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Genetic diversity analysis of Puan Kalianda kopyor coconuts (*Cocos nucifera*) from South Lampung, Indonesia based on SSR markers

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Abstract. *Rahayu MS, Setiawan A, Maskromo I, Purwito A, Sudarsono. 2021. Genetic diversity analysis of Puan Kalianda kopyor coconuts (*Cocos nucifera*) from South Lampung, Indonesia based on SSR markers. Biodiversitas 23: 205-211.* Puan Kalianda kopyor coconut (*Cocos nucifera* L.) is a newly released tall kopyor coconut from Kalianda, South Lampung, Indonesia. The kopyor coconut is an exotic, highly economic value coconut mutant with an abnormal endosperm. This study aimed to analyze the genetic diversity of Puan Kalianda kopyor coconut from South Lampung, Indonesia using SSR markers. As many as 91 Puan Kalianda kopyor coconut accessions were genotyped using 10 SSR marker loci, and the generated data were used to evaluate their genetic diversity and population structure. The results showed a high degree of SSR marker polymorphism (PIC value = 67%), indicating the SSR marker loci are informative for revealing the genetic diversity within the evaluated Puan Kalianda kopyor coconut population. The Puan Kalianda coconut population showed a 70% expected heterozygosity (He) and 60% observed heterozygosity. The phylogenetic analysis formed two main clusters, and each cluster consisted of three sub-clusters. The Genetic structure analysis showed that the population most probably derived from two ancestral origins (K = 2) and can further be clustered into six sub-clusters (K = 6). Therefore, since genetic diversity within the population is relatively high, the Puan Kalianda tall kopyor coconut population can be considered an essential genetic resource for future kopyor coconut development.

Keywords: Abnormal endosperm, *Cocos nucifera,* genetic diversity, kopyor tall coconuts, SSR markers

Abbreviations: PIC: polymorphic information content, He: expected heterozygosity, Ho: observed heterozygosity, SSR: simple sequence repeats

INTRODUCTION

Coconut (*Cocos nucifera* L.) is a tropical palm tree with multi-purpose products (Perera 2013). However, the coconut economic values were recently replaced by the oil palm (*Elaeis guineensis*) (Kasryno 2015; FAOSTAT 2019), driving down the competitiveness of copra and coconut oil. Therefore, finding alternative use of coconut is the focus of various institutions worldwide (Verma et al. 2012; Lima et al. 2015; Directorate General of National Export Development 2017a, 2017b).

Indonesia is known for diverse coconut germplasms with many different phenotypes and specific characters, such as fruit and shapes, husk colors, and endosperm characters (Maskromo et al. 2014). Kopyor coconut is a natural coconut mutant having an abnormal endosperm (Sudarsono et al. 2011; Maskromo et al. 2014) with soft and peeled-off endosperm from the shell (Kumar et al. 2015; Sudarsono et al. 2015). Coconut provenances capable of producing kopyor fruits only exist in limited regions in Indonesia, such as South Lampung district (Lampung), Pati district (Central Java), and Sumenep district (East Java) (Maskromo et al. 2014). The kopyor coconut provenances in Pati (Central Java) are mostly the dwarf kopyor coconut type (Novarianto and Lolong 2012), while in the other areas are tall ones (Maskromo et al. 2014). Since the price of kopyor coconut fruit is many times higher than the normal one (Novarianto and Lolong 2012), the development of kopyor coconuts may become alternative routes for coconut farmers to regain the economic value of coconut (Larekeng et al. 2015a, 2015b; Maskromo et al. 2015).

Information for the phenotypic and genetics of the kopyor abnormal endosperm traits is limited (Maskromo et al. 2014; Rahayu et al. 2019). Therefore, compiling the phenotypic and genetic of kopyor abnormal endosperm traits will benefit future kopyor coconut improvement programs. As in another coconut, kopyor coconut breeding progress is slow because of the perennial nature and the scarcity of phenotypic and genetic diversity. Introgression of kopyor's abnormal endosperm trait into normal coconut genetic background should increase the phenotypic and genetic diversity of kopyor coconut (Rahayu et al. 2019).

