EVALUATION OF FTA CARD PREPARATION FOR SSR MARKER AMPLIFICATION OF mEgCIR3607 FROM MATURE OIL PALM LEAVES

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ABSTRACT: The oil palm (*Elaeis guineensis*) leaves are characterized by high levels of polysaccharides and secondary metabolites, which present significant challenges for effective DNA extraction and Polymerase Chain Reaction (PCR) analysis. The objective of this study is to investigate the applicability of Flinders Technology Associates (FTA) cards to preserve oil palm tissue from mature oil palm leaf for direct PCR. A total of 50 mature oil palm leaf samples were collected from independent smallholder plantations and deposited onto the FTA card. An attempt was made to compare between direct use of FTA card disc and FTA elute as DNA templates for PCR amplification using primer mEgCIR3607. Evaluation of FTA elutes as DNA template on 50 oil palm samples demonstrated successful PCR amplification with high intensity of PCR product. The findings showed that FTA cards effectively preserve DNA from mature oil palm leaves, enabling successful PCR amplification using FTA elute as a DNA template, which demonstrates their suitability for overcoming challenges associated with DNA extraction in *Elaeis guineensis.*

KEY WORDS: *FTA; PCR; Mature leaf; oil palm; mEgCIR3607*

1. INTRODUCTION

Oil palm (*Elaeis guineensis*) have been cultivated and domesticated in Malaysia since its first introduction in the 1870s and began as an ornamental plant in Malaysia. It has the fastest-growing global demand as a raw material for food, personal care products, livestock feed, bioenergy, and other products [1]. Oil palm production has made significant progress over the years, starting from 4.1 million tonnes in 1985 until projected to reach 19.4 million tonnes in 2012. *E. guineensis* has become commercial oil palm in Malaysia and the second highest most consumed in the world as well as has been exported and traded worldwide over

soybean oil [2]. Malaysia's gross domestic product (GDP) contribution from palm oil was found to be 3.7 percent of GDP in 2023. The commodity has contributed to Malaysia's economic growth, more job opportunities, and acts as a primary contributor towards the foreign exchange earnings, Malaysia's Gross Domestic Product (GDP). Roughly, it was reported oil palm has risen 7.8% in Malaysia's GDP with a total of RM72.77 billions of export revenue [3]. The economic impact of oil palm cultivation is profound, particularly for smallholder farmers who rely on this crop for their livelihoods. According to [4], the oil palm sector supports millions of jobs and contributes significantly to rural development in producing countries. Besides, global food security is greatly enhanced by smallholder oil palm growers [5]. Since then, oil palm crops became a part of the industrialised Malaysia's agricultural economy and has grown into a massive industry because of its crude palm oil [6]. However, ensuring high productivity and quality remains a challenge [7] due to various factors, including planting material quality and genetic research study.

DNA-based molecular markers can be classified into several main classifications which are (1) non PCR-based and (2) PCR-based. Molecular markers that are frequently used in oil palm studies can be categorised into (A) PCR-based such as (1) rapid amplified polymorphic DNA (RAPD) [8, 9], (2) amplified fragment length polymorphism (AFLP) [10, 11, 12, 13], (3) single nucleotide polymorphism (SNP-discovery) [14, 15,16], (4) expressed sequence tag-derived simple sequence repeat markers (EST-SSR) [17, 18], (5) randomised amplified microsatellite (RAM) [19], (6) inter-simple sequence repeats (ISSR) [20], (7) Simple sequence repeats (SSR) [21, 22, 23], and (B) non-based PCR which is (1) restriction fragment length polymorphism (RFLP) [24, 25, 26].

PCR-based was widely utilised and gained demand among researchers in extending knowledge of gene mapping and genetic diversity compared to non-PCR-based due to user-friendly features over probe-based. Amplification PCR-based markers mainly consist of (1) arbitrarily and (2) non-arbitrarily primers. Additionally, PCR-based becomes useful as it able to be utilised as (1) co-dominant; target specified locus or (2) dominant; amplified large number loci randomly [27]. PCR-based markers are more effective than those based on hybridization due to the use of hybridization-based probes for genetic diversity detection. However, it is constrained by a series of drawbacks, including the need for large quantities of extremely pure DNA, the use of radioactivity for detection, the needs for trained manpower, and less polymorphism detection. A breakthrough of the polymerase chain reaction (PCR) discovery by Kary Mullis (1984) turned out to be a unique approach that produced a brand-new category of DNA profiling markers. PCR-based molecular markers can be divided into three categories such as (1) PCR-based with probe; AFLP, (2) amplification based which are RAPD; ISSR; SSR, and (3) new generation of molecular markers; such as EST-SSR and SNP.

