphism per primer (Poczai, 2011; Parashami and Thengane, 2012).

The commonly used polymerase chain reaction (PCR) based DNA marker systems includes random amplified polymorphic DNA (RAPD) and most recently simple sequence repeats (SSRs). The major limitations of these methods are low reproducibility of RAPD and the need to know the flanking sequences to develop species specific primers for SSR polymorphism (Reddy *et al.*, 2002; Chandrika and Rai, 2009). Collectively, ISSR is thus a technique that overcomes most of these limitations as they need no prior knowledge or DNA sequence information of desired species (Farsani *et al.*, 2012) and require comparatively small amount of DNA (Ansari *et al.*, 2012) .

Inter-simple sequence repeats (ISSR) markers were introduced in 1994 (Zietkiewicz *et al.*), which is generated from single-primer PCR amplification. The primers are based on dinucleotide or trinucleotide repeat motifs (Wolfe, 2005). ISSR markers consist of longer primer (16-20 bp), and can precisely target the template DNA and thus improve reliability and reproducibility (Xu *et al.*, 2010).

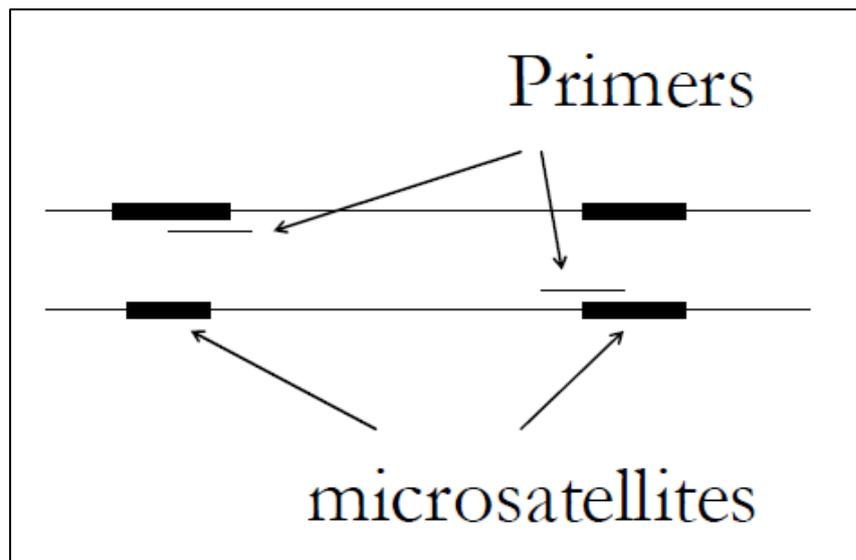


Figure 2.5: The amplification region of genome targeted by ISSR primers.
(Adapted source from Dje *et al.*, 2006)

ISSR markers amplify the DNA region located between two microsatellites locus. It also offers sensitive detection to reveal high polymorphism across the tested samples (Semagn *et al.*, 2006). In this study, ISSR marker was employed for the purpose of genetic fingerprinting, detection of genomic instability and phylogenetic analysis.

2.3 Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) is a molecular technique for unlimited amplification of targeted DNA. This sophisticated technique has led to advancement in the development of molecular biology since it offers a quick, inexpensive and simple approach. It is also referred as one of the most sensitive biological techniques ever devised. The technique amplifies specific DNA fragments from minute quantities of source DNA material, even if the source of DNA used was of relatively poor quality (Erlich, 1989).

For PCR-based marker system, optimization of PCR is essential in order to produce bands that are of similar intensity across the gel. Performing PCR without the optimized conditions will result in some often encountered problems, such as undetectable products and low yield of the desired products.

Therefore, an effective order of optimization PCR reaction condition can be conducted: (1) establish an annealing temperature that produces bands or smears of bands, (2) using the best temperature and systematically increase or decrease the DNA concentration in the reaction mixture, (3) take the best result from step 2 and systematically increase or decrease the concentration of $MgCl_2$ in the reaction mixture. Other PCR reaction condition can also be adjusted, such as the concentration of the thermostable *Taq* polymerase DNA.