Puan Kalianda kopyor coconut is a newly released tall kopyor coconut variety from South Lampung, Indonesia. The abnormal endosperm phenotype of Puan Kalianda kopyor coconut is controlled by one or two recessive genes (Novarianto et al. 2014; Sudarsono et al. 2015; Rahayu et al. 2019). Other than genetic control of abnormal endosperm, genetic information associated with the Puan Kalianda kopyor coconut is poorly reported. Therefore, any genetic studies of the Puan Kalianda kopyor coconuts will benefit future coconut breeding.

Molecular markers are commonly used to study the genetic diversity of many perennial crops, including coconuts (Maskromo et al. 2014; Larekeng et al. 2018; Mahayu and Taryono 2019). Simple sequence repeats (SSR) marker was used more extensively for the genetic analysis of coconuts than RAPD (Randomly Amplified Polymorphic DNA), ISSR (Inter Simple Sequence Repeat), and AFLP (Amplified Fragment Length Polymorphism) markers (Martinez et al. 2010; Ribeiro et al. 2010; Larekeng et al. 2018; Mahayu and Taryono 2019), since it is a codominant, a multi-allelic and are widespread across the plant genome (Zalapa et al. 2012). Therefore, SSR markers are suitable for coconut genetic studies (Zhu et al. 2011; Ting et al. 2014; Larekeng et al. 2015a, 2015b; Larekeng et al. 2018; Mahayu and Taryono 2019).

The kopyor coconuts in South Lampung district, Lampung province, Indonesia are especially interesting since they show a broad phenotype diversity (Maskromo et al. 2014). Therefore, it will be essential to elucidate further the phenotypic and genotypic variations among individuals within the Puan Kalianda tall kopyor coconuts. This study aimed to analyze the genetic diversity of Puan Kalianda tall kopyor coconut from South Lampung, Indonesia using SSR markers. The generated information will help breeding programs develop new coconut varieties having kopyor endosperm in the future. Moreover, the generated phenotypic variation and genetic diversity data are beneficial for germplasm management and conservation of the Kalianda tall kopyor coconut.

MATERIALS AND METHODS

Plant material and DNA isolation

A total of 91 kopyor coconut provenances existed in a sole location in South Lampung, Indonesia was identified and mapped using GPS. Each coconut provenance was tagged and numbered according to the GPS positions. Young leaves were harvested from selected palms and sent to Plant Molecular Biology (PMB) Laboratory, Department of Agronomy and Horticulture, Faculty of Agriculture, Bogor Agricultural University. Total DNA was isolated from leaf samples (0.3 g) using standard cetyltrimethylammonium bromide (CTAB) procedures, routinely used for DNA isolation in the palm species (Larekeng et al. 2015a, b; Natawijaya et al. 2019; Rinawati et al. 2021). The isolated DNA was resuspended in 50-100 µL of aqua bidest and checked for its quantity using 0.8% agarose gel electrophoresis.

Genotyping using Simple Sequence Repeat (SSR) marker

Ten SSR marker loci previously used for characterizing Indonesian coconut genetics (Larekeng et al. 2015a, 2015b; Maskromo et al. 2015) were used for genotyping all kopyor coconut samples (Table 1). The SSR primers used to generate the SSR markers have been used for various genetic analyses of coconut from Indonesia (Larekeng et al. 2015a). The PCR reaction was done in a final volume of 12.5 µL, consisting of a 6.26 µL PCR ready mix (KAPA Biosystem), 0.38 µL each of forward and reverse primers, 2 µL DNA template, and 3.5 µL ddH2O. After pre-denaturation at 94^{o}C for 3 minutes, the amplification was done in 35 cycles. Each cycle consisted of denaturation at 94^{o}C for 30 secs, primer annealing at 48-60^{o}C for 15 secs, and primer extension at 72^{o}C for 5 secs. In the end, one cycle of final extension at 72^{o}C for 10 mins was added. The amplification was performed in a thermal cycler PCR machine (Biorad). Amplified PCR products were checked using 0.8% agarose – TBE buffer gel electrophoresis, and the DNA was visualized using Gel Red. Allele visualization of the SSR marker was conducted using a vertical SB buffer, 0.6% polyacrylamide gel electrophoresis (PAGE), and visualization of the DNA fragments was done using silver staining (Creste et al. 2001). The PAGE was run at a constant 100 mA electric current for 30 mins, and a DNA ladder of 100-200 bp was used as the size control.

