Relationships between Malaysians Cultivars of Tongkat Ali (*Eurycoma longifolia* Jack) Obtained through RAPD Analysis

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Abstract: Random Amplified Polymorphic DNA (RAPD) analysis was done on *Eurycoma Longifolia Jack (ELJ)* cultivars originating from 7 different locations in Malaysia to determine whether the originality of *ELJ* cultivars can be preserved in an uncontrolled cultivation area. Out of 60 arbitrary 10-mer primers, a total of 320 DNA fragments were amplified with an average of 53.3 RAPD markers per primer. 71 (22%) of the 320 DNA-fragments was found to be monomorphic. The remaining 249 (78%) were polymorphic. The approximate size of the largest fragment produced was 3000 base pair (bp) and the easily recognizable fragment produced was 300 bp. Similarity index of RAPD banding patterns was used to investigate the relationships between *ELJ* cultivars. Mantel test showed that the correlation between Jaccard and Dice similarity matrices was high and significant (0.9907). The dendogram showed that *ELJ* cultivars can be clustered into three main clusters. Cluster 1 consisted of Sabah cultivars (East Malaysia), Cluster 2 consisted of Pahang and Terengganu cultivars (middle part of West Malaysia) while cluster 3 consisted of Kedah and Kelantan cultivars (east part of West Malaysia). The results of Principle Coordinates Analysis (PCA) were comparable to the Dendogram by UPGMA. The first two principle coordinates explained 53.62% of total variations and separated the 37 *ELJ* cultivars into three main clusters with a slight overlap between each other.

Keywords: *Eurycoma longifolia* Jack, Random Amplified Polymorphic DNA (RAPD) analysis, uncontrolled cultivation area, clustering.

1. INTRODUCTION

Eurycoma longifolia Jack *or ELJ,* belongs to the family of *Simaroubacea.* It is a single-stem slow growing tree, usually found in the jungles of Malaysia, Indo-China, Thailand, Vietnam, Sumatra, Borneo and other parts of South East Asia [1]. *ELJ* is traditionally used throughout South East Asia as a herbal medicine or tonic for the treatment of malaria, ulcer, fever and male fertility problems [2-5]. It has different common names. In Malaysia, it can be called Tongkat Ali, Payung Ali, Bedara Pahit, Tongkat Baginda, Petala bumi, Setunjang Bumi or Penawar Pahit. Indonesians call it Pasak Bumi while Vietnamese called it as Cay Ba Binh [4].

The plant has been chemically profiled [6-10], hence it is possible to chemically screen plants for genotypes of high quality. The active ingredients are concentrated in the root. The plant takes five years or more to reach reproductive age, and usually the fruiting rate is low [11]. Thus the rate of seed production cannot sustain the existing level of demand for these plant materials. The collection of this plant in the forest has become very difficult and non-economical. As a result of rampant, unlicensed harvesting of *ELJ* in the jungles of Malaysian Borneo, the tree has now been placed on the list of protected plants in Malaysia. To preserve the natural environment and to protect the remaining wild specimens of *ELJ*, Malaysia is currently promoting commercial cultivation of this plant. The Malaysian government is enthusiastic about the future of *ELJ* and is currently providing financial support for its development.

Suitable genetic markers are needed for propagation and breeding programs to support conservation of *ELJ*. However, only a small number of markers currently exist for the species [12]. In addition, land for propagation programs is also quite limited. Re-using old plantation farms could be a good alternative in addressing the cultivation space problem. This study was conducted to determine whether the originality of *ELJ* cultivars can be preserved in an uncontrolled cultivation area. Random Amplified Polymorphic DNA (RAPD) analysis was performed for this purpose.

2. MATERIALS AND METHODS

2.1. Plant Materials

A total of 45 cultivars of *ELJ* originating from 7 different locations in Malaysia (Sabah, Kelantan,

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Terengganu, Melaka, Pahang and Kedah) were harvested from a cultivated area at an old Palm Oil Plantation Farm in Felda Jengka 26, Jerantut, Pahang. Samples were 1.5 to 2.5 years old during harvest time. The amount of cultivars originating from each location is described in Table **1** below.

