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GENETIC ANALYSIS OF DXP ICALIX OIL PALM (*ELAEIS GUINEENSIS* JACQ VAR. ICALIX) USING *TRNL-TRNL-TRNF INTERGENIC SPACER* DNA BARCODE AND RAPD

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SUMMARY

The DxP iCalix oil palm (*Elaeis guineensis* Jacq.) is a hybrid cultivar obtained through the crossing of cultivars Dura Banting from Malaysia and Pisifera AVROS from Africa. The provided study aimed to determine the similarity between the iCalix oil palm plantations made in the community-managed and company plantations through randomly amplified polymorphic DNA (RAPD) and *trnL-trnL-trnF intergenic spacer* (*IGS*) methods. The five replicates of young oil palm leaf samples came from plantations made in the community-managed (10 individuals of iCalix) and company plantations (three iCalix from block I [ICM1 and ICM2] and one from block II [ICMA10]) and two Dura Banting cultivars as female parents (D1 and D3). Then, using standard protocols, proceeded for DNA extraction, electrophoresis, PCR (polymerase chain reaction), sequencing, and data analysis. The *trnL-trnL-trnF IGS* sequences of IC9, ICM1, and D3 were 916 bp in size and found to be 100% similar. The BLASTn analysis showed those sequences had the highest (99.24%) similarity with *E. guineensis*. The RAPD analysis revealed that iCalix in the community-managed plantation formed a group with ICMA10 planted in block II of the company plantations at the similarity value of 72.8%. The said group was also similar to the female parent based on the *trnL-trnL-trnF IGS*.

Keywords: DxP iCalix oil palm, oil palm (*E. guineensis*), Dura Banting, community-managed and company plantations, DNA barcode, RAPD, *trnL-trnL-trnF intergenic spacer,* similarity

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Key findings: The integration of DNA barcoding and RAPD analysis ensured a comprehensive seed quality control system in oil palm (*E. guineensis*). The RAPD marker has determined the similarity of crossbred offspring to their parents. The use of the *trnL-trnL-trnF IGS* sequence enriched the iCalix oil palm genetic information in public databases like GenBank.

INTRODUCTION

Oil palm (*Elaeis guineensis* Jacq.) is a high-value plantation commodity due to its primary role in palm oil production in Indonesia. This crop has a classification of three cultivars: Dura, Pisifera, and Tenera (a hybrid of Dura × Pisifera, DxP). Based on fruit morphology, distinguishing these cultivars from each other can be easy. Oil palm produces crude palm oil (CPO), which plays a pivotal role in the food industry (vegetable oil), chemical industry (oleochemicals), and alternative energy sector (biodiesel) (Gan *et al*., 2021; Alhaji *et al*., 2024).

Progenies of the hybrid cultivar Tenera (derived from Dura × Pisifera) have successful development from oil palm breeders (Swaray *et al*., 2020). One of these progenies is DxP iCalix, a hybrid obtained from crossing Dura Banting (female parent) and Pisifera AVROS (male parent). The cultivar DxP iCalix's official release as a commercial cultivar occurred in November 2019. Its advantages include fresh fruit bunch (FFB) production with a potential of up to 30 t/ha/year and a crude palm oil yield of 8.5 t/ha/year. Moreover, it has a high oil content, relatively slow height increment, high oil-to-bunch ratio, uniform growth, and early harvest at 24 months after field plantation (Jatmiko, 2019).

The genetic uniformity of oil palm planting materials plays a crucial role in maintaining plantation productivity and yield stability. In field conditions, the use of planting materials from different genetic sources, accidental mixing of seedlings during nursery or planting stages, and the lack of genetic verification may result in heterogeneous populations. Such genetic variation can lead to differences in growth performance, fruit production, and harvesting index among individual palms. Furthermore, the legitimacy and purity of oil palm planting materials are critical factors in breeding and commercial plantation systems, as unintended genetic mixtures could reduce plantation performance and compromise yield potential (Teh *et al*., 2019).