Data analysis

Scoring the amplified SSR allele was done manually by observing various sizes of amplified fragments as the allele (Figure 1), and the data were stored in the MS Excel software for further analysis. The genetic parameter such as polymorphic information content (PIC) value, observed heterozygosity (Ho), and expected heterozygosity (He) were calculated using CERVUS software Version 3.0.7 (Kalinowski et al. 2007). The simple matching method was used to calculate the dissimilarity index of allelic data, and the weighted Neighbour-Joining method was used to construct the phylogenetic tree. Both dissimilarity index and phylogenetic tree were determined utilizing DARwin software version 5.0 (Perrier and Jacquemoud-Collet 2006).

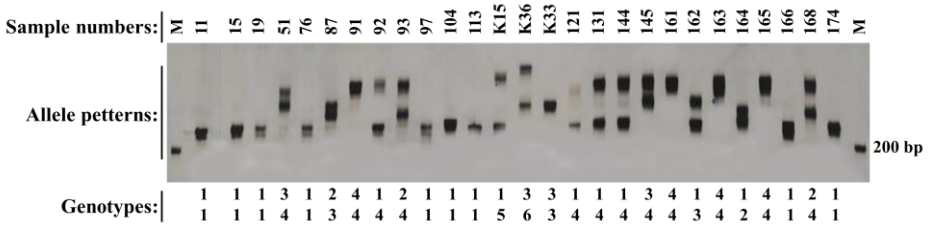

Figure 1. The example of denaturing polyacrylamide gel electrophoresis (PAGE) of SSR allele profiles generated by CnZ 51 SSR primers. M: 100 bp DNA ladder; K15: control profile for allele 1 and 5, K36: allele 3 and 6, K33: allele 3. Sample numbers represent the number of kopyor coconut provenances in the field; Allele patterns: the representative SSR allele patterns among evaluated Kopyor coconut provenances; Genotypes: the predicted genotypes of the coconut provenances based on the allele profiles generated by using the CnZ 51 SSR primers

The STRUCTURE software (version 2.4) was used to determine the population structure (Pritchard et al. 2000). Iteration of Markov Chain Monte Carlo (MCMC) was 100.000 (Porras-Hurtado et al. 2013). The number of sub-group K was figured out based on the criteria of ΔK (Evanno et al. 2005) by using Structure Harvester Software (Earl and von Holdt 2012). The K value was set from 1 to 10 and 10 times running was done for each K.

RESULTS AND DISCUSSION

SSR Polymorphism and genetic diversity

The 10 SSR marker loci used in this study have been used to characterize the genetics of Indonesian coconuts (Larekeng et al. 2015a, b; Maskromo et al. 2015) generated polymorphic markers in the evaluated Puan Kalianda kopyor coconuts (Table 1, Figure 1). This result confirmed previous studies showing the evaluated SSR primers were polymorphic when tested for various Indonesian coconut populations (Maskromo et al. 2012; Larekeng et al. 2018). Based on PCR results, the ten SSR marker loci generated number of total alleles (Na) ranged from 2-8 alleles per locus, and the average number of alleles across loci was 5 (Table 1). The example of polymorphic SSR allele profiles was generated using the CnZ 51 primer pairs, yielding six SSR alleles in the Puan Kalianda tall kopyor coconut population (Figure 1).

The lowest effective allele number (Ne) and the PIC values were 2.0 and 0.4 for CnCir 87, and the highest were 4.9 and 0.8 for CnZ 51. The average Ne and PIC values across the 10 SSR marker loci were 3.4 and 0.6, respectively (Table 2). The number of total alleles (Na) obtained from this tall kopyor coconut was higher than those obtained by Larekeng et al. (2015b) for the Pati dwarf kopyor coconut population (Na max = 6.0). On the other hand, the Na of Kalianda tall kopyor coconuts was lower than Yao et al. (2013) for Mozambique tall, Tahitian tall, and the Gazelle Peninsula tall coconut populations with Na max = 14.

