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Differentiation of Oil Palm Hybrid from Parentals Using As-PCR Marker

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ABSTRACT

Oil palm (*Elaesis guineensis*) is a highly economic oil producing tree that produces fruits of three forms which cannot be differentiated until reproductively matured. The commercially acceptable form is the *Tenera* fruit form because of its high oil-yielding capacity. It is a hybrid of Dura and *Pisifera*. The aim of this study was to determine alleles unique to *Tenera* fruit form of the Nigerian oil palm. Allele Specific Polymerase Chain Reaction (AS-PCR) method was used in this study. It required DNA isolation, common shell gene primer testing, allele specific primer pair investigation as right and left primers and a multiplex PCR investigation consisting of common and allele specific primers for the shell gene. These primer combinations were used to screen the genome of Dura, *Pisifera* and *Tenera* for variants in the shell gene. The findings from this study suggest two alleles of sizes 400 bp and 200 bp unique to *Tenera* when the primer combinations S3F&R and S22&S32 was used. This can serve as a marker for differentiating *Tenera* from Dura and *Pisifera*. The outcome of this research will aid early detection of choice variety for distribution to Oil palm growers.

Keywords: *Oil palm, Tenera, Dura, Pisifera, Allele Specific-PCR.*

Introduction

Oil palm (*Elaeis guineensis* Jacq.) can be classified into separate groups based on its fruit characteristics, and has three naturally occurring fruit forms (plate 1) which vary in shell thickness and oil yield.[1] Dura type palms are homozygous for a wild-type allele of the SHELL gene (Sh/Sh), have a thick seed coat or shell. *Tenera* type palms are heterozygous for a wild-type and mutant allele of the SHELL gene (Sh/sh), it has a relatively thin shell surrounded by a distinct fiber ring.[2] Finally *Pisifera* type palms are homozygous for a mutant allele of the SHELL gene (sh /sh), have no seed coat or shell, and are usually female sterile.[3] Therefore, the inheritance of this single gene controlling fruit shell phenotype is a major contributor to palm oil yield. The recent sequencing of this gene made it possible to identify Single Nucleotide Polymorphism (SNP) mutations that are now used in developing molecular markers for fruit form predictions.[4]

Traditional SNP genotyping methods such as CAPs (The Cleaved Amplified Polymorphic Sequence), dCAPs (derived CAPS), and AS-PCR (Allele-specific PCR) are widely used for low throughput applications in plant research. In application, CAPS and dCAPS are restricted by endonuclease sites that could be inefficient and not cost-effective.[5] AS-PCR is based on the extension of primer only when its 3'end is perfectly complemented to the template.[6] In principle, SNPs can be detected using allele-specific PCR primers based on the 3' terminal nucleotide of a primer that corresponds to a specific SNP site.[7] This method only needs one PCR to distinguish the different genotypes and it has obvious advantages with regards to speed and cost. The choice of molecular marker used was based on the availability of equipment, cost of reagents and reliability of the method unlike SSR (Simple Sequence Repeat), RFLP (Restriction fragment length polymorphism) and some other molecular markers, AS-PCR requires a thermal cycler and agarose gel electrophoresis.[8,9] At present, allele-specific PCR has been successfully applied to plant SNP identification[10] and assay to type *E. coli* strains.[11]

Identification of the fruit form of a given palm is typically performed after the plant has matured enough to produce the first batch of fruits, which typically takes approximately six years after germination. Notably, in the six-year interval from germination to fruit production, significant land, labour, financial and energy resources are invested into what are believed to be *Tenera* trees, some of which may ultimately be of the unwanted low yielding types. By the time these trees are identified, it is impractical to remove them from the field and replace them with *Tenera* trees, and thus growers achieve lower palm oil yields for the 25 to 30-year production life of the contaminant trees. Therefore, the issue of contamination of batches of *Tenera* seeds with dura or *Pisifera* seeds is a problem for oil palm breeding, underscoring the need for a method to predict the fruit form of seeds and nursery plantlets with high accuracy. The aim of this study was to determine alleles unique to *Tenera* fruit form of the Nigerian oil palm.

Materials and Methods

Leaf samples collection and DNA extraction

The tissue source used for DNA extraction were fresh leaves of Dura, *Pisifera* and *Tenera* oil palms obtained from the Nigerian Institute for Oil Palm Research (NIFOR), Benin City, Nigeria. DNA extraction was carried out using Promega wizard genomic DNA purification kit (USA) according to the manufacturers' instruction with modification in the homogenizing step which was done by grinding the tissue with a pestle in a mortar containing the lysis buffer. In order to distinguish between shell alleles, four allele-specific primers (S20, S22, S32 and S33) were adopted and a primer pair targeted to the region of the shell gene believed to contain the mutation responsible for the shell variation in the fruit form of the oil palm was obtained and formulated by Inqaba Biotec West Africa Ltd. Primers used for the study are stated in Table 1.