For genetic purity determination in DxP iCalix seeds, using conventional approaches included morphological, physiological, and agronomic observations. However, at the early growth stage, the varietal identification based on morphology is difficult due to their highest similarity among vegetative traits. Kadam *et al*. (2023) reported that morphological characters in several palm groups appeared to be conservative and provided limited variations. In oil palm, fruit traits, such as bunch components, fruit number per bunch, oil yield, and shell presence, were commonly used indicators (Swaray *et al*., 2020; Sitepu *et al*., 2022). Cultivars Dura and Tenera have thick shells, while the cultivar Pisifera is shell-less (Gan *et al*., 2021). However, these morphological traits can only be visible after the plant enters the generative phase (after 4–5 years), with these parameters also influenced by environmental factors (Roslim *et al*., 2023a).

The DNA-based molecular markers' application is an alternative solution, which the environment does not also affect and can be applicable at early growth stages (Le *et al*., 2020). Several markers have reached usage, including simple sequence repeats (SSR) (Budiman *et al*., 2019), random amplified polymorphic DNA (RAPD) (Basyuni *et al*., 2018), and DNA barcoding (Le *et al*., 2020).

The DNA barcoding technique is effective for identifying organisms using short DNA sequences (500–1500 bp) located at known genomic regions, referred to as DNA barcodes. Beyond species identification, DNA barcoding has been beneficial for the authentication of foods, beverages, and herbal medicines (Galimberti *et al*., 2019), conservation of endemic and endangered species (Safhi *et al*., 2023), forensic analysis

(Park *et al*., 2017), and cultivar identification and intraspecific diversity studies (Samarina *et al*., 2025). Common plant DNA barcodes include *matK*, *rbcL*, *trnL-trnL-trnF intergenic spacer* (*IGS*), *trnH-psbA IGS*, and *trnE-trnT IGS* (Herman *et al*., 2023; Samarina *et al*., 2025).

The *trnL-trnL-trnF IGS* region is a non-coding chloroplast DNA sequence comprising the *trnL* intron and the spacer between *trnL* and *trnF* genes. Given its highest mutation rate, it proved suitable for evolutionary and species identification studies (Herman *et al*., 2023). Being maternally inherited, it can easily trace maternal lineage in plant hybrids. This marker application has been successful in *G.* × *subvillosa* (Takács *et al*., 2020), *Phalaenopsis orchids*, and the species of Solanaceae and Fabaceae (Herman *et al*., 2023).

RAPD is a simple and rapid molecular marker that uses short random primers (10-mers) without requiring prior genomic information. It amplifies random genomic DNA segments and reveals the genetic polymorphism through DNA band profiles. The RAPD markers have been favorable for germplasm characterization, genome mapping, seed purity testing, and early-stage selection in plant breeding programs (Probojati *et al*., 2019; Al-Jayid and Altameme, 2024). Moreover, six RAPD primers (P7, P10, P12, P15, P19, and P28) have succeeded in distinguishing the three oil palm cultivars (Sathish and Mohankumar, 2007). Furthermore, research by Girsang *et al*. (2019) reported a 100% polymorphism percentage in the MTG variety with a DNA fragment size of 424–2572 bp. Additionally, Pasaribu *et al*. (2017) found 43.72% molecular diversity in commercial oil palm genotypes using RAPD markers.

RAPD markers target the nuclear genome and are widely used to detect overall genetic polymorphism and assess genetic diversity among oil palm individuals (Simbolon *et al*., 2017). In contrast, the chloroplast *trnL-trnL-trnF IGS* represents a plastid DNA region that is commonly employed in phylogenetic and DNA barcoding studies because it provides information on maternal lineage inheritance (Baker *et al.,* 1999). Therefore, the combined use of RAPD and *trnL-trnL-trnF IGS* enables a more comprehensive assessment of genetic purity, integrating both nuclear genome variation and maternal lineage information.