The codominant and multi-allelic nature of SSR markers are the two major factors making the SSR more popular for plant genetic analysis than the dominant markers, such as RAPD or AFLP, and codominant markers as RFLP and isozyme (Kalia et al. 2011). This study has confirmed Teulat et al. (2000), Perera et al. (2000), Larekeng et al. (2015a, b), Maskromo et al. (2015), and Yao et al. (2013) on the usefulness of SSR markers for genetic analysis of coconuts.

The high PIC value of the used SSR primers shows their ability to differentiate among individual members of the Puan Kalianda tall kopyor coconut population. In this study, the PIC value ranged from 0.4 for primer CnCir 87 to 0.8 for primer CnZ 51, while the average PIC value across loci was 0.6 (Table 2). The generated SSR markers were very informative based on the PIC values of the 10 SSR primers used since the average PIC value across loci is higher than 0.5. The SSR primers yielding high PIC values are necessary for genetic diversity research, QTL mapping, and parentage analysis (Lebrun et al. 2001; Yao et al. 2013, Maskromo et al. 2014; Larekeng et al. 2015a, b; Larekeng et al. 2018). The PIC value observed in this study (0.6) is comparable to those reported by Loiola et al. (2016) in their genetic diversity study of the tall coconut population (0.71) and almost two times higher than those of Pesik et al. (2017) in their SNAP marker studies for coconut (0.33), which confirmed the usefulness of SSR markers for the coconut genetic analysis.

Table 2. The simple sequence repeat (SSR) locus names in the Puan Kalianda tall kopyor coconut population

SSR locus names	Ne	PIC	Ho	He
CnCir 86	2.5	0.5	0.5	0.6
CnCir B12	3.0	0.6	0.3	0.7
CnCir 56	4.2	0.7	0.7	0.8
CnCir 87	2.0	0.4	0.4	0.5
CnZ 51	4.9	0.8	0.5	0.8
CnZ 18	2.8	0.6	0.6	0.6
CnZ 21	2.2	0.5	0.5	0.5
CnCir 123	4.0	0.7	0.8	0.7
CnCir 147	3.7	0.7	0.6	0.7
CnCir 206	4.7	0.7	0.8	0.8
Average	3.4	0.6	0.6	0.7

Note: Ne: the number of effective alleles, PIC: polymorphic information content, Ho: observed heterozygosity, and He: expected heterozygosity

Table 1. The SSR locus names, forward and reverse primer sequences, melting temperature (Tm), and the number of alleles per locus (Na) in the Puan Kalianda tall kopyor coconut population

SSR locus name	Primer sequences		Tm (°C)	No. of alleles (Na)
	Forward	Reverse		
CnCir 86	CCACTTGAGACTTGAAAC	ACTCACGCAAATATACTCA	52	4
CnCir B12	GCTCTTCAGTCTTTCTCAA	CTGTATGCCAATTTTTCTA	55	5
CnCir 56	AACCAGAACTTAATTGTCG	TTTGAACTCTTCTATTGGG	55	5
CnCir 87	ATAACATCCTCCAACCTG	GACTGAATCCAACCCTT	55	2
CnZ 51	AAAGTGAAGTGGATAATGTG	AGAGAGGATCTAGGGTTGT	55	6
CnZ 18	ATGGTTCAGCCCTTAATAAAC	GAACTTTGAAGCTCCCATCAT	55	5
CnZ 21	ATAACATCCTCCAACCTG	GACTGAATCCAACCCTT	55	5
CnCir 123	AAAGTGAAGTGGATAATGTG	AGAGAGGATCTAGGGTTGT	55	6
CnCir 147	TTTCTCACCAACAAATAAAC	CTTGTGTGTTAGGGTCATC	55	4
CnCir 206	AAAGAGAACGCAACCA	CAAGTTCCAAAGAACCA	52	8
The average allele numbers/locus				5

Estimated observed heterozygosity (Ho) and expected heterozygosity (He) values using the 10 SSR marker loci for the Puan Kalianda tall kopyor coconut population from South Lampung, Indonesia, ranged from 0.3 to 0.8 and 0.5 to 0.8, respectively. In general, expected heterozygosity values were higher than observed heterozygosity, and there was a heterozygous deficit among the studied Puan Kalianda tall kopyor coconut population. The average observed heterozygosity (Ho) value of the 10 primers (0.6) was lower than the average of expected heterozygosity (He) value (0.7) in the studied Puan Kalianda tall kopyor coconut population (Table 2).