Table 1: Number of Cultivars Based on Origins

Location	Number of cultivars
Tanah Merah, Kelantan	4
Kuala Krai, Kelantan	8
Mengkabong, Sabah	8
Kuala Krau, Pahang	8
Merlimau, Melaka	4
Kuala Berang, Terengganu	6
Gunung Jerai, Kedah	7

Only the leaves were used for RAPD analysis. Harvested leaves were kept air tight in sealed plastic bags that were placed in an ice box until they could be stored properly in a laboratory.

2.2. DNA Isolation

Isolation of DNA was performed using Doyle & Doyle protocol [13] with some modifications. Grinded samples were mixed with an extraction buffer and incubated at 65^{o}C for 30 minutes with frequent shaking (every 5 minutes). Then the samples were centrifuged at 8000 rpm for 10 minutes. Proteins were extracted twice with chloroform: Isoamyl alcohol and then centrifuged at 8000 rpm for 10 minutes. Isopropanol and ammonium acetate were added before storing for a short period at -80^{o}C. 2 mg/ml RNase A was then added to the pellet for at least 4 hours at 37^{o}C, and the pellet was later washed using absolute ethanol and kept at -80^{o}C for 2 hours. After centrifuging, the sediment was then washed with 70% ethanol, dried at room temperature and lastly re-suspended in TE buffer.

RAPD reactions were performed on each *ELJ* sample in a 20 µl reaction mixture containing 10× buffer, dNTP mix, $MgCl_2$, Taq Polymerase, random primer and suitable amount of distilled water. For amplification, the thermal cycling conditions set were 40 cycles of 3 minutes at 94^{o}C, 30 seconds at 94^{o}C, 30 seconds at 40^{o}C and 3 minutes at 74^{o}C.

In order to find a useful and suitable primer for identification of *ELJ* at the species level, primer screening was performed. Sixty 10-mer single strands DNA primers were initially screened on six individuals from each location to identify primers revealing polymorphisms.

Amplification products were analyzed using electrophoresis. 1.2 % agarose gel was used. The gel was stained with ethidium bromide, and visualized under UV light to justify nanodrop spectroscopy result. The RAPD bands were recorded according to the presence (1) or absence (0) of a DNA band at the same location on the gel. Similarity Index was calculated using RAPDistance software. Similarity index matrices imported from RAPDistance software underwent a clustering analysis. Mantel test was applied in the program through MXCOMP procedure in the comparison of original matrices.

2.3. Correlation Analysis

The correlation coefficients calculated with the Mantel test enabled the finding of correlation between the similarity matrices. Relationships among cultivated *ELJ* were studied using Principal Co-ordinates Analysis (PCA). It was done to estimate genetic structure or degree of differentiation among populations and it was done using Analysis of Molecular Variance (AMOVA), as described by Excoffier *et al*. [14]. The analysis was performed using Genalex software [15].

3. RESULTS

Out of 60 primers, 25 primers showed good amplifications and 35 random primers did not give any amplification products or give poor amplification products. Of 25 primers which showed amplifications, the intensity of three primers fragment were very low and only twenty-two primers with good fragment intensity (OPA-1, OPA-2, OPA-3, OPA-4, OPA-7, OPA-10, OPA-11, OPA-13, OPA-18, OPB-5, OPB-7, OPB-8, OPB-12, OPB-14, OPB-15, OPB-17, OPC-2, OPC-5, OPC-6, OPC-8, OPC-11, OPC-20) were selected.

Table 2: Usable Primers for Further Analysis

Primer	Sequence (5' to 3')
OPA-3	AGTCAGCCAC
OPA-4	AATCGGGCTG
OPA-13	CAGCACCCAC
OPA-18	AGGTGACCGT
OPC-5	GATGACCGCC
OPC-6	GAACGGACTC
Total	6 Primers

Table 3: Correlation Coefficients from Mantel Test of Original Matrices

	Simple matching	Jaccard	Dice
Simple matching	****	****	******
Jaccard	0.69285	********	******
Dice	0.65929	0.9907	******

However, after careful screening only six primers were selected for further analysis as shown in Table **2**. These six primers were chosen because of its high reproducibility for every test. To determine the relationship between *ELJ* populations, Similarity index were calculated based on three types of coefficient: Dice, Jaccard and Simple matching coefficient.

In order to determine which type of similarity index gave the best result, the entire index was compared by applying Mantel test [16] in NTSYS PC program. The correlation values obtained are shown in Table **3**.