The emergence of implementation of molecular markers in plant genomics began in the 1980s, followed by the development of PCR-based DNA markers in the 1990s, marking a significant milestone in plant genomic research [28]. Since then, molecular markers have been widely acknowledged in various aspects of plant genomics and molecular breeding [29]. Molecular marker methods have significantly advanced and are now invaluable tools, complementing traditional approaches for plant identification [29]. These methods are essential for improving oil palm productivity and quality by efficiently and accurately analysing genetic material. These markers reveal genotypic differences, known as monomorphism and polymorphism, among individuals or species. SSR markers have

become the preferred choice in molecular diagnostics due to their high polymorphism, minimal DNA requirements, ease of automation, transferability between populations, and effectiveness in understanding genetic structure and diversity [30, 31]. A crucial step in molecular marker implementation is selecting the most suitable markers and optimizing PCR conditions for microsatellite systems. Recent advancements in molecular biology have provided robust tools for verifying oil palm varieties and assessing genetic uniformity. SSR markers offer detailed insights into genetic diversity within plantations, reliably identifying genetic variations and ensuring consistent crop performance [32, 29].

Understanding the foundation of leaf structure is crucial for DNA extraction and amplification of molecular markers especially in oil palm research [33]. Young leaves are often preferred for DNA extraction since they generally contain higher concentrations of metabolites [34] which can yield higher-quality genomic material for molecular analyses, but mature leaves are more readily accessible for routine sampling in plantations [35]. However, due to the high polysaccharides, secondary metabolites, lipid and polyphenol content in mature oil palm leaves, PCR amplification of microsatellite markers can be challenging [36]. Previous oil palm research [37, 38, 39] that uses commercial DNA extraction kit focused on usage of young leaves as DNA sources and mature oil palm leaves only successful been used with conventional DNA extraction kit. Therefore, there is need to explore the alternative of conventional DNA extraction methods that can effectively extract DNA from mature oil palm leaves and evaluate its potential for subsequent PCR analysis. Traditional PCR analysis methods, widely applied in previous studies [32, 23], often have limitations such as complexity, time consumption, and the need for specialized equipment and extensive sample handling [40, 41, 42]. Additionally, the transportation and storage of genetic samples, critical for research collaboration and diversity studies, pose logistical challenges that extend research time and restrict access to genetic analysis in resource-limited regions [43]. Reliable preservation and extraction methods are also necessary to ensure DNA quality [44].

In response to these challenges, this research endeavors to initiate a paradigm shift in the way we approach PCR analysis of oil palms. Central to this effort is the integration of Flinders Technology Associates (FTA) card technology, a compact and versatile device specifically designed for the collection, preservation, and transport of nucleic acid samples [45]. FTA cards are a valuable tool for the preservation and analysis of plant DNA. They facilitate the collection and long-term storage of DNA samples without the need for refrigeration [45], making them particularly useful for fieldwork [46]. The successful use of FTA cards in various studies IN agricultural plants such as tomato, cassava, *Nicotiana bethamiana,* maize [46], and various non-agricultural plants such as Cactaceae, Magnoliaceae, Pinaceae, Poaceae and Vitaceae [47]. The only attempt of using FTA card from oil palm was from [48] but only focused on young oil palm leaves. While previous studies have demonstrated the potential of FTA cards for DNA extraction and PCR amplification from various plant species, their efficacy in amplifying specific markers, such as the crucial mEgCIR3607 microsatellite, from mature oil palm leaves remains to be comprehensively assessed.

The objective of this study is to investigate the applicability of Flinders Technology Associates (FTA) cards for preserving mature oil palm (*Elaeis guineensis*) leaf tissue for direct PCR analysis. By comparing between direct use of FTA card disc and FTA elute as DNA templates for PCR amplification, this research aims to establish a reliable and efficient

method to simplify genetic studies. The study also seeks to enhance the preparation processes for FTA cards, further improving DNA preservation and analysis efficiency. This approach is expected to benefit oil palm breeding programs and broader agricultural research, promoting advancements in sustainable agricultural practices worldwide.