Despite marker successes, no studies have compared the genetic fidelity of DxP iCalix across community and commercial plantations, nor verified conformity to Dura Banting using these markers. This study aimed to use the RAPD and *trnL-trnL-trnF IGS* for determining the genetic similarity and kinship among DxP iCalix obtained from plantations made by community-managed and a company and their conformity with the maternal parent Dura Banting. Therefore, the genetic analysis was essential to ensure the authenticity of the iCalix hybrid, supporting sustainable productivity and plantation management.

MATERIALS AND METHODS

Plant materials

The fronds collection comprised 10 from the community plantation in Siabu Village, Salo District, Kampar Regency (IC). Meanwhile, two fronds came from Dura Banting female parent oil palm plants (D) and three fronds from iCalix plants in a company plantation (Block I: ICM and Block II: ICMA) in Riau Province, Indonesia. Five young leaflets received sampling from each frond. The number of samples analyzed was not identical among the studied groups because the objective of the study was to compare representative oil palm materials from a community-managed plantation, a company plantation, and the Dura Banting female parent. The inclusion of a total of 15 individuals underwent the RAPD analysis. These consisted of 10 iCalix palms from the community-managed plantation, three iCalix palms from the company plantation (ICM1, ICM2, and ICMA10, where ICM refers to Block I and ICMA refers to Block II of the plantation), and two Dura Banting parental palms (D1 and D2). For the *trnL–trnL–trnF IGS* sequencing analysis, the study selected only three representative samples (IC9, ICM1, and D3).

DNA extraction

Total genomic DNA extraction used the Plant Genomic DNA Mini Kit (Geneaid, Taiwan) following the manufacturer's protocol. Approximately 0.2 g of leaf tissue, cut into small pieces with sterile scissors, incurred grinding into a fine powder in liquid nitrogen using a mortar and pestle before transferring into a 1.5 mL microtube. The extracted total DNA then served as a template for PCR amplification. Determining the quality and quantity of total DNA utilized electrophoresis on a 1% agarose gel. The DNA bands are large, thick, and do not smear, indicating good quality and massive quantity.

The *trnL-trnL-trnF IGS* analysis

DNA barcoding analysis continued on three samples, including one each from the community and company plantations and the Dura Banting parent. The PCR, as performed, employed the primer pair of B49317_F2 (5'-CGA AAT CGG TAG ACG CTA CG-3') and A50272_R3 (5'-ATT TGA ACT GGT GAC ACG AG-3') (Herman *et al*., 2023).

The PCR reactions' preparation consisted of a total volume of 50 µL containing 1× PCR buffer (+Mg^{2+}), 0.2 mM dNTPs, 2.4 µM forward primer, 2.4 µM reverse primer, 2 U DreamTaq DNA polymerase, 2 µL genomic DNA, and nuclease-free water. The amplification procedure had the following conditions: initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 95 °C for 45 s, annealing at 49.2 °C for 45 s, and extension at 72 °C for 90 s. A final extension ensued at 72 °C for 10 min, with the reactions held at 15 °C for an unlimited time (Roslim *et al*., 2024).

The PCR products sustained visualization by electrophoresis on a 1.2% agarose gel, loading a mixture of 2 µL PCR product and 2 µL loading dye into each well, with a 1 kb DNA ladder (Thermo Scientific) loaded into the first well. Electrophoresis in an electrophoresis tank containing 1X TBE buffer solution occurred at 50 V for 45 min. The agarose gel preparation process involved the addition of 2 µl of ethidium bromide, with the DNA bands visualized under UV light using a UV transilluminator and documented with a UV-filtered camera. Successful PCR products (40 µL), along with 30 µL each of forward and reverse primers (separately), entailed sealing with parafilm before sending to PT Genetika Science Indonesia. Meanwhile, sequencing procedures took place at First Base Laboratories, Malaysia.

