GENETIC RELATIONSHIP OF *Jatropha curcas* L. FROM SAMARAHAN, LUNDU AND KALIMANTAN DETECTED BY RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD) MARKERS

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Bachelor of Science with Honours (Plant Resource Science and Management) 2012
Genetic Relationship of *Jatropha curcas* L. From Samarahan, Lundu and Kalimantan Detected by Random Amplified Polymorphic DNA (RAPD) Markers

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This thesis is submitted in partial fulfillment of the requirements for the degree of Bachelor of Science with Honours Plant Resource Science and Management

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I hereby declare that no portion of the work referred to in this thesis has been submitted in support of an application to another degree or qualification to this university or any other institution of higher learning.

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<td>AFLP</td>
<td>Amplified Fragment Length Polymorphism</td>
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<tr>
<td>CTAB</td>
<td>Cetyl-trimethylammonium bromide</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ISSR</td>
<td>Inter-Simple Sequence Repeat</td>
</tr>
<tr>
<td>NTSYS-pc</td>
<td>Numerical Taxonomy and Multivariate Analysis System for personal computer</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<tr>
<td>RAPD</td>
<td>Random Amplified Polymerase DNA</td>
</tr>
<tr>
<td>RFLP</td>
<td>Restriction Fragment Length Polymorphism</td>
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<tr>
<td>TAE</td>
<td>Tris-acetate-EDTA</td>
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<tr>
<td>UPGMA</td>
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ABSTRACT

*Jatropha curcas* L. from family Euphorbiaceae are also known as “Jarak” is getting attention nowadays even in Malaysia also this plant are being study from every aspects. A total of five samples were collected from Samarahan, Lundu and Kalimantan and planted in UNIMAS green house. Fresh *Jatropha*’s leaf sample was taken for the DNA extraction, DNA quantification (gel electrophoresis), and PCR amplification of DNA samples to gain the genetic relationship. From the result, a total of 48 alleles were generated from 9 OPF primers with 54.2% were polymorphic. OPF-08 primer produced highest polymorphic bands while OPF-17 and OPF-18 shows all monomorphic bands. Two cluster was constructed which unknown UNIMAS samples was in cluster I while the rest samples were in cluster II, the genetic relationship was analyzed through dendogram using NTSYS-pc 2.1 software. An UPGMA dendogram based on the polymorphic bands showed highest genetic similarity between Kg. Rembus 1 and Kg. Rembus 2 while the lowest similarity is between unknown UNIMAS sample with Kg. Rembus 2. In conclusion, *Jatropha curcas* L. in Samarahan, Lundu and Kalimantan have quiet high genetic variation among accessions studied. This study may provide genetic information for next study on *Jatropha curcas* L. in Sarawak.

Key word: *Jatropha curcas* L., Euphorbiaceae, RAPD markers, genetic relationship.

ABSTRAK


Kata kunci: *Jatropha curcas* L., Euphorbiaceae, penanda RAPD, hubungan genetik.
1.0 INTRODUCTION

1.1 Research Background

*Jatropha curcas* L. or also known as physic nut is a bush or small tree (up to 5 m height) and belongs to the Euphorbiaceae family. Genus *Jatropha* contains approximately 170 known species. Genus name *Jatropha* derives from the Greek *jatrós* (doctor), *trophé* (food), which implies medicinal uses. *Curcas* is the common name for physic nut in Malabar, India. The plant is planted as a hedge by farmers all over the world, because it is not browse by animals (Henning & Rothkreuz, 2004).

Worldwide interest in using *Jatropha curcas* L. as a feedstock for the production of biodiesel is rapidly growing. The properties of the crop and its oil have persuade investors, policy makers and clean development mechanism (CDM) project developers to consider *Jatropha curcas* L. as a substitute for fossil fuels to reduce greenhouse gas emissions (Achten et al., 2008). In Madagascar, Cape Verde and Benin, *Jatropha* oil was used as mineral diesel substitute during the Second World War (Agarwal, 2007; Akbar et al., 2009).