Ho and He values obtained from the Puan Kalianda tall kopyor coconut was higher than those obtained by Larekeng et al. (2015b) for Pati dwarf kopyor coconut population (Ho=0.50; He=0.54); Larekeng et al. (2018) for a mixed population of Pati dwarf kopyor and Pati tall normal coconut population (Ho=0.63; He=0.68); Maskromo et al. (2015) for a mixed population of Pati dwarf kopyor, Jember dwarf kopyor, Lampung tall kopyor, Pati tall kopyor, Sumenep tall kopyor, and Jember tall kopyor coconut (Ho=0.37; He=0.57); and comparable to those of Yao et al. (2013) for the three population of tall coconut from Mozambique, Tahiti, and Gazelle Peninsula (He = 0.73). Ho and He value of a primer show a primer's power to distinguish between heterozygous and homozygous individuals of a population. On the other hand, PIC reflects the relative informativeness of a primer. The Ho value of this study ranged from 0.3 (primer CnCir B12) to 0.8 (primer CnCir 123 and primer CnCir 206). This result shows that primers CnCir 123 and CnCir 206 are three times more effective for heterozygous individual identification in the evaluated coconut population than the primer CnCir B12.

Genetic diversity within a population can be reflected from heterozygosity value. Hence, if the heterozygosity value is low, the genetic diversity of a population is usually also low. Gunn et al. (2011) reported that the heterozygosity value of the tall type coconut collection originated from Southeast Asia, the tall type originated from Panama, and the dwarf coconut type from Southeast Asia was 53.0%, 23.0%, and 9.9 %, respectively. The average heterozygosity value of the studied Kalianda Tall kopyor coconut population was 0.7 (70%) (Table 2). This figure supports the nature of tall type coconut, considered the cross-bred plant (Teulat et al. 2000; Larekeng et al. 2015a, b).

Phylogenetic analysis and population structure

Genetic diversity can also be reflected by the population member's degree of similarity or dissimilarity. Given no information on the origin of the Puan Kalianda tall kopyor coconut population, this study could provide an initial view on the genetic complexity of the Kalianda tall kopyor coconut population from Lampung, Indonesia. Subsequently, phylogenetic analysis was performed to cluster among the individual member of the population. By this analysis, an individual with high dissimilarity will fall into a different cluster and vice versa. Cluster analysis of 91 individual provenances using Darwin software version 5.0 shows that the Kalianda tall kopyor coconut accessions belonged to two clusters (Figure 2). Both clusters are further divided into three sub-clusters. Cluster 1 was further divided into sub-clusters 1A, 1B, and 1C, while cluster 2 was divided into sub-clusters 2A, 2B, and 2C. Sub-cluster 1A consisted of 18 accessions, sub-cluster 1B of 15 accessions, and sub-cluster 1C of 10 accessions (Figure 2).

In contrast, sub-cluster 2A consisted of 23 accessions, sub-cluster 2B of 11 accessions, and subcluster 2C of 14 accessions (Figure 2). Correlation between the clusters and the origin of the Kalianda tall kopyor coconut population members cannot be concluded as recently no information on the source of the Puan Kalianda kopyor tall coconut population was available. The phylogenetic tree could provide helpful information for the researcher to study kopyor coconut. For instance, to conserve the Kalianda tall kopyor coconut germplasm, reducing the sample number will not cause a loss of vital allele diversity if the samples were selected from different clusters instead of taking many representatives from the same cluster. Whatever tool is implemented for a breeding program, selection could have no genetic gain if the population's genetic diversity understudy is low. The success of the selection relies heavily on the availability of genetic diversity of the germplasm (Govindaraj et al. 2015; Swarup et al. 2021). Therefore, the cluster analysis of Puan Kalianda tall kopyor coconut could provide coconut breeders with an essential hint for parental selection.