RAPD analysis

Six RAPD primers, namely, P7 (GGCGGACTGT), P10 (GGACCCAACC), P12 (TCTGGTGAGG), P15 (TTGGCACGGG), P19 (AGCGCCATTG), and P28 (ACCCGGTCAC) (Sathish and Mohankumar, 2007; Basyuni *et al*., 2018), received initial screening using five previously selected DNA specimens. Primers that produced polymorphic DNA bands were options for subsequent application to all studied samples.

The PCR for RAPD analysis proceeded in a total reaction volume of 25 µL following the manufacturer's protocol (Thermo Scientific), containing 1× PCR buffer (+Mg^{2+}), 0.2 mM dNTPs, 1 µM primer, 3 U DreamTaq DNA polymerase, 2 µL genomic DNA, and nuclease-free water. The amplification program comprised an initial denaturation at 94 °C for 3 min, followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 36 °C for 30 s, and extension at 72 °C for 2 min. A final extension performed at 72 °C for 5 min had reactions held at 15 °C for an unlimited time (Sathish and Mohankumar, 2007). The PCR products' separation continued on 2.5% agarose gels before being documented.

Data analysis

The obtained *trnL-trnL-trnF IGS* sequences upon editing used BioEdit software, and their analysis utilized BLASTn (Basic Local Alignment Search Tool for nucleotides). Phylogenetic tree construction engaged MEGA version 11. The nine closest species identified by BLASTn, together with the three sample sequences, served in constructing the neighbor-joining phylogenetic tree based on the Kimura 2-parameter model with 1,000 bootstrap replicates. Phylogenetic analysis of the *trnL*–

trnL–trnF IGS barcode ensued using the neighbor-joining (NJ) method with the Kimura 2-parameter (K2P) model because this combination is a standard approach in plant DNA barcoding studies. The NJ method uses algorithmic principles for genetic distances between nucleotide sequences in similar regions, making it suitable for monophyletic analysis (Rizal *et al.,* 2020). Meanwhile, the K2P model accounts for differences in transition and transversion substitution rates, resulting in more accurate estimates of evolutionary distance.

The RAPD bands' scoring as binary data has '1' indicating the presence and '0' indicating the absence of a band. Only bands ≥ 250 bp proceeded in the analysis, with the bands smaller than 250 bp excluded because too small fragments tend to be less stable, difficult to distinguish, and have a low level of reproducibility. Small fragments (< 200–250 bp) often appear as faint bands, non-specific amplification results, or PCR artifacts to prevent bias in dendrograms or kinship analysis (Wulan *et al.,* 2024). The binary matrix analysis engaged NTSYSpc version 2.11 software with the Dice similarity coefficient to generate a genetic similarity matrix. A dendrogram illustrating genetic relationships among the iCalix oil palm samples underwent construction using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA).

RESULTS AND DISCUSSION

The *trnL-trnL-trnF intergenic spacer* sequence analysis

Obtaining the *trnL-trnL–trnF IGS* sequences was successful from oil palm samples originating from the plantations of community (IC9), company (ICM1), and the Dura Banting parent (D3). All sequences were 916 bp in length and were 100% identical among the three studied samples. The study deposited the sequences in the GenBank under accession numbers PV030961, PV030962, and PV030963, respectively.

The absence of polymorphic sites among IC9, ICM1, and D3 indicates that the *trnL–trnL–trnF IGS* region was not sufficiently variable to detect intraspecific variation within the studied oil palm materials. Although it successfully confirmed their identity as *Elaeis guineensis* and suggested a shared maternal origin. Chloroplast DNA regions, including *trnL–trnL–trnF IGS*, are generally more conserved than nuclear markers and therefore have frequent uses for species identification and phylogenetic reconstruction rather than for detecting fine-scale variation among closely related individuals. Furthermore, because plastid genomes undergo maternal inheritance in most angiosperms, identical *trnL–trnL–trnF IGS* sequences may reflect a common maternal ancestry shared by the iCalix and Dura Banting planting materials (Sakamoto and Takami, 2024).

