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Comparative Analysis of DNA Extraction Approaches to Verify Hybrid Purity and Genetic Relationships in Maize

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Abstract: Genetic purity in hybrid maize is essential to ensure the stable combination of parental genetic materials, which underpins heterosis and on-field hybrid performance. Conventional field-based tests for assessing hybrid purity are labor-intensive, time-consuming, and less efficient compared to molecular marker-based approaches. Alternatively, DNA-based markers such as simple sequence repeats (SSRs) provide a feasible alternative. DNA-based markers assay requires the DNA extraction method that produces sufficient amount and quality of DNA. Therefore, this study compared three extraction methods—SDS-based, CTAB-based, and a commercial DNA extraction kit—using leaf tissue from maize seedlings as the DNA source. The extracted DNA samples were assessed for yield, quality, and suitability for SSR-based polymerase chain reaction (PCR) assays. Results demonstrated that all three methods successfully produced DNA of adequate quality and quantity for SSR-PCR analysis. These findings confirm the applicability of multiple extraction techniques for molecular based hybrid purity testing. The study contributes practical guidance for optimizing DNA extraction in maize, supporting the broader adoption of molecular markers in hybrid breeding programs.

Keywords: DNA extraction, Hybrid, Maize, Molecular marker, Simple Sequence Repeats (SSR)

1. Introduction

Maize (*Zea mays* L.) is one of the world's major staple foods. It plays an important role in global food security after rice and wheat. Over the past decade, the national demand for maize has reached about 20 million tons, an increase of 3.7 per cent per year (Alya et al., 2022). As the human population and the food and feed industry grow, global corn demand is projected to continue to expand in the future (Adnan et al., 2024). Increasing maize productivity has been achieved by various methods, one of which is the use of hybrid maize seeds (Alya et al., 2022). Hybrid seeds of maize are produced by crossing two parental varieties with a wide genetic distance, combining their genetic material. This genetic combination produces a heterosis effect and stimulates improved vigor, resistance, and productivity in F1 seeds (Hochholdinger & Baldauf, 2018).

This heterotic effect is only observed when the F1 maize seed inherited a set of genetic material from each distantly related parent (Rajeev et al., 2005; Xiao et al., 2021). Therefore, the field performance of F1 maize seeds heavily depends on the seed industry's ability to maintain and ensure the genetic purity of the seeds it produces. The genetic purity of F1 seeds can be determined by the Grow Out Test method (GOT). This method relies on the morphological observation of the F1 maize seed and the comparison of its physical characteristics with its parental seeds (Shinde et al., 2021). This is considered the gold standard for checking F1 seed purity. The GOT method involves planting the F1

production seeds alongside both parental lines. Subsequently, each growing plant is characterized by its morphological traits from the seedling stage up to maturity. This method, however, has several limitations including the long duration for cultivation and maintenance, the need for planting land, and the requirement for routine care, similar to those of production crops (Daniel et al., 2012). As the number of test samples increases, more resources, a longer cultivation time, and more personnel are required for maintenance. Consequently, there is a need for a rapid, reliable, and highly accurate method that provides results comparable to the GOT, such as the approach using molecular markers (Daniel et al., 2012; Kovincic et al., 2023). Molecular markers were useful indicators to test the genetic purity of hybrid seeds. There is a wide variety of molecular markers, including Single Nucleotide Polymorphisms (SNPs), Random Amplified Polymorphism DNA (RAPD), and Simple Sequence Repeats (SSRs) (Kumar et al., 2022). SSR markers are short, tandem-repeated DNA sequence motifs that are dispersed across several chromosomes' loci in the maize plant. The robustness of SSR markers is attributed to a more stable nature of tests thus not affected by environmental conditions. Compared to RAPD markers, SSRs are relatively stable against variations in PCR conditions and machine settings. Furthermore, compared to SNPs, the collection and application of SSR markers are generally more cost-effective as they do not require Whole Genome Sequencing (Hasan et al., 2021).

Additionally, SSR markers are co-dominant, allowing the unique band patterns of both the paternal and maternal parents to be simultaneously visible in the hybrid plant DNA sample. SSR markers have been widely applied in various plant genetic studies, including genetic diversity analysis, variety and cultivar identification, genetic mapping, evolutionary studies, and seed purity testing (Abhijit et al., 2022). Henceforth, using SSR markers allows detection of genetic differences at a much earlier time (Shayanowako et al., 2018). This is in contrast with the phenotypic character differences in the GOT which are only completely observable upon plant's mature stages (Varshney et al., 2005). The stages of SSR-based seed hybridity testing usually involve Polymerase Chain Reaction (PCR) to amplify the maize seed DNA fragments containing the SSR sequences, followed by gel electrophoresis to separate the SSR fragments based on size. Differences in SSR sequence size among individuals/varieties result in DNA fragments of different sizes. This difference allows the SSR method to easily detect unique DNA patterns for each variety during gel electrophoresis analysis. In previous research, SSR markers have been successfully applied for genetic purity detection (Daniel et al., 2012) and the detection of several important traits in maize plants (Bocianowski et al., 2021). Therefore, the very first requirement to fulfil is to provide good quality and quantity of DNA extracted for PCR SSR template.

