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Development of a bidirectional PCR tool for Tenera oil palm (*Elaeis guineensis* Jacq.) identity

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SUMMARY. Three forms of fruit are distinguished in the Oil palm (*Elaeis guineensis* Jacq.): Dura, Pisifera and Tenera. The most cultivated high yielding oil palm variety, the thin shell Tenera is produced from the cross between the thick shelled Dura and the shell-less Pisifera. Due to the perennial nature of the oil palm, it is impossible to determine the fruit phenotype until it is reproductively mature. Oil palm farmers are therefore faced with the challenge of uncertainty of the kind of fruits and yield output expected until production time. The recent sequencing of the shell gene in the oil palm made it possible to identify Single Nucleotide Polymorphism (SNP) mutations that are now useful in developing molecular markers for fruit form predictions. In this study, Allele Specific PCR (AS-PCR) was used to assess regions of the shell gene containing SNPs for oil palm fruit form discrimination. Three primer pairs (S1, S2 and S3) were designed as common primers while four AS-PCR primers (S20, S22, S32 and S33) were adopted as inner primers. Possible combinations of common and AS primers were investigated in Dura, Pisifera and Tenera genomes. A 300bp fragment was observed to be unique in the Tenera shell gene when S3 was combined with S20 and S32. Other inner primer combinations investigated with S3 generated similar assays in Dura, Pisifera and Tenera. The result from this study detects a marker for the Tenera fruit form of the oil palm.

Keywords: Allele Specific PCR, Dura, Fruit forms, Oil palm, Pisifera, Shell gene, Tenera.

Introduction

Oil palm (*Elaeis guineensis* Jacq.) is a diploid, monocotyledonous plant, belonging to the family Arecaceae. It is economically an important tree, as it is the source of palm oil.

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Palm oil is the most traded vegetable oil in the international market (Corley and Tinker, 2003). Oil palm is widely cultivated in the tropical zones which include Malaysia, Indonesia, Nigeria, Ivory Coast, Columbia and Thailand (Wahid *et al*., 2005).

It is the highest producing oil seed crop in the world. Some cultivars produce as much as 5-9 tonnes of oil per hectare per year; three times the yield of coconut and ten times that of soya bean per hectare. In addition, another 0.5 tonnes of kernel oil per hectare per year can also be obtained (Jalani *et al.,* 1997). The large amount of oil produced in the oil palm fruit is a unique biological characteristic of this palm species (Hartley, 2000). The oil palm fruit can be classified into three forms: Dura (thick shelled), Pisifera (shell-less) and Tenera (thin shelled). The Tenera form is the accepted commercial cultivar with higher palm oil content. In conventional breeding practices, the time taken to differentiate between the oil palm fruit forms has been a major limitation. Molecular studies are carried out in oil palm research laboratories to investigate the genetic makeup of this crop in relation to its oil yield character. The fruit shell thickness is an important trait because it defines oil yield of the crop. This important trait is controlled by the SHELL gene (Singh *et al*., 2013).

The recent sequencing of this gene made it possible to identify Single Nucleotide Polymorphism (SNP) mutations that are now useful in developing molecular markers for fruit form predictions. Some molecular markers have been used to investigate the genetic diversity in the oil palm these include: Simple Sequence Repeat (SSR) (Ihase *et al*., 2014), Restriction fragment length polymorphisms (RFLPs) of ribosomal DNA (Singh *et al*., 1993), Random amplified polymorphic DNA (RAPD) (Sathish and Mohankumar 2007). In this study, a traditional SNP method known as the Allele Specific PCR (AS-PCR) was used in assessing the shell gene of the three fruit forms of the oil palm. It was aimed at developing a marker for the high yielding Tenera at the vegetative stage.

Materials and methods

Plant material for DNA extraction

DNA was isolated from fresh leaves of 4 Dura, 4 Pisifera and 13 Tenera oil palms obtained from the Nigerian Institute for Oil Palm Research (NIFOR), Benin City, Nigeria. DNA extraction was done using promega plant DNA extraction kit according to the manufacturers' instruction.

Primer design

In order to distinguish between shell alleles (Sh/sh), four allele specific primers (S20, S22, S32 and S33) were adopted from a previous study (Reyes *et al*., 2015) and combined as right and left primer. Three primer pairs specific to the shell gene

(S1, S2, S3) forward and reverse respectively were designed using primer blast on NCBI website. All 10 primer sequences were obtained and formulated by Inqaba (Table 1).

AS-PCR conditions

PCRs were performed with 10 µL volumes using Thermo Fisher Scientific PCR mix. 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.

Table 1.