The BLASTn analysis revealed that the iCalix and Dura Banting samples indicated the highest similarity (99.24%) with *E. guineensis* (accession number OR125049.2) and the lowest similarity (98.14%) with *Euterpe edulis*, with an E-value of 0 and query coverage of 100% (Table 1).

The results of the BLASTn analysis disclosed that the samples had similarities to *E. guineensis* with the highest sequence similarity (99.24%), confirming the studied plants were indeed oil palm. It is implied as most closely related to *E. guineensis* and clearly distinct from other taxa in the dataset. The similarity value below 100% suggested varietal differentiation from the African oil palm reference sequence. The lower similarity with *E. edulis* (98.14%) further revealed the sufficient nucleotide divergence to distinguish the genera within the family Arecaceae. An E-value of 0 indicates the alignment did not occur by chance, while a query coverage of 100% confirmed the complete alignment of the sample sequences with reference sequences in GenBank. Comparable past findings have also had reports for *Champereia manillana* var. *manillana* Merr. from Riau, which exhibited less than 100% similarity with *C. manillana* var. *longistaminea.* Thus, confirming the ability of the *trnL-trnL–trnF IGS* sequence to differentiate various plant cultivars (Roslim *et al*., 2024).

Table 1. The BLASTn analysis result of the iCalix oil palm *trnL-trnL-trnF intergenic spacer* sequence.

No.	Species	Accession number	Max. score	Total score	Query cover (%)	Identity (%)
1	*Elaeis guineensis*	OR125049.2	1596	1596	100	99.24
2	*Veitchia arecina*	NC_029950.1	1578	1578	100	98.80
3	*Wodyetia bifurcata*	NC_067836.1	1573	1573	100	98.69
4	*Dictyosperma album*	NC_067835.1	1571	1571	100	98.58
5	*Archontophoenix alexandrae*	NC_046017.1	1563	1563	100	98.47
6	*Euterpe precatoria*	NC_065201.1	1562	1562	100	98.36
7	*Euterpe oleracea*	ON533739.1	1560	1560	100	98.36
8	*Areca catechu*	NC_050163.1	1555	1555	100	98.15
9	*Euterpe edulis*	ON533738.1	1551	1551	100	98.14

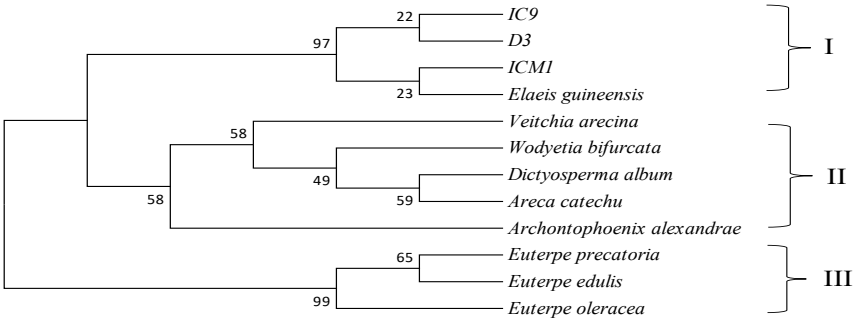

Figure 1. Dendrogram constructed using the neighbor-joining method with 1000 bootstrap replicates, based on *trnL-trnL-trnF intergenic spacer* sequences in iCalix oil palm and other accessions studied.

Phylogenetic analysis resolved three major groups. The three samples clustered together with *E. guineensis* (accession number NC_017602.1) in Group I and showed obvious separation from other genera. The species of the genus *Euterpe* formed a distinct cluster in Group III, while Group II comprised the species from different genera (Figure 1). The results further revealed that the *trnL-trnL–trnF IGS* barcode effectively discriminates the species within the same genus from those belonging to different genera.

