Genetic Diversity of Sugar Palm Populations from Cianjur and Banten revealed by Simple Sequence Repeat (SSR) Markers

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Abstract. Sugar palm (Arenga pinnata Merr.) originating from Indonesia, is classified as underutilized plant but has potential as water conservation and high productivity sugar producer. Therefore, it is important to have baseline genetic diversity information for this plant species. In this study, seven simple sequence repeat (SSR) markers were used to analyse genetic diversity of sugar palm trees among the populations collected from their natural areas, especially in Cianjur Regency located in West Java and Banten Province. To measure their genetic diversity, phylogenetic tree and principal coordinate analysis were performed using recommended softwares. The results revealed that high allele variation (23-33 alleles) was observed among sugar palm samples tested, with an average allele number and polymorphism information content (PIC) value was 27.143 and 0.942 (0.937-0.951) respectively. All of SSR markers showed PIC value >0.9 which indicated that these markers were suitable for sugar palm diversity studies with a high differentiation. The UPGMA cluster analysis based on Nei’s standard genetic distance and principal coordinate analysis produced two main groups that were Cianjur and Banten populations, indicating the geographical differentiation. Taken together, the generated information about genetic diversity of sugar palm in this study could importantly contribute to assist sugar palm breeding program in Indonesia.

Keywords: Sugar palm, genetic diversity, simple sequence repeat

1. Introduction
Sugar palm, botanically known as Arenga pinnata Merr. is a palm species belongs to the Arecaceae family and monoecious in nature. Sugar palm trees not only produce sap but also multipurpose products (edible fruits, stem wood, building materials, fuel, fibres, etc.) [1][2]. Sugar palm can produce sugar 2-4 times higher than sugarcane from its sap. According to habitat, sugar palm is widely grown in tropical rainforests of and grown best in warm conditions with a maximum amount of light and abundant water supply on very fertile soils. Nevertheless, it is also well adapted under various environments and climates, from sea level up to 1400 m altitude, on all soil types from heavy loam to loamy sand and lateritic soils that are not regularly inundated [3]. This plant can also serve for land and water conservation plant due to its capability to prevent erosion, improve soil conditions and porosity, and trapping water from the rain [3–6]. As a consequence, sugar palm has been used to support ecological restoration and land rehabilitation in Indonesia, suggesting its high
potential to be developed [7]. In this regards, sugar palm has been intensively studied [4,8–12] and need deeper investigation addressing to phenotypic and genotypic characters.

Sugar palm is classified as underutilized perennial plant which has not been cultivated in a commercial scale although it provides a lot of benefits. Most farmers in Indonesia utilize this plant directly from the forest or those which are grown in their backyard whereas the fruit and sap productivity is relatively low. Providing superior varieties derived from selected high yielding parental trees through plant breeding programs can increase the productivity of sugar palm [6]. Information of estimated genetic diversity in perennial plant species such as of sugar palm would be very beneficial in parental lines selection for breeding and other genetic improvement programs for for desired traits [13]. Therefore, it is important to have baseline genetic diversity information for its genetic improvement programs in Indonesia due to wide plantation of sugar palm across the country including those from Cianjur and Banten as centers of production areas of sugar palm in Java’s island.

The use of phenotypic character for genetic diversity analysis of perennial plant is not sufficient due to it only possess small numbers of distinctive characters which do not provide an accurate classification [14]. On the contrary, the molecular markers have been proven to be an effective tool to get high precision of genetic diversity information and to point up relationship among perennial plant genotype [15,16]. The molecular markers are highly heritable, stable, less time required and exhibit sufficient polymorphism to discriminate very closely related genotype at any developmental stage without environmental interference [17]. There are very limited reports utilizing molecular markers in sugar palm, such as a study by using isozyme markers to study genetic diversity of four populations of sugar palm [18] and some others study used RAPD markers to analyse genetic diversity of sugar palm [19]. The first genome analysis of sugar palm and developed some simple sequence repeat (SSR) markers was already reported [20]. Concisely, genetic diversity information of sugar palm is very limited at the molecular level both domestically and abroad.