This research aims to compare 3 methods of DNA extraction: 1.) SDS-based, 2.) CTAB-based, and 3.) Kit-based DNA extraction. The DNA subsequently subjected to PCR using SSR markers to differentiate male parent, female parent, and F1 hybrid SSRs. SSR-based seed hybridity testing offers significant advantages, including a relatively short analysis time and independence from environmental growing conditions, allowing flexible implementation in various locations with adequate laboratory facilities. The integration of SSR-based molecular technology into the hybrid maize seed production system is expected to increase the efficiency and effectiveness of seed quality control, which will ultimately contribute to improving the quality and competitiveness of seeds in the market and contribute to national food security.

2. Materials and Methods

Plant Materials

Plant materials obtained from a local seed company (PT Jafran Indonesia) encompassed three varieties: male, female, and hybrid seeds. Plants were cultivated in fields in Politeknik Negeri Jember and maintained to mature stage.

Primer Design

Primer designs were primarily obtained from (Elci and Hancer, (2015) and Kovincic et al., (2023)) and compared to available online maize genome databases (maizeGDB: https://maizegdb.org). The target for selected primers was ability to work specifically on genomic DNA template and avoid the cDNA template. The selected primers were further sent to primer blast analysis (https://ncbi.nlm.nih.gov/tools/primer-blast/) to estimate PCR products and potential off-target products. Following the completion, primer designs were sent into primer synthesizing company (PT Widya Life Science, Indonesia) to produce the designed primer sets.

DNA Extraction and PCR analysis

Leaves samples from 5 days after planting (DAP) plants were freshly sampled using 70% alcohol-sterilized blades to obtain about 0.1 g leaves tissue. The sampled tissues were subsequently placed into sterile 2 ml microtubes containing sterile steel beads. Afterwards, leaves tissues were plung into liquid nitrogen and ground into fine powder using vortex 3000 rpm for at least 2 minutes. Following the grinding completion, 3 methods of DNA extraction were utilized: CTAB-based (Doyle et al., 1990), SDS-based, and Commercial Kit (FastPure DNA Extraction Kit-Vazyme). Subsequently, PCR analysis was performed using standard protocol of One-PCR™ kit (GeneDirex) and according to the melting temperature (Tm) of designed primers. PCR product then being subjected to electrophoresis and visualized in a UV-transilluminator. The visualized band pattern of DNA was captured and compared to the 1 kb DNA marker (GeneDirex).

3. Results and Discussion

Primer List

The successful application of SSR markers in this study was facilitated by careful consideration of primer design principles that are essential for reliable amplification across different DNA extraction methods. The primer designs originated from list of primer from (Elci and Hancer, 2015 and Kovincic et al., 2023). All designed primers were provided in Table 1. Effective primer design for maize SSR analysis requires attention to several critical factors such as primer length, melting temperature, and GC% that directly influence the quality and reproducibility of molecular marker results. The current design has 15-23 base of primer length, melting temperature of 42 – 68 °C, GC % content 31.6-60% which come into the standard range of primer design.

Table 1. List of primer used in this study

Chromosome Number	SSR Motifs	Primer	
		A	T
1	(ACG)4	AAGTGTTGGCAGAAAGCAA	CTCCCCTGTACATGAATTA
2	(CA)19	CATACAAAGCTCACA	AAACCGTTTCCACAA
4	(GGC)4	TCGAGGGGATGTACAAG	ACTGGCACAGAGACG
6	(TC)7	ACCAAGAGTGCAGCAAGAG	TCTGGAATTCTCCTCTGTT
7	(GTCTTTG)4	CATCACTGGTAAGAGCATGGC	GACACACCCATACTTCCAACAAG
8	(TA)8	TCTCCTTGCTGACCAGTTT	CAAATTAAAGCAAAGGCAG