The result from Mantel test showed that the correlation between Jaccard and Dice similarity matrices was high and significant (0.9907). However, correlations between Jaccard, Dice and Simple Matching coefficient were very low compared to Jaccard and Dice correlations. As a result, Dice and Jaccard coefficients were used for *ELJ* cultivars dendogram construction (Figure **1**). Principal Co-ordinate Analysis (PCA) was used to estimate genetic structure or degree of differentiation among ELJ population. PCA plot of the relationship is shown in Figure **2**.

4. DISCUSSIONS

Genetic polymorphic markers such as isozymes, RAPD and simple sequence repeat (SSR) can be used to study genetic diversity, population structures and

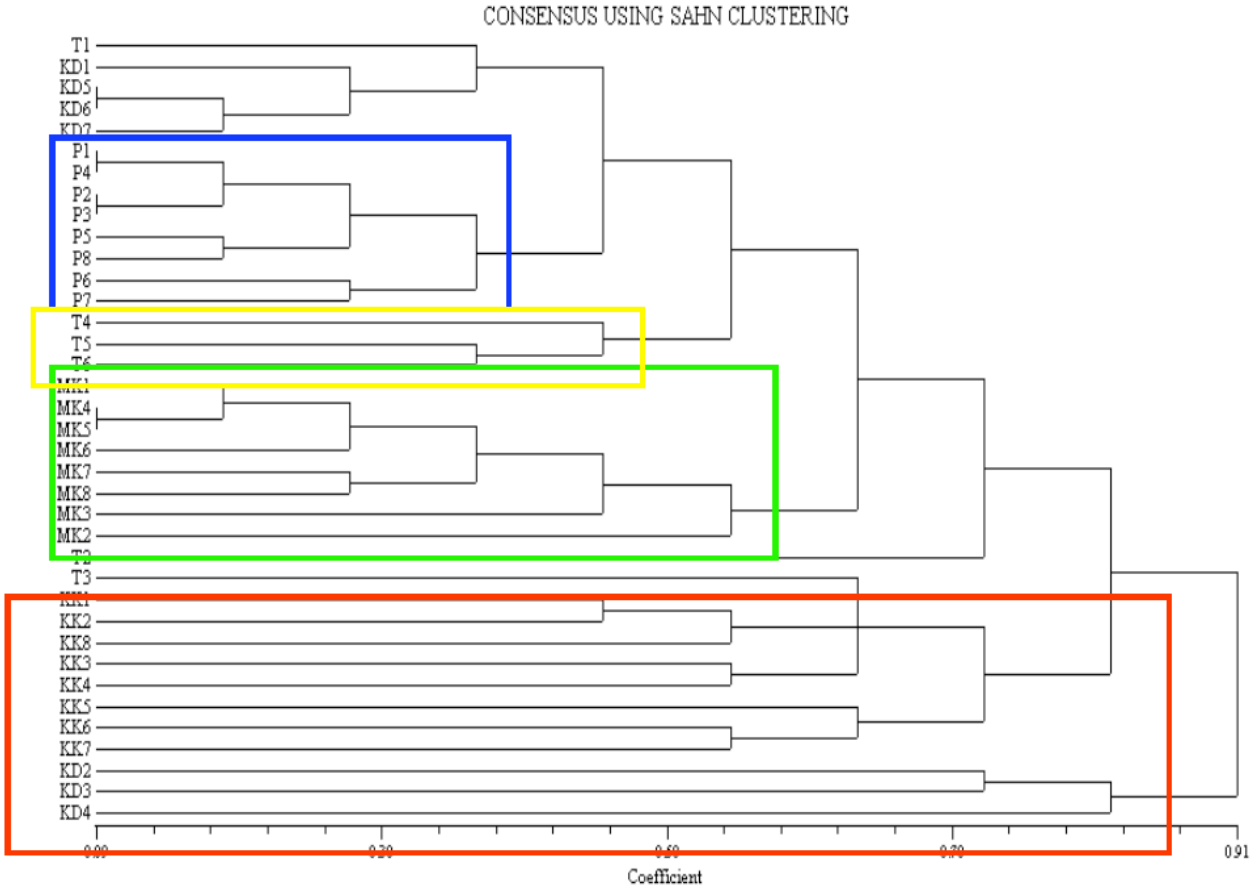

Figure 1: Dendogram based on Sequential Agglomerative Hierarchical Nonoverlapping (SAHN) clustering. T = Terengganu; P = Pahang; MK = Mengkabong, Sabah, KK = Kuala Krai, Kelantan; KD = Kedah.