The identical sequences observed among the three samples confirmed that iCalix IC9 cultivated in community plantations originates from the same maternal origin as those planted in company plantations and their female parent (Dura Banting). These results received further support from the phylogenetic tree (Figure 1) and the presence of three diagnostic nucleotide positions characteristic of *E. guineensis* (Table 2).

The *trnL-trnL–trnF IGS* region is part of the chloroplast genome and has had wide uses as a DNA barcode due to its ease of amplification and informative variation at the genus and species levels. In most angiosperms, the inheritance of chloroplast DNA was uniparental, typically through the maternal line, proving it particularly suitable for tracing maternal lineage (Chung, 2025).

In the presented study, three diagnostic nucleotide positions (215, 400, and 669) succeeded in their identification as characteristic of *E. guineensis*, including iCalix (Table 2). These sites were likely arising from the substitution mutation and represent stable genetic polymorphisms, which can be useful to distinguish the closely related species (Prasetya *et al*., 2020). Nucleotide variation resulting from mutation is a key driver of genetic diversity and plays a primary role in evolutionary processes and phylogenetic inference (Wathon *et al*., 2023). Reports of

Table 2. Critical nucleotides based on *trnL-trnL-trnF intergenic spacer* sequences.

Accessions	Nucleotide number		
	215	400	669
IC9	C	C	G
ICM1	.	.	.
D3	.	.	.
Elaeis guineensis	.	.	.
Veitchia arecina	T	A	A
Wodyetia bifurcata	T	A	A
Dictyosperma album	T	A	A
Archontophoenix alexandrae	T	A	A
Euterpe precatoria	G	A	A
Euterpe oleracea	G	A	A
Areca catechu	T	A	A
Euterpe edulis	G	A	A

Table 3. Number of polymorphic and monomorphic bands based on five RAPD primers.

Primers	Polymorphic band number	Monomorphic band number	Total
P7	13	2	15
P10	16	1	17
P12	9	0	9
P19	6	1	7
P28	9	2	11
Total	53	6	59
Average	10.6	1.2	11.8
Percentage	89.83	10.17	

similar diagnostic nucleotides have also come out in other plant taxa using the *trnL-trnL–trnF IGS* region, including *Benstonea* spp., *Cleome gynandra* (Roslim *et al*., 2023b), and species within the families Solanaceae and Fabaceae (Herman *et al*., 2023).

RAPD analysis

Out of the six RAPD tested primers, five produced polymorphic bands, whereas primer P15 failed to amplify any bands (Figure 2). Primer P10 generated the highest number of bands (17 bands) (Table 3), enunciating it as the most informative primer, likely due to its ability to anneal to multiple complementary sites within the oil palm genome. The failure of primer P15 to produce bands could refer to the absence of complementary target sequences or suboptimal annealing conditions.

The appearance of RAPD bands incurred influences from several factors, including the DNA template quality and quantity, primer sequence, and annealing temperature. The number of amplified bands depends on the primer binding to complementary genomic regions, which can also be due to mutation (Probojati *et al*., 2019). Variations in banding patterns reflect the differences in genomic DNA sequences at primer binding sites, resulting in differential amplification and varying levels of polymorphism (Wahyuningsih *et al*., 2025).

Overall, polymorphic bands totaled 53 (89.83%), generated using five primers, with an average of 10.6 bands per primer. Meanwhile, only six monomorphic bands emerged, averaging 1.2 bands per primer (Table 3). The highest proportion of polymorphic bands revealed the substantial genetic variability among studied samples, despite their classification within the same iCalix cultivar. Polymorphism levels among accessions may be because of differences in the extent of genetic variation present within each accession (Poerba and Achmad, 2010).