As demonstrated by many researchers the huge number of SSR in various plant species, the developed SSR molecular markers are widely applied in genetic diversity analysis and marker assisted selections (21). These markers are short DNA tandem sequences distributed across the plant genome [22]. The advantages of these markers are codominant, high level polymorphism and reproducibility, and easier application through PCR (Polymerase Chain Reaction) [23]. To date, no any genetic diversity assessment of sugar palm using molecular markers has been reported elsewhere. The objective of the present study was to analyse genetic diversity of sugar palm trees and genetic distance among its populations collected from its natural distribution in Cianjur Regency, West Java and Banten Province based on SSR markers.

2. Material and Methods

A total of 40 sugar palm individual trees comprised 10 samples originally collected from Cianjur, West Java Province, 30 samples from Banten province which consisted of 15 samples from Cibaliung, and 15 samples from Rangkasbitung were used in this study. All these sugar palm samples were selected from local farmers based on geographical different places, where Cianjur and Banten could represent the producing centre producer in West Java and its nearby province.
Figure 1. A representation of local origin of sugar palm trees which are from Cianjur, Rangkasbitung and Cibaliung on West Java and Banten Province map. The number wrote inside the box is representing the number of samples collected from those areas.

Extraction of genomic DNA was carried out in the Molecular Biology Laboratory of Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development. Total genomic DNA was extracted from fresh and young leave, grounded into fine powder using pestle and mortar with liquid nitrogen. The DNA isolation was isolated using Cetyl Trimethyl Ammonium Bromide (CTAB) according to standard procedure as described previously [24]. Dry pellet of DNA was dissolved in 100 µl TE solution (10 mM Tris pH 8.0 and 1 mM EDTA) and then was diluted to a working concentration of 10 ng/µl for DNA amplification. The quality and quantity of DNA were determined by electrophoresis on a 1% agarose (w/v) gel, and using a NanoDrop™ 2000 Spectrophotometer (Thermo Fisher Scientific, USA). Furthermore, DNA of all samples was visualized under Gel Doc-UV Imager (Thermo Fisher, USA).

PCR amplifications were carried out using seven SSR markers developed based on genomic variation of dwarf and tall sugar palm which are available at Pusat Genom Pertanian Indonesia (http://genom.litbang.pertanian.go.id) (Table 1). DNA amplifications were performed in a T1 Thermocycler (Biometra, Germany). Amplifications were carried out in 10 µl reaction solution containing 2 µl of 10 ng DNA template, 5 µl Kapa2G Fast Ready Mix (Kapa Biosystems, USA), 0.5 µl each of the forward and reverse primers, and 2 µl sterile ddH2O. Conditions for amplification of the region consists of initial denaturation at 94°C for 5 min, 35 cycles consisting of denaturation at 94°C for 30 s, annealing at 55°C for 1 min, elongation at 72°C for 1 min, then final extension at 60°C for 15 min followed by hold temperature at 4°C. The PCR products for each sample were separated on 8% polyacrylamide gel in Tris-Borate EDTA buffer at 90 V for 110 min and then stained with ethidium bromide. These were visualized and photographed under a UV Light Transilluminator (Biorad, USA). The sized DNA products were calculated by comparison them with 100 bp DNA ladder (Vivantis, Malaysia).

Table 1. List of seven SSR primers of sugar palm and their sequences used in this study.

| Primer names | Primer sequences (5’ → 3’) | Repeated Motif |
|--------------|----------------------------|--------------|
| AD159        | F: GAAGCTAGAGGTTTTGAAGGAG  | (AG)18       |
|              | R: AAATGCCCTCTTTTTATTCAC   |              |
| AD187        | F: GCAAGCCTCGAAACAATTAAA   | (ATA)27      |
|              | R: CAGTGCTTCTATCATCCAAG    |              |
Clearly selectable and easily countable DNA bands of each SSR primer on all individual sample were scored as binary data and SSR allelic size was determined using Gel Analyzer software [25]. The binary data was analysed then using three different software packages, including PowerMarker V3.25 [26], GenAlEx 6.5 [27] and Mega5 [28]. The major allele frequency (Na), Nei's gene diversity (Gd), expected heterozygosity (He) and Polymorphic information content (PIC) values for each marker was estimated using Power Marker V3.25 software [29].