The Puan Kalianda tall kopyor coconut population structure analysis was also conducted. The population structure was another kind of clustering based on Bayesian Monte Carlo Methods (Pritchard et al. 2000). One of the methods used to determine the best K is based on Δ K, and the K value is indicated by apparent peak Δ K (Evanno et al. 2005). Assessing k value from 1 to 10 and ran for 10 x independently for each k, resulting in the peak of Δ K at K = 2 and K = 6 (Figure 3A). Results of the structure analysis at K = 2 was presented in Figure 3B, while at K = 6 was shown in Figure 3B. Population structure analysis enables us to have an idea about the genomic composition of each sample (Figures 3B and 3C). The population structure analysis result agreed with the phylogenetic analysis results, which clustered the 91 accessions into two clusters, as presented in Figure 2. Therefore, the studied Kalianda tall kopyor coconut population was from two different genetic backgrounds or ancestors (K = 2) (Figure 3A). Moreover, further analysis also divides the population into six sub-clusters (K = 6), in line with the phylogenetic analysis results (Figure 2). Hence, gene diversity in the population can be considered high, as indicated by its high value of He (70%) and by many sub-clusters (6 sub-clusters).

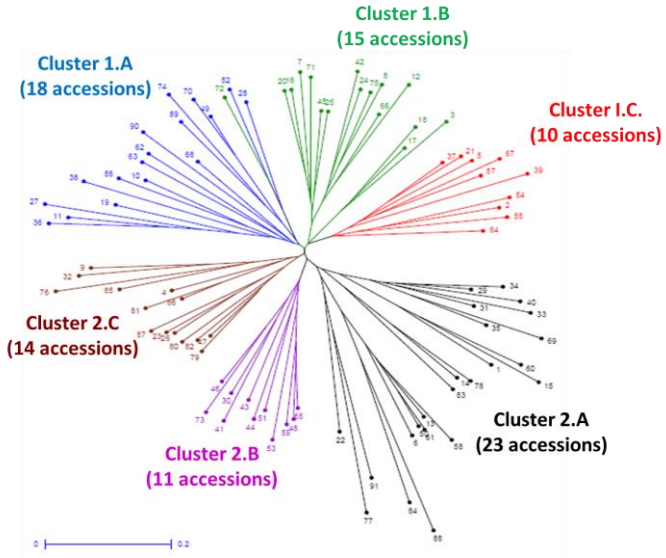

Figure 2. Phylogenetic tree generated by 91 Puan Kalianda tall kopyor coconut accessions based on the Weighted Neighbor-Joining method using DARwin software version 5.0. The genetic distances were calculated using the simple matching dissimilarity index method of allelic data