In Malaysia, this plant can be found at Terengganu, Selangor, Kedah and also in East Malaysia which are Sabah and Sarawak. Based on Burkill (1996) and Heller (1996) assumes that the Portuguese brought the physic nut to Asia: “Perhaps it did not reach Malacca until a date when the Dutch were in possession, for the Malays call it by a name meaning Dutch castor oil”. Nevertheless, the Portuguese transported it to the Old World. The biggest constraint to
cultivate *Jatropha* in Malaysia is the small number of fruits produced per inflorescence and the different ripening time of fruits on the same inflorescence (Camellia *et al.*, 2012). Based on Camellia *et al.* (2012) also, an intensive conservation program has to be carried out and the germplasm must be characterized to enhance utilization for varietal development. Assessment of genetic diversity using molecular marker is one of the major key for efficient management and conservation of plant genetic resources in gene banks.

*Jatropha* in the form of a plantation for seed production is not common. Farmers look for the dual advantage of crop protection combined with seed production. Plantations of several hectares have only been reported in Mali, West Africa, and in Nicaragua and Belize, Central America. Recently plantations are reported from Ghana, India, Nepal and Zambia (Henning, 2000). There were few studies done in characterization of natural diversity of *Jatropha curcas* germplasm with various multilocus markers systems and reports resulted in narrow diversity limiting the species for various genetic improvement programs (Sudheer *et al.*, 2010).

Prebreeding knowledge is limited. In particular, the regeneration ecology and the degree of genetic diversity among and within natural populations in and outside the center of origin are poorly studied. There is only a limited understanding of the *Jatropha* breeding system and the effect of inbreeding and out breeding (Achten *et al.*; Charles *et al.*, 2010).

Limitation with the currently used germplasm is the lack of knowledge on the genetic base, poor yield, low genetic diversity and vulnerability to a wide array of insect pests and diseases under monoculture. No systematic breeding program for breeding superior high yielding
genotypes has been initiated. Vast scope lies for genetic improvement of the *Jatropha* through genetic diversity study was done to understand the diversity in different germplasms for assessment and creation of diverse lines for future breeding (Gohil & Pandya, 2008). An understanding of the extent of genetic diversity is critical for the success of a breeding programme. Traditional methods using morphological characteristics for establishment of genetic diversity and relationships among accessions were largely unsuccessful due to the strong influence of environment on highly heritable seed traits like 100-seed weight, seed protein and oil content in *J. curcas* (Heller, 1996). Thus, selection based on genetic information using neutral molecular markers is essential as it is more reliable and consistent.

1.2 Research Objective

Objectives of this study is to detect the genetic relationship of *Jatropha curcas* L. between accessions at Samarahan, Lundu and Kalimantan as preliminary study to better understand the chance of genetic improvement for this species at Sarawak in future.
2.0 LITERATURE REVIEW

2.1 Botanical description

*Jatropha curcas* L. are also known as the “physic nut” or “Jarak” in some country. It is a drought-resistant species which is widely cultivated in the tropic as a living fence. *Jatropha* is a small tree or large shrub which can reach a height of up to 5 m. It showed articulated growth, with a morphological discontinuity at each increment. Dormancy is induces by fluctuation in rainfall and temperature or light. Branches of *J. curcas* contain latex. Normally, five roots are form from seedlings, one central and one peripheral. A tap root is not usually formed by vegetatively propagated plants (Kobilke, 1989; Heller, 1996). *Jatropha* is monoecious and flowers are unisexual. Pollination is by insects. The life-span of the *Jatropha curcas* plant is more than 50 years (Henning, 2000). *Jatropha’s* propagation through seeds naturally results in high degree of variation which is by cross-pollinating. It is also can live almost everywhere at varieties climatic conditions without harm, thus producing relatively variable and unpredictable yield.

2.2 Origin and distribution

*Jatropha curcas* L. is belong to the family Euphorbiaceae. It is a low growing tree, native to Central and South America and is widely present throughout Central America, Africa and Asia (Francis, 2005). *J. curcas* L. trees can grow in arid, semiarid and wastelands. It has a high-seed yield and high oil content (Wood, 2005). This shrub species is an alternative biofuel plant and thrive well in a tropical and subtropical climate and is well adapt to the Malaysia climate.
and soils (Rosdi Koter et al., 2000). *Jatropha* was introduced to Angola and Mozambique by Portuguese seafarers, from where it spread to the neighbouring countries. In the middle of last century, it was cultivated in a large extend in Madagascar, Benin and Guinea, from where it was exported to France as raw material for the famous "Savon de Marseille" (Henning, 2000).