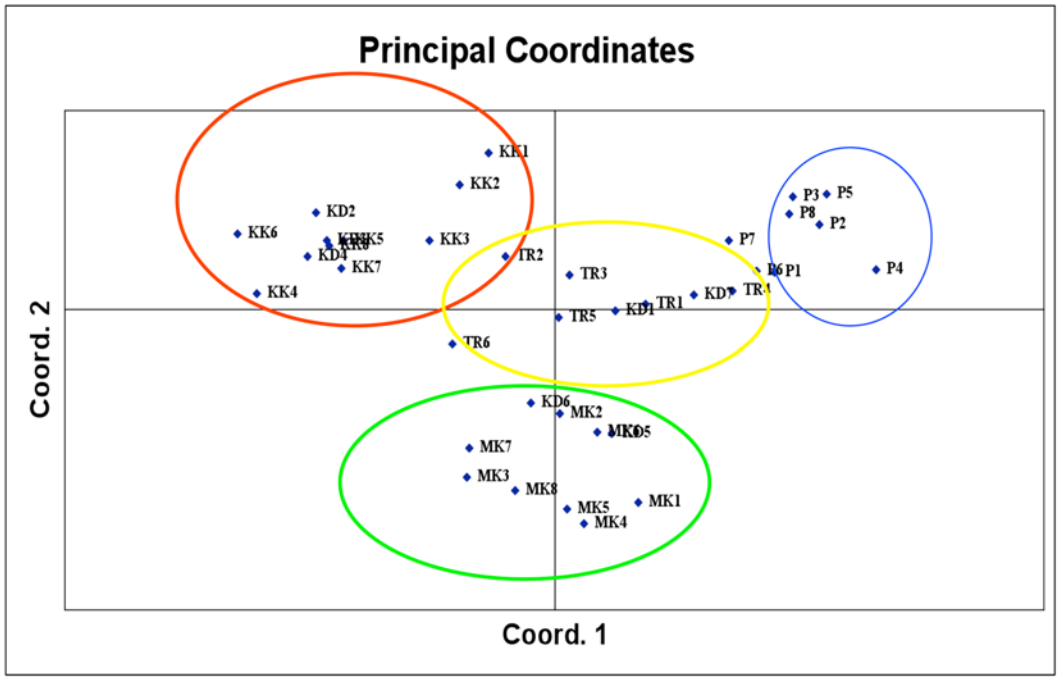

Figure 2: Principle Coordinate Analysis for *ELJ* cultivars.

subdivisions [17-21]. In this work, RAPD was used to characterize the seven populations of *ELJ* and elucidate the relationships between them. A total of 320 DNA fragments were amplified with an average of 53.3 RAPD markers per primer. Out of 320 DNA-fragments, about 71 (22%) was found to be monomorphic. The remaining 249 (78%) were polymorphic. The approximate size of the largest fragment produced was 3000 base pair (bp) and the easily recognizable fragment produced was 300 bp. However, Milelia *et al.* [22] suggested that fragments below 500 bp in size were rarely reproducible. As a result, only product sizes 500 bp onwards that yield strong, sharp and repeatable bands were scored to build a binary matrix.

The relationships between the populations of ELJ were estimated from similarity index analysis and PCA. Dendogram based on Sequential Agglomerative Hierarchical Nonoverlapping (SAHN) clustering, (Figure **1**) shows that *ELJ* cultivars can be clustered into their original locations, especially cultivars from Mengkabong, Sabah. All of the cultivars were clustered well. The same results were obtained for cultivars from Kuala Krai, Kelantan and Kuala Krau, Pahang. However some samples from Gunung Jerai, Kedah and Kuala Berang, Terengganu were seen to also be correlated to other cultivar locations.

Cultivars from Sabah, Pahang and Terengganu created three sub-clusters of own cultivar while samples from Kedah and Kelantan created the fourth sub-cluster. In view of all cultivars, *ELJ* cultivars can be clustered into three main clusters. Cluster 1 consisted of Sabah cultivars, Cluster 2 consisted of Pahang and Terengganu cultivars while cluster 3 consisted of Kedah and Kelantan cultivars. Location wise, two cultivars from east part of west Malaysia formed Cluster 3, populations from middle part of West Malaysia (Pahang and Terengganu) formed cluster 2 and cultivars from East Malaysia (Sabah) formed cluster 1. Figure **3** shows the locations of the origin of the cultivars.