Primer information for this study

Primer	Sequence	Amplified target	Reference
S1 (f)	AGTGCTGCCAAAGAAGGCT		This study
S1 (r)	TAAGTGACCAGGGTTGGCTG	*SHELL*	This study
S2 (f)	GGCGGTTTACAGGAGCAGAT		This study
S2 (r)	TAGCCTTTCTTTGGCAGCACT	*SHELL*	This study
S3 (f)	TTTGTTGCTTTTAATTTTGCTTGAATACCTTT		Reyes *et al*., 2015
S3 (r)	TGGCTTGGCCATAGAACAAA	*SHELL*	Reyes *et al*., 2015
S20	TCAGCATCACAAAGGACAGACAACTCATAATCT	Sh/Sh, Sh/sh	Reyes *et al*., 2015
S22	CAGCATCACAAAGGACAGACAACTCATAAGCA	sh/sh	Reyes *et al*., 2015
S32	GCCGAAATGGACTGCTGAAGCAT	sh/sh, Sh/sh	Reyes *et al*., 2015
S33	GCCGAAATGGACTGCTGAAGAAA	Sh/Sh	Reyes *et al*., 2015

Results and discussion

A regular PCR was carried out to test the primers (S1and S2) for their responsiveness to the genome of Dura, Pisifera and Tenera palms (Figure 1). The allele specific primers adopted were also paired per allele (Sh/Sh, sh/sh, Sh/sh) and used to amplify the gene coding the three fruit forms. Products obtained were the same in the first and second combination (S20 and 33, S22 and 32). Amplification products in the third combination (S20 and 32) were the same in Pisifera and Tenera but different in Dura (Table 2).

Table 2.

Paired *SHELL* Allele Specific (A.S) primers as forward and reverse on Dura, Pisifera and Tenera genomes.

A.S primer	Shell allele	Pairs per Allele	Amplification in the fruit forms		
S20	Sh/Sh,Sh/sh	S20 &S33 (Sh/Sh)	Dura (100bp)	Pisfera (100bp)	Tenera (100bp)
S22	sh/sh	S22 &S32 (sh/sh)	Dura (100bp)	Pisfera (100bp)	Tenera (100bp)
S32	sh/sh, Sh/sh	S20 &S32 (Sh/sh)	Dura (100bp)	Pisifera (100bp & 300bp)	Tenera (100bp & 300bp)
S33	Sh/Sh				

S1 primer combined with AS primer pair S20&33 showed no difference between the fragments of Dura, Pisifera and Tenera S1 primer combined with AS primer pair S20&33 showed no difference between the fragments of Dura, Pisifera and Tenera. For the second set, (S1{S22&32}) the Tenera generated two bands of 600bp and 80bp which was different from the Pisifera (one band of 80bp). In the third set, (S1{S20&32}) similar banding pattern were observed in the three fruit forms. S2 in second and third combination with AS primers shows similar band patterns in all fruit forms (Figure 3). In the first combination, the fragment size of 400bp was not observed in the Pisifera form. S3 pimer in combination with AS primr pairs generated a unique band of 300 bpin Tenera fruit (Figure 4). A unique band of 300bp was observed in only the tenera fruit form when primer S3 was combined with primer S20 & S32. S3 primer combination with S20 and S32 generated a different PCR pattern as showed in Figure 5. The allele observed in the Tenera palms sampled was absent in Dura and Pisifera.

In order to find markers linked to the SNP mutation responsible for the variation in shell thickness of the oil palm, the present study employed a simple Allele Specific (A.S) PCR tool. This involved designing primer pairs from the shell gene and adopting shell Allele Specific (A.S) primers. The A.S primers (S20 and S32) used in identifying a marker for Tenera in this study were adopted from a previous research (Reyes *et al*., 2015) But there was no evidence for the discrimination of Tenera genotype with this prime pair. Our results showing the variantion in Dura could be due to homozygote dominant allele (Table 2), S20 & S32 is intended for the heterozygous allele (Sh/sh). This was previously shown by Corkey and Tinker, 2003.

Given that primer S20 & S32 is intended for the heterozygous allele (Sh/sh). According to Patent WO2016205240A3, Pisifera trees may be heterozygous at the SNP position of SEQ ID NO: 922 and SEQ ID NO: 923 as a result of having contribution from both Zaire and Nigerian derived lines. Several differences were

observed in the Pisifera genotype in the palms sampled for the second set of primers (2nd D4-T38) from Tenera being that there were two bands (600bp and 80bp) in Tenera and only one band (80bp) in Pisifera. This possibly maybe as a result of the primers tendency to target homozygote recessive allele in the Pisifera palms sampled.

Amplicons generated with S2 (S20&S33) in Dura and Tenera have the same size (Fig. 3). This may be due to the fact that primer S20 and S33 are for the heterozygote and homozygote dominant allele respectively (Reyes *et al*., 2015). It is also important to state that the Dura and Tenera genotypes are the shelled fruit forms, as such it is expected that they share similarities in alleles (Sathish and Mohankumar, 2007).

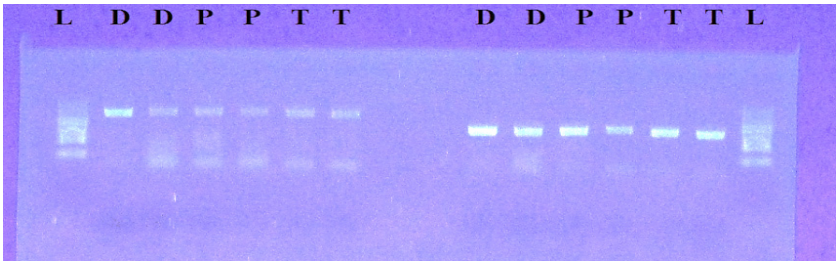

Figure 1. Electrophoregram of amplicons generated with S1 and S2 primers. (S1 and S2) designed. Primer S1: Right side, Primer S2: Left side.
Keys: L: 100bp ladder; **D**: Dura; **P**: Pisifera; **T**: Tenera.