2. METHODOLOGY

2.1 Materials and reagents

The reagents and chemicals for that were used in this study are phenol, 99% ethanol, distilled water, boric acid, molecular biology grade water, Tris-base, and EDTA (Ethylenediaminetetraacetic acid), 5x Hot FIREPol blend master mix, QIAGEN QIAcard FTA wash buffer, 1x TE buffer (Tris-EDTA buffer), 10x TBE buffer, gel red nucleic acid staining 10000x water, Quickload 100bp DNA ladder, Solis Biodyne 100bp DNA ladder ready to load, SSR primer (mEgCIR3607), 6x DNA loading dye.

2.2 Sample collection

A total of 50 mature oil palm leaves were collected from an unrelated tree of independent smallholder plantation (2°56'46.6"N, 101°27'33.5"E; 2°56'40.3"N, 101°27'31.5"E). The easily reachable mature frond: frond 32-40 were chosen, harvested, cut, placed, and labelled in the zip lock bag individually according to Fairhurst, (2015). The samples were placed in the box and brought back to the laboratory immediately. The samples must be prepared immediately within a 24-hour collection period.

2.3 Sample preparation using direct PCR-based

Each leaf tissue was carefully cleaned to eliminate any dust or dirt that could interfere with the DNA extraction process. Precisely 0.4g of the leaf tissue was weighed using an analytical balance. A few drops of water were added to moisten the tissue, which was then crushed using a mortar and pestle. The crushed leaf material was transferred onto the circle area of the QIAcard FTA classic (QIAGEN, Düsseldorf, Germany) using a pair of tweezers. To prevent the leaf material from adhering to the FTA card cover, small pieces of parafilm were placed over the crushed leaves. A pestle was then used to press and roll over the FTA card to ensure the extraction of the green leaf extract. Upon completion, the FTA card containing the sample was labelled, placed in a zip-lock bag, and stored at room temperature.

2.4 FTA preparation for direct PCR-based

Based on optimised the washing step [49], the modified procedure begins with placing clean, sterile PCR tubes on a rack and labelling them according to the PCR gradient

temperature. For example, samples from plantation 1 are labelled P1S1 denoted as E1, P1S2 denoted as E2, P1S3 denoted as E3, ..., P1S25 denoted as E25, and samples from plantation 2 are labelled P2S1 denoted as E1, P2S2 denoted as E2, P2S3 denoted as E3, ..., P2S25 denoted as E25. The Harris MicroPunch was sterilized by punching clean paper. Two discs from the FTA card were punched using the Harris MicroPunch and inserted into labelled PCR tubes. Each sample was washed with 200 µL of QIAcard FTA wash buffer, vortexed, and centrifuged at 6100 rpm and 2°C for one minute. The wash buffer was discarded, and this step was repeated, with the total of two washes with QIAcard FTA wash buffer (QIAGEN, Düsseldorf, Germany). Afterwards, 200 µL of TE buffer (containing Tris-base, and EDTA (Ethylenediaminetetraacetic acid)) was added to the PCR tubes, vortexed, and centrifuged at 6100 rpm and 2°C for one minute. The TE buffer was discarded, and this step was also repeated, making the total of two washes with TE buffer. The FTA elute was then incubated at 95°C for 5 minutes and air-dried for an hour at room temperature or for 20 minutes in a drying cabinet.

2.5 Polymerase Chain Reaction (PCR) via FTA card

PCR was performed in a 25 µL reaction volume containing 4 µL of FTA elute, 1 µL of reverse primer (5'- GAGGGGGTTGGGACATTAC-3'), 1 µL of 10mM forward primer (5'- TAGCTCACAACCCAGAACTAT-3'), 4 µL of 5x Hot FIREPol Blend master mix, and 15 µL of sterile distilled water. The gradient temperature was set between 5°C below the melting point (Tm). The PCR cycling conditions started with an initial denaturation at 94°C for 4 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at an optimized temperature gradient for 30 seconds and elongation at 72°C for 1 minute. This was followed by a final extension at 72°C for 10 minutes, with the PCR held at ∞ for 10 to 12°C. The amplification was carried out using a thermal cycler. The PCR products were then separated using 1.8% agarose gel electrophoresis with 1x TBE (containing 50mM Tris-base; pH 8 (Thermo Fisher Scientific, USA), Boric acid(Merck, Germany) and Na2EDTA (J.T. Baker, USA).