The genetic similarity (GS) and distance value among sugar palm populations was calculated based on Dice coefficient of similarity [27]:

$$\text{GS} = \frac{2N_{ij}}{N_i+N_j}$$

where $N_{ij}$ is the number of allele types presented in both population $i$ and $j$, $N_i$ is the number of allele types presented in population $i$, and $N_j$ is the number of allele types presented in population $j$. Cluster analysis was performed by UPGMA (Unweighted Pair Wise Methods with Arithmetic Averages) to generate a dendogram. GenAlEx 6.5 was used to determine the banding patterns among groups and for principal coordinates analysis (PCoA). Due to unbalanced genotype numbers per group and to confirm the results of the cluster analysis, principal coordinates analysis was carried out using Nei’s unbiased genetic distance.

3. Result and Discussion

3.1 Molecular Marker Polymorphisms Analysis

In this study, all seven pairs of SSR markers used produced a total number of 190 repeatable and scorable polymorphic bands (Table 2, Figure 2). DNA fragments ranged from 122 to 184 bp. Results of the analysis using SSR markers across 40 samples of sugar palm from three different areas indicated that there were 23-33 alleles per locus, with an average of 27.143 alleles. Expected heterozygosity (He) or Nei's gene diversity (Gd) per locus is used to quantify the genetic variation and to evaluate genetic divergence and population relationship. Nei's gene diversity estimated using each of the evaluated SSR markers among 30 samples of sugar palm from three different areas, ranged from 0.941 (AD159) to 0.953 (AD303), with an average of 0.945. Heterozygosity is obtained from the calculation of gene frequencies at each locus [30]. Furthermore, all SSR markers are able to distinguish heterozygosity with heterozygosity values varied from 0.100 to 0.725, with an average of 0.364. The value of heterozygosity close to 0 is low, while the value of heterozygosity close to 1 is high [31] AD303 and AD159 SSR loci exhibited the highest and lowest heterozygosity, respectively. Varied heterozygosity existed was possibly due to the high outcrossing in sugar palm genotypes as a result of allogamy, as also found in other palmae like coconut, oil palm and date palm [32–34].
Figure 2. Examples of DNA banding patterns produced by AD359 and AD187 markers separated by 8% polyacrylamide gel. The well on the left side represents M: 100 bp DNA ladder; 1-30: samples from Banten Province; 31-40: samples from Cianjur.

Table 2. Statistics of the 7 SSR loci across 40 sugar palm samples.

| Locus  | Allele size range (bp) | Na<sup>a</sup> | He<sup>b</sup> | Gd<sup>c</sup> | PIC<sup>d</sup> |
|--------|------------------------|----------------|--------------|--------------|---------------|
| AD159  | 149-175                | 23             | 0.100        | 0.941        | 0.938         |
| AD187  | 128-184                | 27             | 0.225        | 0.948        | 0.945         |
| AD303  | 122-167                | 33             | 0.725        | 0.953        | 0.951         |
| AD363  | 138-178                | 27             | 0.300        | 0.946        | 0.943         |
| AD675  | 150-172                | 27             | 0.525        | 0.940        | 0.939         |
| AD359  | 141-168                | 25             | 0.275        | 0.940        | 0.937         |
| AD75   | 149-180                | 28             | 0.400        | 0.945        | 0.942         |
| Mean   |                        | 27.143         | 0.364        | 0.945        | 0.942         |

<sup>a</sup>Na: number of alleles; <sup>b</sup>He: heterozygosity; <sup>c</sup>Gd: Nei's gene diversity; <sup>d</sup>PIC: polymorphic information content.