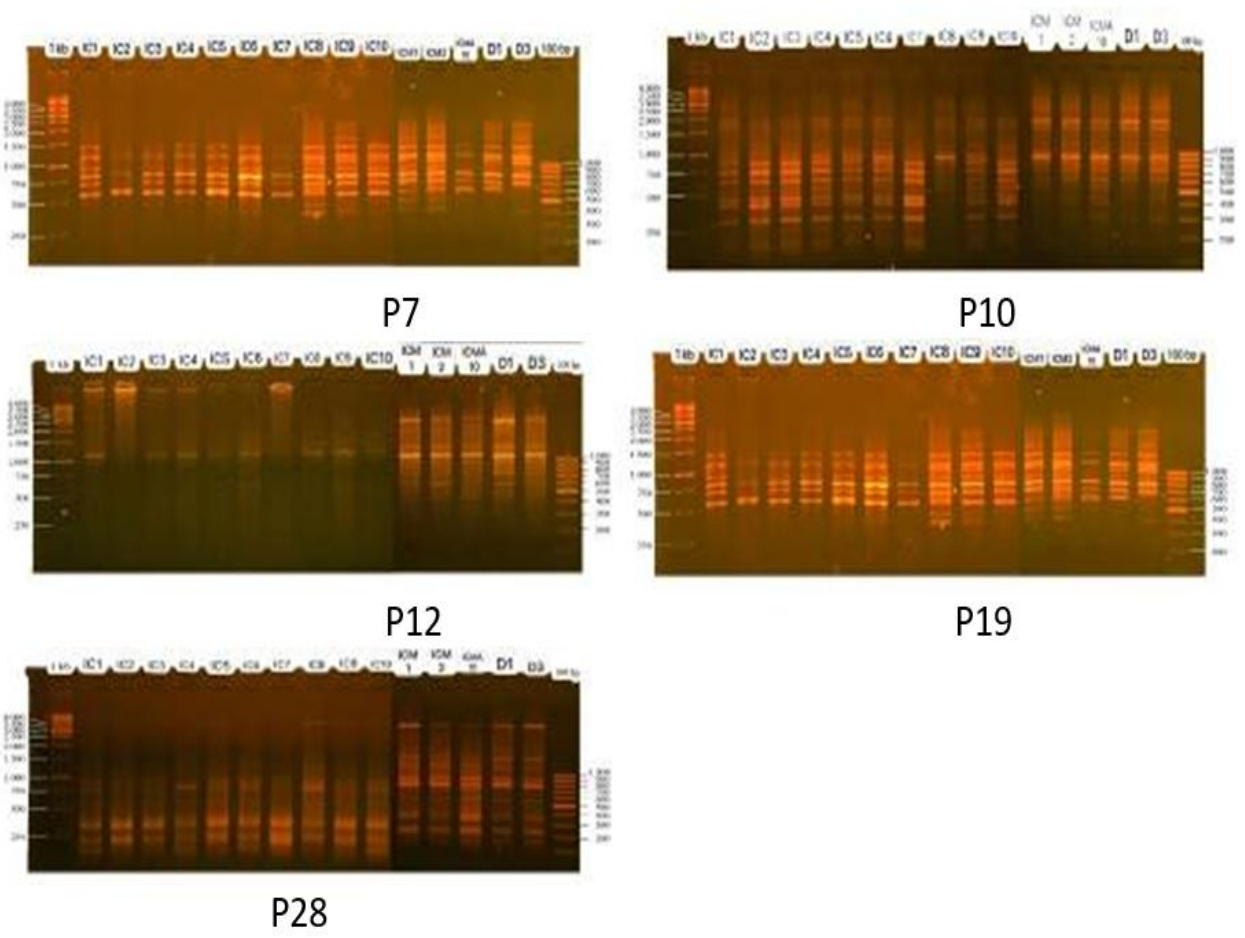

Figure 2. The RAPD DNA band profiles on iCalix oil palms amplified by using P7, P10, P12, P19, and P28 primers. 1 kb DNA ladder (Thermo Scientific).