2.6 Gel electrophoresis for primer evaluation and primer validation

Approximately 2 µL of Quickload DNA ladder was used as a marker, and a mixture of 2.0 µL loading dye and 5.0 µL of PCR product was pipetted into the wells of a 1.8% agarose gel. The SSR products were electrophoresed on a 1.8% agarose gel in a 1x TBE buffer at either 90V, 200mA for 75 minutes (20-well gel casting electrophoresis; 10.3 cm x 15.3cm) or 90V, 200mA, 55 minutes for 8-well gel casting tray; 10cm x 7.5cm. The gel was stained with 10x gel red nucleic acid staining (diluted from 10000x red nucleic acid staining in water) by using ratio 1:1 to 1x TBE buffer poured into agarose powder and allowed it to be solidified. The gel was then photographed using a Bio-Rad gel documentation system.

2.7 Validation across 50 samples

To assess genetic uniformity across the plantation, genotyping was conducted using SSR markers: mEgCIR3607. This marker was chosen for their ability to provide detailed information on genetic basis and consistency within the oil palm plantation [32, 23]. The

SSR fragments were amplified using a PCR thermocycler in a reaction volume of 25 µL, which included 5× Hot FIREPol Blend master mix (containing Taq DNA polymerase, dNTPs, MgCl2, and reaction buffer), 10 µM forward primer, 10 µM reverse primer, and FTA discs. The PCR conditions were set as follows: an initial denaturation at 94°C for 4 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at the optimized temperature for 30 seconds, extension at 72°C for 1 minute, and a final extension at 72°C for 10 minutes. The amplified products were separated on a 1.8% agarose gel using 1× TBE buffer. After electrophoresis, DNA bands were visualized with GelRed™ staining and imaged using a Bio-Rad Gel Doc™ UV transilluminator.

3. RESULTS

Manufacturer's protocol [50] suggested that FTA discs were directly used as DNA source after FTA washing steps. To ensure the DNA content in the FTA discs fully unbound during the washing process, several modifications and optimization in FTA card preparation steps are required especially for the high-level polysaccharide plants such oil palm samples [48,51,47]. Based on the modification of FTA card preparation steps, FTA elute was developed to become as DNA source for PCR amplification. To develop an optimized FTA card preparation process for direct amplification of SSR in oil palm, both FTA discs and FTA elute were used as source of DNA for oil palm PCR amplification without changing any PCR components. Electrophoresis is one of the best ways to show PCR products through base pairs on gel by channelling electric current through the gel to move the molecule which is the DNA product. The DNA fragments are negatively charged, so they move towards the positive electrode. Because all DNA fragments have the same amount of charge per mass, small fragments move through the gel faster than large ones [52]. The gel was stained with DNA-binding dye which made them visible during observation under UV light. The band was produced when primers bind to the specific active site of the DNA extracted from the oil palm sample. The band was produced when primers bind to the specific active site of the DNA extracted from the oil palm sample. The polymerase chain reaction were amplified the DNA sequence which then observed through gel electrophoresis [28]. Based on the Figure 1, it shows the two different approaches for using FTA card which are (1) direct use of FTA discs and (2) FTA eluted from FTA discs.

Referring to (A) in Figure 1, this method involves directly use FTA disc by placing several small discs cut from FTA cards as a PCR component. The gel visualization of direct-use FTA card demonstrates that the unable to be amplified, as evidenced by the presence of primer-dimer, or also known as non- specific bands with no PCR product. This indicates that the PCR inhibitors typically associated with direct sample input from FTA cards resulting the DNA not fully unbound from the FTA card matrices and were not sufficient to have successful PCR amplification.