2.4 Tissue culture-derived plantlets

Plant tissue culture has become one of the fundamental tools of plant science research. It is extensively employed in the production, conservation and improvement of plant resources (Chandrika and Rai, 2009; Bairu *et al.*, 2011).

This technique has often been regarded as a potential source for production of large-scale genetic copies of plants in a relatively small period of time. Regeneration of whole plants from cultured cells is essentially an extension of micro propagation and therefore should result in clonal uniformity.

Regeneration of plantlets via tissue culture is able to occur due to the characteristic of the plant itself, by having the ability to regenerate into whole organisms which depends upon the concept that all plant cells is able to express the total genetic potential of the parent plant given the correct stimuli. This term is known as totipotency, which refers to the capacity of a cell to give rise to an entire organism, provided by all the necessary genetic potential contained in its genome.

The positive impact of tissue culture technique was demonstrated in the micropropagation of Rhizome *Curcuma longa L.* (Panda *et al.*, 2007). Approximately 400 million plantlets could be produced from a single explant in a year, whereas multiplication rate through conventional method is only a maximum of 8 plants per annum.

However, implementation of this alternative technique may possibly causes variation to arise in cell culture, regenerated plants and their plantlets (Abd-Alla, 2010; Nayak *et al.*, 2011). The genetic fidelity of micropropagated plants is often questioned because there are frequent reports on the occurrence of somaclonal variations (Chandrika

and Rai, 2009). The genetic variation may occur as a result of *in-vitro* culturing of somatic cells which might be distracted by the phenomenon of mutagenesis (Bairu *et al.*, 2011).

For commercial utilization via tissue culture technique, development of newly elite lines of true-to-type is greatly essential (Nayak *et al.*, 2011). Hence, the assessment of the genetic stability of *in-vitro* derived clones is an essential step in the application of biotechnology for micropropagation of true-to-type clones (Chandrika and Rai, 2009).

2.5 Previous studies on genetic stability using ISSR markers

Several techniques have been developed to assess the genetic purity of tissue culture derived plantlets which includes molecular studies (Chandrika and Rai, 2009). At present, molecular techniques are recognized as powerful and valuable tools used in analysis of genetic fidelity of *in-vitro* propagated plants (Chandrika and Rai, 2009).

ISSR technique has successfully been used for the assessment of genetic fidelity in *Robina ambigua* (Guo *et al.*, 2006) and medicinal herb *Swertia chirayita* (Joshi and Dhawan, 2007). Other than that, a previous study by Huang *et al.* (2009), on the determination of genetic stability of long-term micropropagated plantlets of *Platanus acerifolia* using ISSR marker had determined a very low percentage of polymorphism, and detected 91% similarity level.

On the other hand, an assessment of genetic variation in *Passiflora* via ISSR markers by Santos *et al.* (2011) had detected as high as 98% of polymorphism. ISSR markers are considered suitable to detect variations among tissue culture-produced plants, since a simple sequence repeat-based primer targets the fast evolving hypervariable

sequences (Lopes *et al.*, 2006; Abd-Alla, 2010; Mahdizadeh *et al.*, 2012; Mohanty *et al.*, 2012).

In this study, ISSR marker is chosen to assess the genetic stability of regenerated *kelampayan* plantlets due to its specialty in giving a sensitive method for detection of polymorphism level. Moreover, ISSR had also been proven useful for genetic variation studies (Santos *et al.*, 2011; Farsani *et al.*, 2012). Hence, this point of advantages aid in providing practical information for breeding programs and eventually assists conservation strategies of this genuine species.

MATERIALS AND METHODS

3.1 Selection of stock plants

The selection of stock plants was done in the Tissue Culture Laboratory, Botanical Research Centre (BRC), Semengoh. Stock plants were defined as the *in-vitro*-grown mother plant seedlings originated from matured *N. cadamba* tree of Landih population. In this project, 40 selected stock plants of height ranging from 4-6 cm were chosen. Selected stock plants were labeled randomly and noted for future reference and further analysis.



Figure 3.1: *N. cadamba* grown conventionally in soil.



Figure 3.2: Selected *N. cadamba* stock plant.