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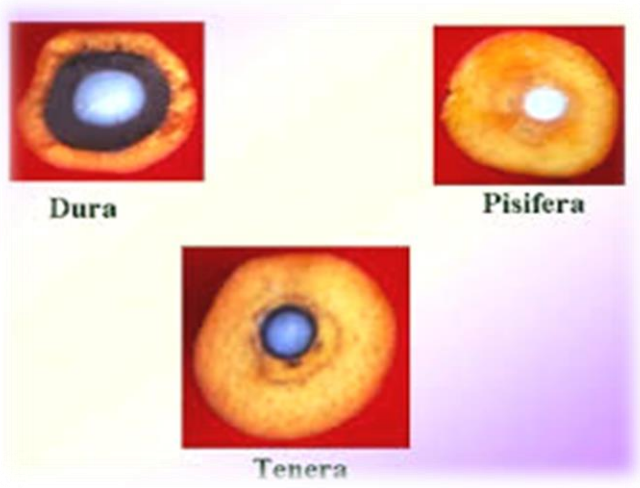


Plate 1: The three fruit forms of the oil palm.

Table 1: Primer information for AS-PCR.

Primer	Sequence
S3F	TTTGTTGCTTTTAATTTTGCTTGAATACCTTT
S3R	TGGCTTGGCCATAGAACAAA
S20	TCAGCATCACAAAGGACAGACAACTCATAATCT
S22	CAGCATCACAAAGGACAGACAACTCATAAGCA
S32	GCCGAAATGGACTGCTGAAGCAT
S33	GCCGAAATGGACTGCTGAAGAAA

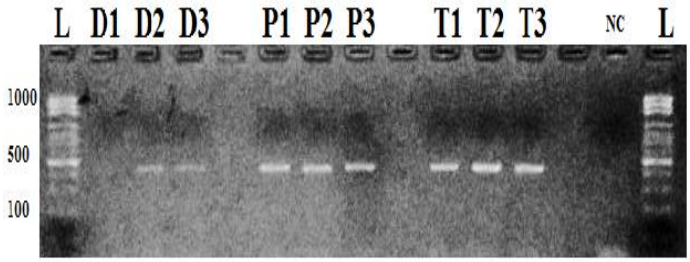
AS-PCR analysis and Agarose gel Electrophoresis

PCRs were performed with 10 µL volumes using Thermo Fisher Scientific PCR mix (USA). The final concentrations were as follows: 1X Taq buffer, 2.5 mM, MgCl2, 0.32 mM, dNTP mixture, 0.25 µM for each primer, 0.5 U Taq and 10 ng genomic DNA. All reactions were performed using a Wagetech Projects Master Cycler (Eppendorf, Hamburg, Germany) with an initial denaturing cycle of 5 min at 95°C, 40 cycles of 30 s at 93°C, 1 min at 59°C, 1 min at 72°C, and a final extension cycle of 10 min at 72°C. The PCR products were visualized using GR green dye in 2% agarose gels at 90 volts for 15mins.

Results and Discussion

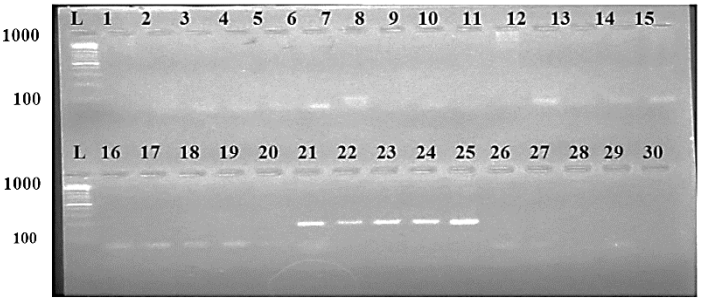
In this study, multiplex PCR was used. Each reaction had primer S3F & S3R and a pair of allele-specific primers (Table 2). PCR amplification of a region of the shell gene was carried out to test the primer on the genome of the oil palm fruit forms and to confirm the expected size of the fragment which is 446 bp (Figure 1). The allele-specific primers were also tested as right and left primers to the forward and reverse primer. Eight combinations (S3R&S20, S3R&S22, S3F&S20, S3F&S22, S3R&S33, S3R&S32 S3F&S33, S3F&S32) were investigated but there was no amplification in two of the combinations (S3F&S33, S3F&S32). (Figure 2) of which combination 5 (S3R and S33) generated clear amplicons of 200bp in dura, *Pisifera* and *Tenera*. An additional fragment of 80bp was present in the dura form which was absent in *Pisifera* and *Tenera* (Figure 2). The four allele specific primers under study were paired per allele and multiplexed with primer S3 (F&R) (Table 2). Amplicons of sizes 400, 200 and 80 bp were obtained from the combination of S3 (S22 and S32) in the *Tenera* fruit form while only the 80bp fragment was observed in the Dura and *Pisifera* forms (Figure 3).