2.3 Importance of *Jatropha curcas* L.

2.3.1 Economic importance

*Jatropha* is well-known of its valuable roles in economic sector. Based on Rosdi Koter *et al.* (2000), *Jatropha* can yield about 1,000 barrels of oil per year per square mile. In such quantities, *Jatropha* is like other biofuel crops in general which can become a partial replacement for oil as it requires minimal inputs, stabilizes or even reverses deforestation, and can be use in a variety of products after the biofuel is extract. Biodiesel can be blend with diesel fuel for use in diesel engine as proposed by Malaysian Government that 5 percent biodiesel mixture (B5) is mandatory. Biodiesel is an alternative fuel produce from renewable vegetable oils, animal fats or recycle cooking oils by trans-esterification reaction. Biodiesel has drawn significant attention due to increasing environmental concern and diminishing petroleum reserves (Ma & Hanna, 1999).

Wood and fruit of *Jatropha* can be used for numerous purposes including fuel. Seeds of *Jatropha* contain viscous oil, which can be used for manufacture of candles and soap, in cosmetics industry, as a diesel or paraffin substitute or extender. This latter use has important implications for meeting the demand for rural energy services and also exploring practical
substitutes for fossil fuels to counter greenhouse gas accumulation in the atmosphere. These characteristics along with its versatility make it of vital importance to developing countries (Akbar et al., 2009; Kumar & Sharma, 2008). More than that, *Jatropha* oil is also useful in soap production. Based on Henning (2000), most interesting and economically viable use of the *Jatropha* oil is soap production.

### 2.3.2 Medicinal value

Other than economic benefit, *Jatropha curcas* L. also known of its medicinal value. Refer to report of Drury (1985), latex of *J. curcas* L. is used to possess a healing and the expressed oil which prepared from the seeds is useful in cutaneous diseases and chronic rheumatism when apply externally. Native in India also use the *Jatropha* leaves for inflammation by warming and rubbing the leaves with castor-oil.

### 2.3.3 Role as anti-erosion

Cutting down the trees may lead to soil erosion and cause flash flood during heavy raining day. Thus, another side of *Jatropha*’s importance is it helps to hold soil and prevent soil erosion. Based on Henning (2000), if not cut *Jatropha* may grow up to 5 m in height. In this way *Jatropha* hedges around gardens and fields will reduce wind speed on the ground, and therefore wind erosion. Since *Jatropha* plants have lateral roots near the surface, they can be used to fix small earth dams which reduce the flow of run-off water. Water erosion can be further reduced by planting vetiver or lemon grass between the *Jatropha* trees.
2.4 Genetic diversity of *Jatropha curcas* L.

Recent years, analysis on genetic diversity of genus *Jatropha* has become infidelity due to limited research and information available about this species. Also due to the increasing popularity of *J. curcas* as a feedstock for biodiesel, there is an immediate need to generate nontoxic and high yielding varieties of the plant through genetic improvement (Zhengyin et al., 2011) in order to gain success in breeding *J. curcas* as it has a lot of advantages.

In order to gain genetic diversity information, many markers are available such as using RAPD markers, ISSR markers, RFLPs, AFLPs and some others. Use of DNA marker will help in providing more efficient, accurate, and reliable data because it is not influence by environmental factors compared to morphological or biochemical markers that are highly affected by environmental conditions (Ganesh Ram et al., 2008).

Study on genetic diversity and phylogenetic analyses had begun almost two decades ago and keep continuous until nowadays. One of the previous studies is on genetic diversity among *Jatropha* species as revealed by RAPD markers which is done by Ganesh Ram et al. (2008). Other than that, study also have been done for the newly developed biotechnological processes related to the exploitation of *J. curcas* include the genetic improvement of the plant, biological pest control, enzyme-supported oil extraction, anaerobic fermentation of the press cake and the isolation of anti-inflammatory substances and wound-healing enzymes (Gubitz et al., 1999). In general, RAPD markers suffer from a lack of reproducibility, but to check the consistency of
the electrophoretic patterns and the polymorphism detect, every PCR reaction disclosing polymorphism among accessions is repeated (Basha & Sujatha, 2009).