As for (B) in Figure 1, this method begins with elute the FTA discs with elution buffer before being used in PCR amplification. The gel visualization of (B) shows clear, consistent bands with no presence of primer dimer indicating successful PCR amplification. This suggest that the elution process is crucial in releasing the nucleic acid from the FTA

card matrices to maximise DNA yields obtain for further downstream applications [53]. This suggests that the elution process does not compromise DNA quality and may reduce the potential impact of any inhibitors that might incorporate in the FTA discs. Besides, additional step such elution method is required especially when working with high-level polysaccharides plant samples.

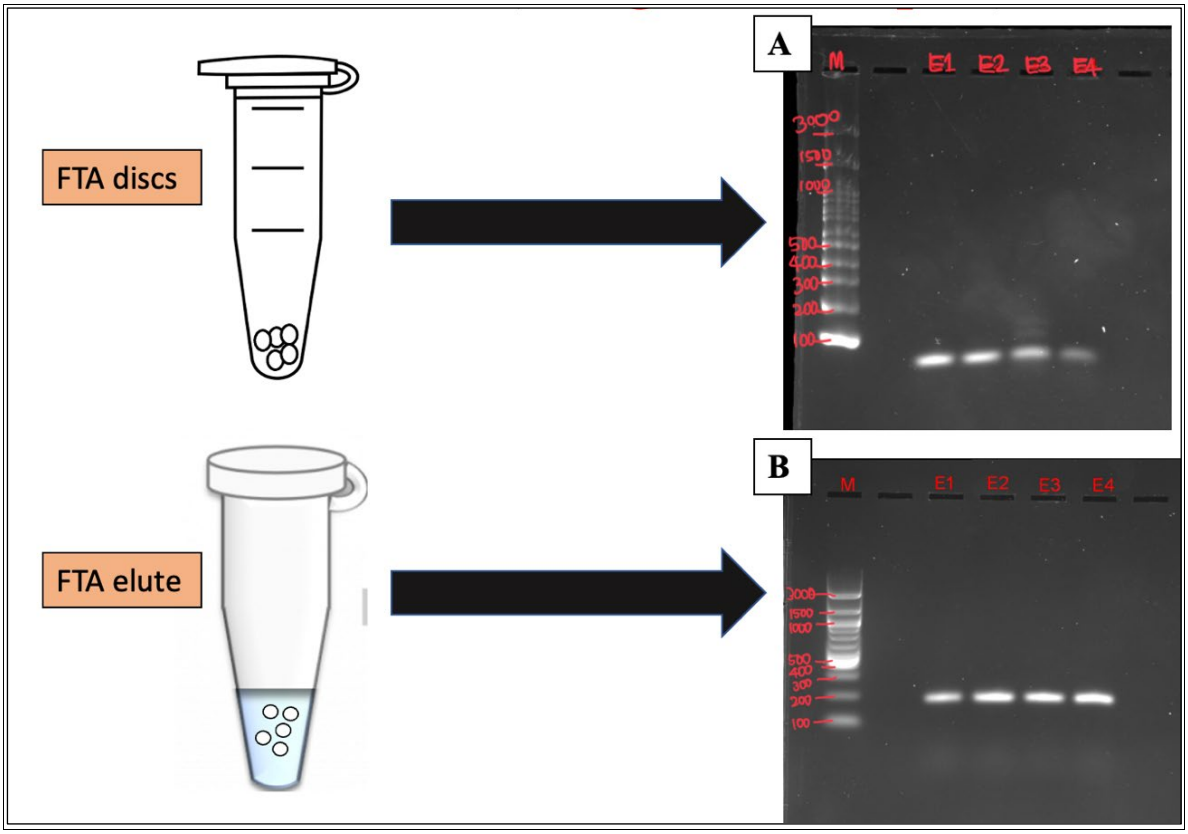

Figure 1: Comparison of outcome for oil palm PCR amplification using DNA source from different FTA card preparation steps. (A) Photograph of gel visualisation of using FTA discs as DNA sources (standardised protocol) and (B) Photograph of gel visualisation of using FTA elute as DNA sources (optimised protocol)

The FTA elute could proceed for validation using 50 samples from two different oil palm plantations of independent smallholder. Throughout the primer validation, the gel visualization across all samples PCR amplification was consistent with clear, distinct band in all lanes that meet expected result which is 190-238bp with no presence of primer dimer across 50 samples used. This indicates that the SSR marker mEgCIR3607 is successfully amplified in both plantation 1 and plantation 2. No non-specific bands or primer dimers are observed, indicating that the PCR conditions and the primers used are highly specific for the mEgCIR3607 marker. The figure demonstrates the successful and consistent amplification of the SSR marker mEgCIR3607 across multiple oil palm samples, validating the efficiency and reliability of the PCR conditions and preparation methods of FTA elute used.