Figure 2. Electrophoregram of amplicons generated with S1 and AS primers.
Keys: L: 100bp ladder; Upper part of gel (1st D4-T38): S20&S33;
(2nd D4-T38): S22&S32; Lower part of gel (3rd D4-T38): S20&S32

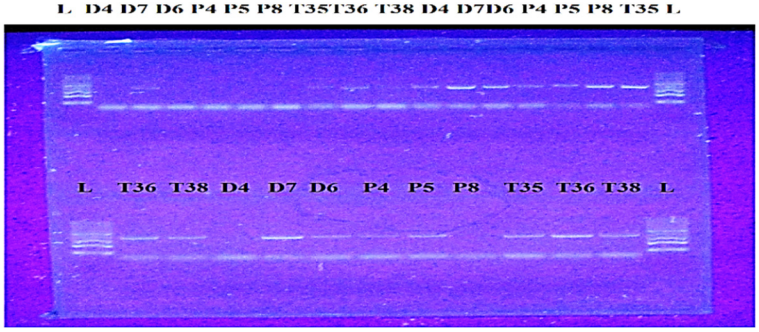

Figure 3. Electrophoregram of amplicons generated with S2 and AS primers.
Keys: L: 100bp ladder; Upper part of gel (1st D4-T38): S20&S33;
(2nd D4-T38): S22&S32; Lower part of gel (3rd D4-T38): S20&S32.

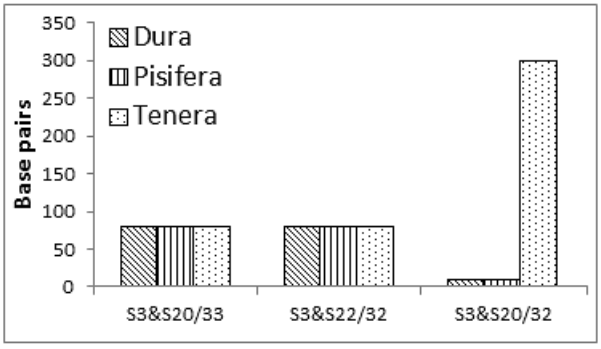

Figure 4. PCR product sizes obtained from using primer S3 in combination with the three allele specific primer pairs.

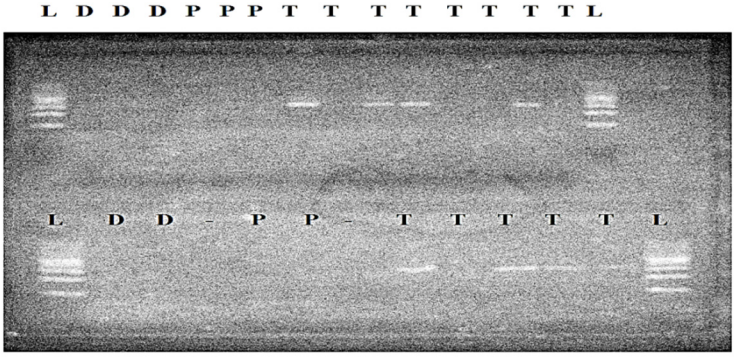

Figure 5. Electrophoregramm of amplicons generated with S3 and AS primers.
Keys: L: 100bp ladder; **-** : Nothing loaded; **D**: **Dura**; **P: Pisifera**; **T: Tenera**.

A unique marker for the Tenera fruit form was observed when primer S3 was used in combination with A.S primers S20 and S32. This amplification was done severally and in all the cases, a fragment size of 300bp was observed in about 75% of the Tenera palms sampled (Fig. 5). Researchers from the Indian Institute of Oil palm Research have developed a CAPS (Cleaved Amplified Polymorphic Sequence) marker which produced three alleles of sizes 550, 280 and 250bp in Tenera genotypes but their method required the use of a restriction enzyme for a digestion step (Babu *et al*., 2017). In the present study, PCR is all that was required to generate a reproducible marker for the Tenera fruit form.

Conclusions

The goal of any breeding programs, particularly in oil palm is to produce planting materials with higher oil yield; the Tenera fruit form is the preferred choice for commercial planting. Despite of the many advances which have been made in the production of hybrid oil palm seeds, the challenge of seed contamination is still significant, hence the need for the development of a molecular tool for accurate identification of Teneral forms.This study therefore provides a marker that can serve as an early screening aid to complement and certify the efforts of the hybrid seed producers who most times rely on morphological charcteristics, which can be very deceptive, for identification. This will dramatically reduce and possibly eradicate contaminants among the oil palm hybrid planting materials and eventually translate into increased income for growers and investors in the industry.

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