Other than genetic diversity study using RAPD marker, another markers also have been used for study genetic. Such as ISSR marker which has been done by Senthil Kumar et al. (2009) entitled molecular characterization of *Jatropha* genetic resources through inter-simple sequence repeat (ISSR) markers. This study shows 100% polymorphic patterns which indicate high level of genetic variation among the genotypes studied. Besides, study using SSR and AFLP markers which reveal low genetic diversity in the biofuel plant *J. curcas* in China (Sun *et al.*, 2008). In this study, the genetic relationships of 58 *J. curcas* accessions were assessed based on simple sequence repeat (SSR) and amplified fragment length polymorphism (AFLP) analyses, only one SSR primer was polymorphic with two alleles and seven AFLP primer combinations amplified 70 polymorphic loci in total, 14.3% of which were polymorphic.

Instead of many markers present nowadays, RAPD markers are commonly used to study genetic diversity due to some advantages. Referring to Cregan (2008), random amplified polymorphic DNA (RAPD) are polymerase chain reaction (PCR) based, no prior knowledge of DNA sequence are required and RAPD analyze as simply as the presence or absence of an amplicon via agarose gel electrophoresis. In spite of the limitation, RAPD markers has the greatest advantage of its capability to scan across all regions of the genome hence highly suited for phylogeny studies at species level (Ganesh Ram *et al.*, 2008; Wilkie *et al.* 1993; Demeke, 1992). Generally, main advantages of the RAPD technology include its suitability
for work on anonymous genomes, applicability to problems where only limited quantities of DNA are available, and the efficiency and low expense (Hadrys, Balick, & Schierwater, 1992).

2.5 Polymerase chain reaction (PCR)

PCR is a useful technique for amplifying many copies of specific DNA fragments. It is a technique of in vitro nucleic acid synthesis where any DNA segment may be replicated in a short time. It involved two oligonucleotide primer which squeeze DNA pieces that want to be amplified and cycles repeated include denaturation of DNA, annealing primer with DNA polymerase, and primer extension. Genetic diversity of particular organism can be explored through the organism's DNA. Amounts of DNA necessary are small enough that adequate samples may be obtained from a small sample of tissue. It is also possible to carry out the PCR analysis on unpurified DNA so that time-consuming DNA preparation and purification are avoided (White, 1993). Some advantages of using PCR are rapid amplification of DNA regions, facilitating of nucleotide sequencing, simple amplification technique and it is inexpensive method.

PCR method optimization contains some component which included Taq DNA polymerase, dNTPs, MgCl₂, and other component such as Tris-HCl turn over. Too higher concentration of Taq DNA polymerase will lead to inaccurate product background, while too low concentration will make insufficient product produced. Meanwhile for dNTPs, all four dNTP must used in same concentration to prevent binding error or mis-incorporation error. It is preferable to use
low dNTP concentration as it will increase the specification and accuracy of PCR because mis-priming at non-target site can be prevented.

Other important component in PCR is primer. Primer is a short segment of nucleotide, synthetic and single stranded DNA molecules used to prime DNA synthesis. It used to determine the DNA fragment to be amplified by the PCR process. Primer will annealed to the denatured DNA template thus providing an initiation site for the elongation of new DNA molecule.

Some previous study on PCR are inter and intra-population variability of *Jatropha curcas* L. characterized by RAPD and ISSR markers and development of population-specific SCAR markers (Basha & Sujatha, 2007). Their studies indicated that an immediate need for widening the genetic base of *J. curcas* germplasm through introduction of accessions with broader geographical background. Next, the genetic analysis study of *Jatropha* species and interspecific hybrids of *J. curcas* using nuclear and organelle specific markers (Basha & Sujatha, 2009) found that the use of 100 primers revealed 61.8% polymorphism with 7.62 polymorphic bands per primer indicating more genetic diversity in the accessions from diverse regions. However, use of ISSR primers failed to reveal higher polymorphism in the world collection when compared with Indian accessions. Latest study on *J. curcas* L. is done by Camellia et al. (2012) which found close genetic relationship among *Jatropha* accessions in Malaysia thus indicated that the accessions were derived from the same source when they were introduced to Malaysia. However, their studies accessions is excluded Sarawak.
Ganesh Ram et al., (2008) also have done PCR during their study in genetic diversity among *Jatropha* species using RAPD markers which shows that the overall grouping pattern of clustering corresponds well with principal component analysis confirming patterns of genetic diversity observed among the species. Their result provides valid guidelines for collection, conservation and characterization of *Jatropha* genetic resources. Based on Shahzadi et al. (2010) their study on optimization of DNA extraction of *Tagetes minuta* for PCR analysis concluded that DNA isolation is a primary and critical step for molecular analysis of any plant species. This process becomes even more difficult when the plant species contain high amounts of secondary metabolites and essential oils like *Jatropha*. These compounds that usually found in medicinal plants, are considers as contaminants which cause DNA degradation during preparation of genetic extraction. Therefore the extraction of genomic DNA from medicinal plant is difficult.
3.0 MATERIALS AND METHODS