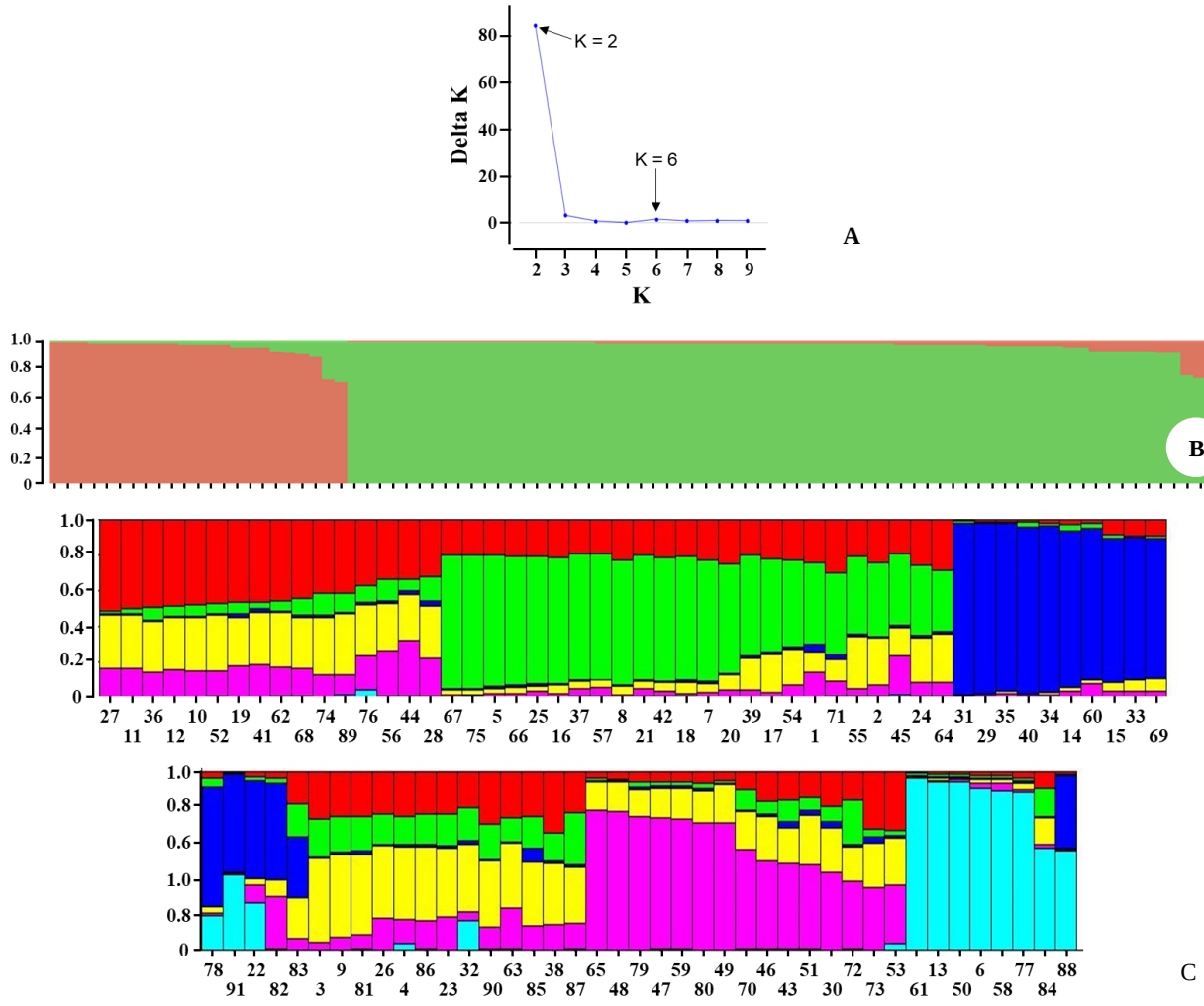

Figure 3. Population structure of 91 provenances of Puan Kalianda kopyor tall coconuts from Lampung, Indonesia. A. The values of Δ K in the population under study showed the prominent peak at K = 2 and K = 6. B. Population structure at K = 2. C. Population structure at K = 6. The arrangement of the accession numbers in B was the same as in C

In conclusion, the SSR average PIC value of 62% reflects that the utilized SSR markers are an effective molecular marker for the genetic diversity study of kopyor coconut. Phylogenetic analysis indicates that the Kalianda tall kopyor coconut population genetically consists of two main clusters, and each cluster was divided into three sub-clusters. Genetic structure analysis reveals that the Δ K peak was at K = 2 and K = 6, which indicates that the coconut population could be derived from two distinct genetic backgrounds of ancestry (K=2) and further structured into six genetic backgrounds (K = 6). Therefore, the Puan Kalianda tall kopyor coconut can be considered a valuable genetic resource for crop improvement. Puan Kalianda tall kopyor coconut can also be a divergent population as indicated by its high gene diversity (He = 70%).

As a preliminary effort, the information generated from this study could advantage for Puan Kalianda kopyor coconut breeding. If we use a molecular marker that links to an important agronomic trait as a basis for genotyping, we could identify which individuals are most promising to be selected for kopyor coconut improvement.

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