The results of Principle Coordinates Analysis (PCA) were comparable to the Dendogram by UPGMA (Figure **2**). The first two principle coordinate explained 53.62% of total variation and separated the 37 *ELJ* cultivars into three main clusters with a slight overlap between each other. However, samples from Mengkabong, Sabah (MK) appeared to be distinct from the other samples in PCA.

5. CONCLUSIONS

The primary objective of this work was to analyze the relationships among *ELJ* cultivars in an uncontrolled cultivated area using genetic approaches. This work has proven that identification of *ELJ* from various cultivars can be obtained using PCR-RAPD, with the help of some analytical software.

Before RAPD analysis was performed, a modified DNA extraction method especially for plant with waxy

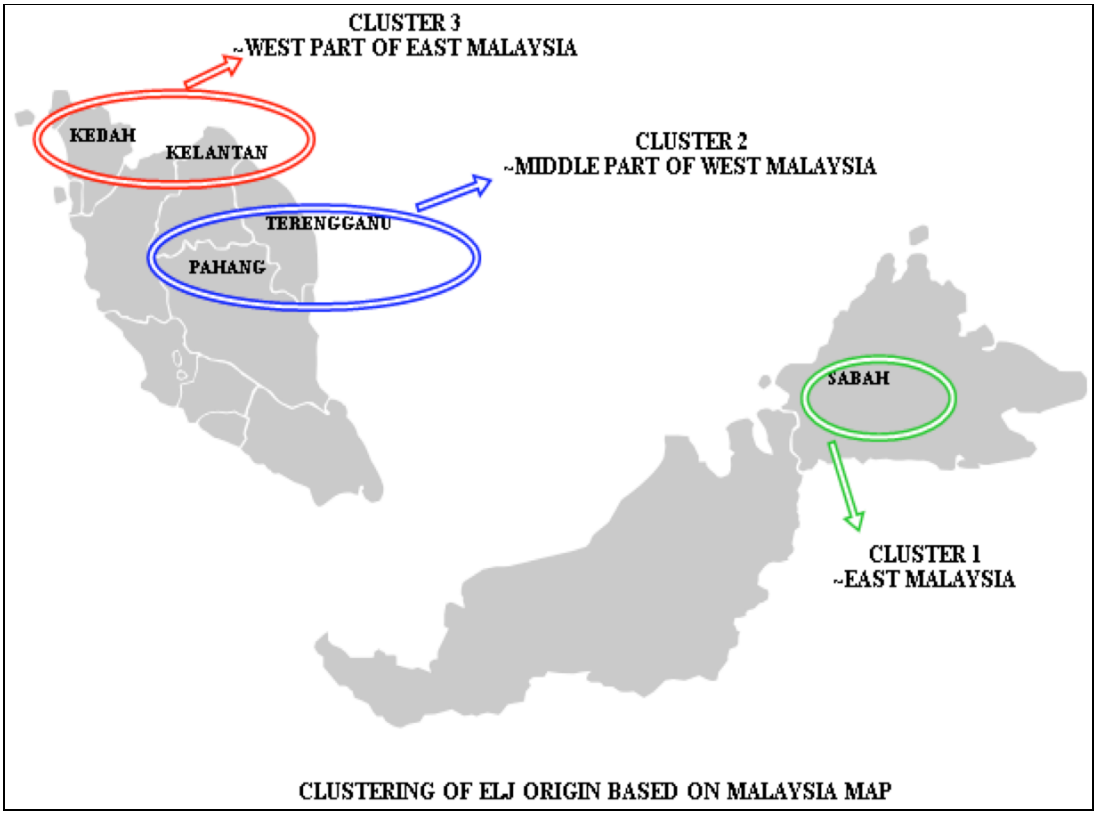

Figure 3: Map of Malaysia and the locations of the origin of the *ELJ* cultivars.

surfaces such as *ELJ* was successfully developed. The method yielded high quality and quantity of DNA. Six random primers (OPA-3, OPA-4, OPA-13, OPA-18, OPC-5 and OPC-6) were found to give good amplifications of *ELJ* DNA samples. However, only OPA-13 primer was chosen as the specific marker for *ELJ.* Clustering analyses detected almost the same classifications as the original cultivars. This showed that in an uncontrolled cultivated area, the *ELJ* samples could be characterized based on their cultivars origins.

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