Usually the observed outcome of designing primers from a gene sequence is expected but the observed prospective response of the primers designed may not be expected by the experimental. Thus, it was necessary to test run the forward and reverse primer (S3F & S3R) used in this study on the genome of the three fruit forms of the oil palm even though they were adopted from a previous research. The primer pair was responsive and the expected size of target region was confirmed to be the same as reported by Reyes and colleagues in 2015. Figure 1 shows the 446 bp amplicons



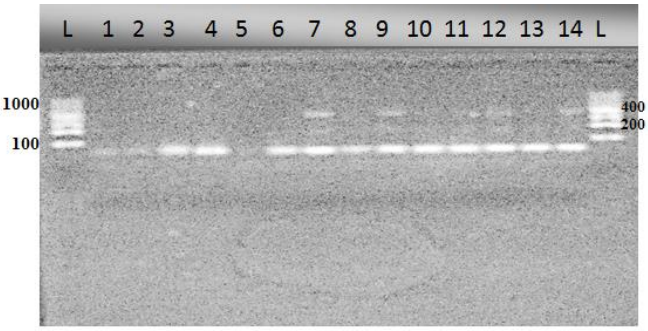
Key: **L**: 100bp Ladder 100-1000bp); **D**: Dura, **P**: *Pisifera*, **T**: *Tenera*, **NC**: Negative control. D, P &T were replicated thrice.

Figure 1: PCR amplification of a region of the SHELL gene in the oil palm using common primer S3 (F&R)



Keys: L: **100bp ladder (100-1000bp)**; 1, 6, 11, 16, 21, 26: **Dura**; 2, 7, 12, 17, 22, 27: ***Pisifera***; 3,4,5,8,9,10,13,14,15,18,19,20,23,24,25,28,29,30: ***Tenera*.**
Primer combinations:
1,2,3,4,5- **S3R and S20**
6,7,8,9,10- **S3R and S22**
11,12,13,14,15- **S3F and S20**
16,17,18,19,20- **S3F and S22**
21,22,23,24,25- **S3R and S33**
26,27,28,29,30- **SR3 and S32**

Figure 2: Allele Specific primer testing on fruit forms as right and left primers to S3forward and S3reverse.



Keys: L- 100bp Ladder (100-1000bp); **1,2,3**- Dura; **4,5,6**- *Pisifera*; **7,8,9,10,11,12,13,14**- *Tenera*. 200bp and 400bp alleles observed in all *Tenera* palms sampled.

Figure 3: Gel photograph of PCR products using primer S3 multiplexed with S22 & S32.

obtained. Other primers adopted as allele-specific primers were used as right and left to the forward and reverse primers. This was done to test them as pairs to the outer primers (S3F & S3R). From the result above in Figure 2, there was amplification in six of the combinations investigated (S3R&S20, S3R&S22, S3F&S20, S3F&S22, S3R&S33, S3R&S32) but only combination 5 (S3R&S33) generated a distinct band of 200 bp in Dura *Pisifera* and *Tenera*. Although there was another band of about 80 bp in the Dura fruit form which maybe as a result of primer S33 present in the

Table 2: Effect of PCR multiplexing with Allele Specific primer pairs and S3 primer in Dura, Pisifera and Tenera genomes

A.S primers	Shell Allele targeted	Primer pairs per Allele	Amplification product after multiplexing with primer S3
S20	Sh/Sh,Sh/sh	S20 &S33 (Sh/Sh)	80bp in Dura (D), Pisifera (P) and Tenera (T)
S22	sh/sh	S22 &S32 (sh/sh)	80bp in D & P, 400, 200 & 80bp in Tenera
S32	sh/sh, Sh/sh	S20 &S32 (Sh/sh)	Optimization ongoing
S33	Sh/Sh		

reaction targeting the homozygote dominant allele (Sh/Sh) in Dura.[4] This possible variant observed in Dura may probably be treated as primer dimer until confirmed by sequencing.

Allele specific PCR method can be done in two ways; nested PCR or a multiplex PCR. The nested involves the use of more time and supplies while multiplexing helps to conserve DNA, manage time and minimize expense if the cocktail is well prepared.[12] In this study, multiplex PCR was used. Each reaction had primer S3F & S3R and a pair of allele specific primers (Table 2). Three alleles were observed in the *Tenera* genotype (400 bp, 200 bp & 80 bp) of which two were unique to *Tenera*, only one shared with dura and *Pisifera* (80 bp) thus, portrays *Tenera* as a heterozygote. Only similarities were observed between the parental genotypes. In a previous study, three alleles were also identified in the *Tenera* fruit form only that their method involved a restriction enzyme and the alleles identified were on a different locus of the shell gene compared to the observation in this study.[2] Optimization is ongoing to identify alleles that differentiate Dura and *Pisifera*.

Conclusion

This study highlights the effectiveness of AS-PCR as a traditional method for identifying SNP mutations. The findings suggest that *Tenera* can be molecularly detected using this cost-effective method requiring basically a thermal cycler. Depending on availability of funds, sequencing of PCR product obtained will further confirm this research finding. The outcome of this research will be applicable in plant breeding programs and seedling production of the Nigerian Institute for Oil Palm Research. This study will aid early detection of choice variety for distribution to Oil palm growers in the Industry.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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