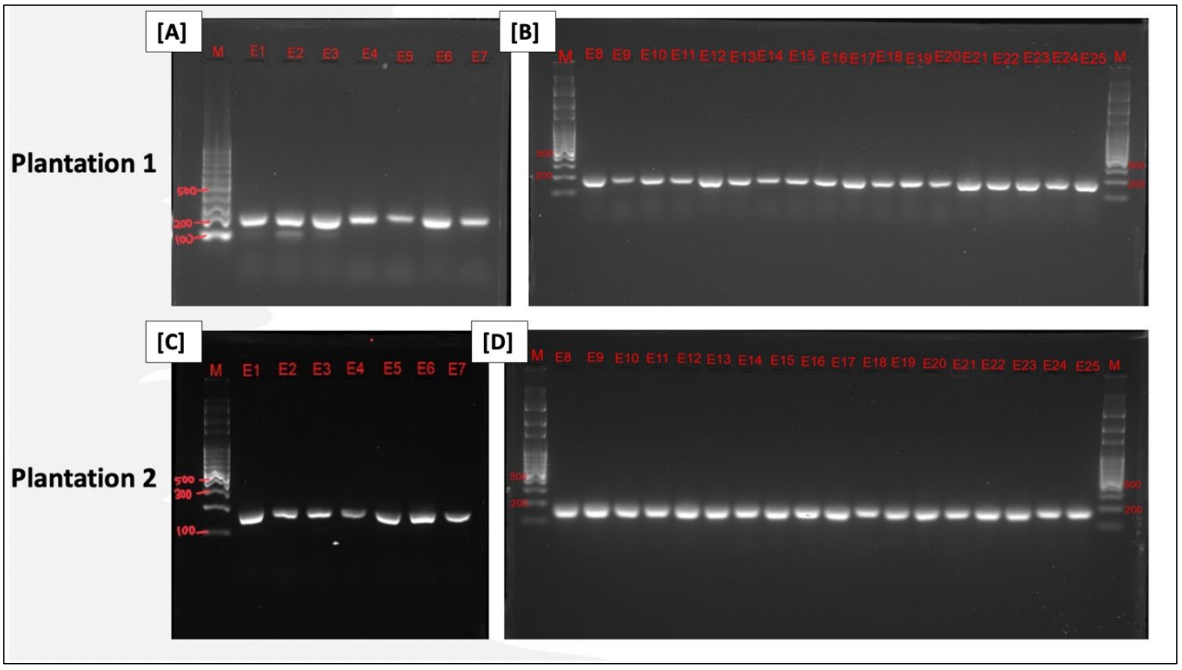

Figure 2: Primer validation of SSR marker mEgCIR3607 using optimised protocol across two different oil palm independent smallholder plantations. Plantation 1 refers to sample collected from (2°56'46.6"N, 101°27'33.5"E), and Plantation 2 refers to sample collected from (2°56'40.3"N, 101°27'31.5"E). [A] Photograph of gel visualisation of sample 1 to 7 collected from plantation 1, [B] Photograph of gel visualisation of sample 8 to 25 collected from plantation 1, [C] Photograph of gel visualisation of sample 1 to 7 collected from plantation 2 and [D] Photograph of gel visualisation of sample 8 to 25 collected from plantation 2.

4. DISCUSSION

In Figure 1, it compares the developed FTA elute technique by [49] with the conventional FTA disc preparation method. The results show that the FTA elute approach successfully amplified the SSR marker, providing distinct and precise bands without non-specific amplifications, but the FTA disc method produced no detectable PCR products and primer dimers. This shows that the FTA elute techniques is substantially better at extracting DNA from oil palm tissues for use in PCR applications downstream, especially when handling high polysaccharide content, which has the potential for hindering PCR reactions.