Moreover, the detection of a high proportion of polymorphic bands in PCR amplification products suggests a high degree of genetic diversity among the species analyzed (Probojati *et al*., 2019). According to Radwan *et al.* (2021), elevated polymorphism levels are valuable in germplasm management because they facilitate the identification of genetic diversity and provide insights into the genetic relationships among genotypes. The low number of monomorphic bands indicated the RAPD primers employed were highly effective in distinguishing the individual oil palm samples. The highest levels of DNA band polymorphism reflect substantial genetic diversity, whereas low polymorphism signified the limited genetic variation. The diversity of loci amplified by different primers arises from variation in target DNA sequences, resulting in distinct RAPD profiles. Cluster analysis based on RAPD data revealed two major groups at the similarity coefficient of 72.8% (Figure 3). The first group comprised 10 iCalix samples from community plantations (IC1–IC10), and one sample was from the company plantation block II (ICMA10). The second group included two iCalix samples from the company plantation block I (ICM1 and ICM2), along with two female parent samples (D1 and D3). The results discovered that iCalix seedlings grown in community plantations share a close genetic relationship with those from the company plantation block II. The observed genetic divergence (27.2%) may refer to differences in the planting block origin and seed selection practices.

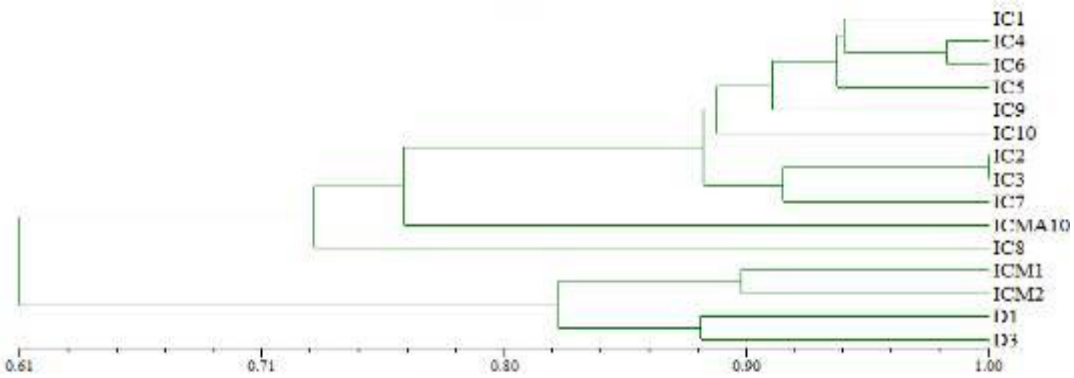

Figure 3. UPGMA dendrogram based on Dice's similarity coefficients derived from RAPD marker data.

The clustering pattern of ICM1 and ICM2 with D1 and D3 showed that samples from Block I were genetically closer to the female parent samples based on RAPD banding profiles. The close clustering of ICM1 and ICM2 with D1 and D3 highlighted a considerable maternal influence on iCalix in the company plantation block I. The separation between the samples of community-managed and company plantations may reflect the differences in the production blocks and seed selection strategies employed by companies for internal purposes.

CONCLUSIONS

The RAPD analysis showed community-grown DxP iCalix was genetically at par with company-grown ICMA10 at >72.8% similarity, while ICM1 and ICM2 (Block I) were remarkable at >81.8% similarity with Dura Banting. The relatively high genetic similarity value (>72.8%) indicates that iCalix individuals in community-managed plantations still have a close genetic relationship with company plantations and come from similar maternal or ancestral lines. The *trnL-trnL-trnF IGS* sequences confirmed maternal lineage consistency. The results assured the farmers and industry stakeholders that iCalix seeds in the community were genetically authentic, met commercial standards, and were not illegal. The integration of DNA barcoding and RAPD provides a comprehensive seed quality control system.

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