3.1 Plant Material

Samples of *Jatropha curcas* from different accessions as showed in Table 3.0 were used in this study. *J. curcas* were taken from UNIMAS, Samarahan, Lundu and Kalimantan and were planted at greenhouse UNIMAS. Fresh young leaves was plucked and taken to the laboratory for extraction on the same day.

Table 3.0: List of *Jatropha curcas*’s accessions use in this study

<table>
<thead>
<tr>
<th>No.</th>
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<tr>
<td>1</td>
<td>UNIMAS</td>
<td>L1</td>
</tr>
<tr>
<td>2</td>
<td>Kg. Rembus 1, Kota Samarahan</td>
<td>L2</td>
</tr>
<tr>
<td>3</td>
<td>Kg. Rembus 2, Kota Samarahan</td>
<td>L3</td>
</tr>
<tr>
<td>4</td>
<td>Lundu</td>
<td>L4</td>
</tr>
<tr>
<td>5</td>
<td>Kalimantan</td>
<td>L5</td>
</tr>
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3.2 DNA Extraction

3.2.1 Reagents and Equipments

Reagents used in genomic DNA extraction were CTAB extraction buffer, chloroform:isoamyl alcohol (CIA 24:1); β-mercaptoethanol, pure cold (-20°C) isopropanol, wash buffer (76% ethanol, 10 mM ammonium acetate), TE buffer and 70% ethanol. CTAB extraction buffer used consists of 100 mM Tris-HCl, 20 mM EDTA, 1.4 mM NaCl, 3% CTAB and 1% polyvinylpirrolidone (PVP). β-mercaptoethanol was added to the CTAB buffer just before used. Tools and equipment used during this laboratory works were PCR machine (BIORAD-
MyCycler), centrifuge machine (Hettich Zentrifugen), water bath (PROTECH, Model-903), micropipettor (LABMATE), tips, 1.5 mL Eppendorf tube, mortar and pestle.

3.2.2 CTAB DNA Extraction Method

Genomic DNA of *Jatropha curcas* from five accessions at Sarawak was extracted based on mini CTAB extraction method of Doyle and Doyle (1987) with minor modification. Leaves were washed with distilled water and 70% ethanol for sterilization. 500 μl CTAB extraction buffer were added into a 1.5 mL Eppendorf tube and preheated to 60°C in a water bath about 15 minutes. Samples were then grinded in liquid nitrogen using a mortar and pestle. Approximately 0.2 g of the ground samples was added into the preheated CTAB buffer and added up 10 μl (2%) β-mercaptoethanol. Mixture is incubated at 60°C for about 45 minutes with occasional mixing every 15 minutes. After the incubation period, the mixture was left to cool down to room temperature for about 10 minutes. Next, an equal volume of CIA (24:1) was added into the Eppendorf tube and briefly vortex before centrifuge at 13,000 rpm for 15 minutes.

Upper aqueous layer was transferred into a new 1.5 ml Eppendorf tube and mixed again with equal volume of CIA. Mixture was centrifuged about 5 minutes at 13,000 rpm. Upper layer was then transferred into a new tube and added up with an equal volume of chilled isopropanol. Mixture was stored at -80°C for 25 minutes. The DNA is then recovered by centrifugation for 13,000 rpm for 15 minutes. Supernatant was discarded and DNA pellet was left to dry about 20 minutes. Then, DNA pellet was washed with 800 μl wash buffer and