For figure 2, it provides additional evidence of the PCR amplification's consistency and reliability across a broader range of samples collected from different plantation blocks. All 50 samples showed consistent amplification of the mEgCIR3607 marker, with distinct bands that aligned the expected product size (190-238 bp). The reliability of the optimised FTA card preparation procedure is highlighted by this uniformity between different samples and preparation techniques among samples from plantation 1 and plantation 2. The fact that non-specific bands and primer dimers are absent in every lane is additional evidence of the primers' specificity and the efficiency of the optimised procedure.

Comparing the results for P1 and P2, the patterns seem to be similar, indicating that

both preparation conditions yielded consistent results. The intensity of the bands is quite uniform across most lanes, indicating consistent amplification. However, there is a slight variation in intensity between the P1 and P2 conditions. There may be slight differences in band intensity or sharpness, which could be due to variations of DNA concentrations among individuals [54].

This indicates that the primers are well-suited for targeting the desired SSR loci in oil palm DNA. The consistency of the results across multiple samples underscores the robustness of the primers in various genetic backgrounds [21,23]. The reproducibility of the PCR results across the 50 samples was noteworthy. Each sample, regardless of its origin within the plantation blocks, yielded similar amplification patterns. This reproducibility is essential for reliable genetic analysis and suggests that the primers and the direct PCR-based method using FTA elute are reliable for routine genotyping in oil palm research [55, 47]. Our findings demonstrate several key aspects of primer performance and the practicality of using FTA elute for field-based DNA sampling and subsequent PCR analysis.

Oil palm genotyping will be greatly impacted by the SSR-mEgCIR3607 marker's effective amplification via the optimised FTA elute technique. The ability to accurately amplify SSR markers straight from FTA elute lowers sample handling, streamlines the DNA extraction procedure, and lowers the possibility of contamination [23] This is especially helpful for field-based research when quick and accurate genotyping is needed. Furthermore, the optimised method's usefulness in regular genetic analysis is further enhanced by its compatibility with current PCR methods without the need for extra changes [56, 57].

Notwithstanding the efficacy of the optimised FTA elute technique, it is imperative to take into account possible constraints. The FTA disc method's early failure raises the possibility that different plant species—especially those with high polysaccharide or secondary metabolite levels—may respond differently to different FTA card formulations [58, 49]. This underscores their effectiveness of FTA elute in maintaining DNA quality for subsequent molecular analyses, such as PCR and sequencing [59,47]. This method allows for accurate genetic profiling and verification, contributing to reliable assessments of planting material authenticity especially for remote area plantations. Besides, this innovative approach aims to eliminate the shortcomings of conventional methods while providing reliability, quick and convenience in oil palm genetic analysis. By using the optimised techniques of FTA elute preparation, it may be suitable for other difficult plant tissues that possess similar leaf structure characteristics as oil palm leaves. Furthermore, even though the optimised approach performed well for the SSR-mEgCIR3607 marker, more research might be required for other SSR marker, different types of another molecular marker and even in other plant species.

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5. CONCLUSION

This study evaluated the applicability of Flinders Technology Associates (FTA) cards for preserving mature oil palm (*Elaeis guineensis*) leaf tissue and enabling direct PCR amplification of the mEgCIR3607 marker. The findings demonstrated that FTA elute

provide an effective and convenient solution for stabilizing DNA from mature oil palm leaves, facilitating its use in downstream molecular applications.

The optimized protocol for PCR amplification using DNA preserved via FTA elute approach proved reliable, producing consistent and reproducible results. The study highlights the potential of FTA elute as a user-friendly and efficient tool for genetic research, particularly in oil palm breeding programs where rapid and accurate DNA analysis is crucial.

Furthermore, improving FTA card preparation techniques enhances the efficiency of DNA preservation and reduces potential inhibitors that could compromise PCR outcomes. This not only streamlines the workflow for molecular studies but also reduces reliance on complex DNA extraction procedures, making the method accessible for broader agricultural applications. Overall, the application of FTA elute for direct PCR analysis represents a significant advancement in oil palm genetics and agricultural research.

It supports initiatives to enhance breeding techniques and advance sustainable practices in the oil palm sector by providing a viable and sustainable method of managing genetic resources. The adaptability and influence of FTA elute for direct PCR approach in agricultural research may be increased by future research that improves these techniques and broadens their use to different crops and molecular markers.

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