Further analysis of the polymorphic alleles of each SSR primer used demonstrated the level of primers informativeness which was determined by the calculation of polymorphic information content (PIC). PIC value provides an estimate of the distinguishing power of a marker by computing not only the number of alleles in one locus but also the relative frequency of the alleles of an identified population. PIC value becomes the standard to evaluate the genetic markers based on PCR amplified DNA pattern [35]. The average PIC value ranged from 0.937 (AD359) to 0.951 (AD303), with an average of 0.942 (Table 2), which means that all of the SSR markers which were developed from genome variations of dwarf and tall sugar palm are highly informative [36] and considered to be suitable markers for genetic diversity differentiation among individuals and populations based on geographic origin. Referring to previous study, sugar palm populations from Banten and Cianjur were categorized as tall type and dwarf type, respectively [37]. Prior to the present study, genetic diversity studies on sugar palm populations in South Tapanuli using ten RAPD markers indicated low genetic
diversity inferred by 31.57% of gene diversity and 0.852 PIC value [38]. Compared to which, this study showed a higher genetic diversity level with the higher values of PIC. These differences may be as a result of molecular markers [39] and the nature of sugar palm type in different environment used in this studies.

3.2 Genetic Diversity Analysis

To identify the genetic diversity between the 40 sugar palm individuals from three different areas (Cianjur, Cibaliung and Rangkasbitung) in this study, a phylogenetic tree was subsequently constructed from seven pairs of SSR markers based on their binary scores using the UPGMA cluster analysis. Sugar palm populations can be clearly distinguished into two main groups (Figure 3) which represented far genetic distance between main groups, in contrast to those in the same group which reflected their closer genetic [40,41]. The first group consisted of Cianjur Regency, West Java populations, while second group included the populations of Banten (Rangkasbitung and Cibaliung). This indicated that the genetic variations in sugar palm are based on geographical distribution area. We assumed that the genetic diversity varied among the sugar palm in different geographical areas was generated by mountains isolation between Banten and Cianjur Regency, which may have effectively blocked gene flow, consequently influence the genetic diversity as well as the genetic relationship among different groups of sugar palm [42]. The second group can be further divided into two sub groups comprising Rangkasbitung and Cibaliung, indicating the close relationship. This was not surprising as Rangkasbitung and Cibaliung are located in the same province, which are around 100 km apart from each other. We assumed that sugar palm populations of Rangkasbitung and Cibaliung may be from the same source, and they have similar genetic background. Therefore, hybridization between two closely related individuals should be avoided as it would lead to inbreeding depression [43].

Figure 3. A phylogenetic tree of three populations of sugar palm based on UPGMA program using Mega5 software.
To determine the relationships between sugar palm populations, principal coordinate analysis (PCoA) was used. PCoA is frequently used to indicate genetic similarity among populations, with populations clustered according to their geographical location [44]. PCoA revealed that the first two components determined 65.67% of total variance. Similarly with UPGMA cluster analysis, the PCoA results as seen in Figure 4 which is based on population genetic distance, also revealed that sugar palm populations could be clearly divided into two groups reflected two provinces where the samples collected from. This analysis revealed strong geographic patterning of sugar palm populations grown in West Java and Cianjur with certain environment. UPGMA clustering and PCoA analysis results in this study are giving a clue that genetic diversity between sugar palm populations is important as initial information on the genetic material selection for plant breeding hybridization,

4. Conclusions

Molecular characterization using seven pairs of SSR markers based on Nei’s standard genetic distance and principle coordinate analysis separated 40 sugar palm samples from three different areas (Cianjur, Rangkasbitung and Cibaliung) into two groups. The grouping of these sugar palm population tended according to their geographical origin. The first group consisted of Cianjur Regency, West Java populations, while second group consisted of the populations of Banten (Rangkasbitung and Cibaliung). All of SSR markers used were highly informative and suitable for sugar palm diversity studies in the future.

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