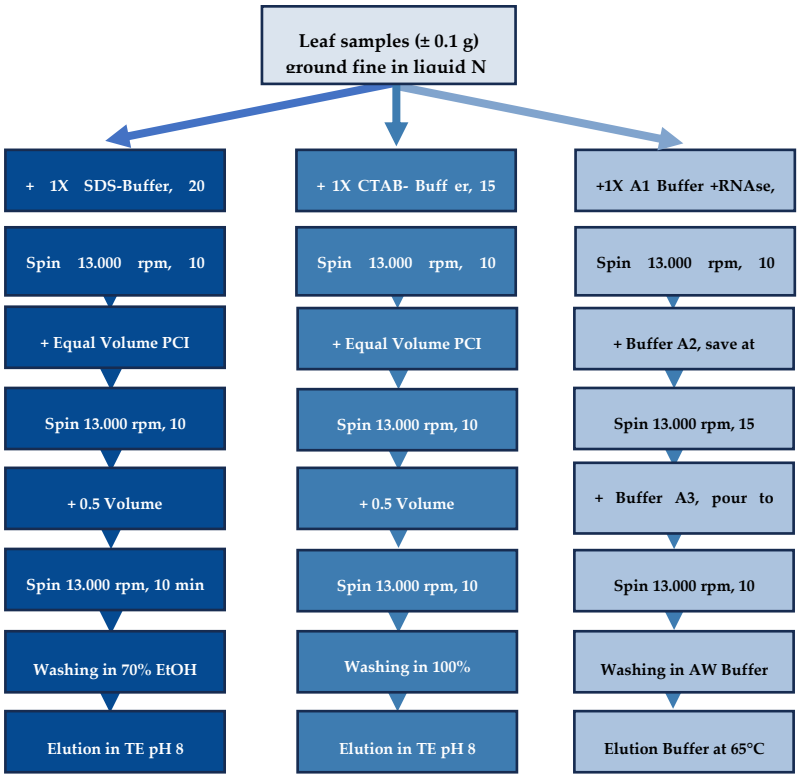
Notes: Primers were obtained from the study of (Elci and Hancer, 2015 and Kovincic et al., 2023) with few modifications. The designed primers were then verified using maize genome database (maizeGDB) (https://maizegdb.org) and blast in primer blast https://ncbi.nih.gov/primerblast).

Optimization of 3 DNA Extraction Methods

The choice of DNA extraction method has direct implications for primer design and PCR optimization strategies. Different extraction protocols yield DNA with varying degrees of purity and quantity, all of which affect the success of SSR amplification (Wanere et al., 2023, Sabriu-Haxhijaha et al., 2020). Studies comparing CTAB, SDS, and

commercial kit extractions have demonstrated that DNA quality parameters, particularly A260/280 ratios approaching 1.8-2.0, correlate strongly with reliable PCR performance (Wanere et al., 2023, Abdel-Latif et al., 2017). When DNA extracts contain inhibitors such as phenolics, polysaccharides, or other secondary metabolites, modified PCR conditions become critical for successful amplification (Doyle et al., 1990, Barra et al., 2012, Elec et al., 2022). We proposed and executed three protocols for DNA extraction based on SDS, CTAB, and Commercial Kit (Figure 1). Overall, the three extraction protocols yielded quantity (114.55 - 1735.05 ng/ul) and quality (A260/A280 1.8-2.0, and A260/A230 > 2.0) of the extracted DNA (Table 2). Additionally, the successful amplification of SSR markers using DNA extracted by all three methods indicates that each protocol yielded DNA with sufficient purity and integrity for downstream PCR applications. This observation aligns with a report demonstrated that both CTAB and commercial kit extractions produce DNA suitable for SSR genotyping and clear variety discrimination (Tan et al., 2013; Nurul et al., 2024). The CTAB method, while traditionally considered the gold standard for plant DNA extraction, showed comparable performance to the commercial kit, supporting previous reports that highlight CTAB's robust removal of polysaccharides and phenolics when properly modified with additives such as polyvinylpyrrolidone (PVP) (Karaca and Ince, 2018). The SDS-based method, though less commonly reported for maize SSR applications, demonstrated adequate performance in our study. This finding extends previous work showing that optimized SDS protocols can produce high-purity DNA with good A260/280 ratios and successful PCR amplification from plant tissues (Sabriu-Haxhijaha et al., 2020). However, it should be noted that most published SDS optimizations have been conducted on other plant species (Shaaban et al., 2022; Tan et al., 2013), and our study provides valuable validation for maize tissue specifically.

Suming up all extraction processes, each extraction method presents distinct advantages and limitations. The CTAB method offers the most cost-effective solution for small-scale projects, with inexpensive reagents and reliable DNA production, though it requires more hands-on time and occasional cleanup steps (Tan et al., 2013; Habibi et al., 2022). This makes it particularly suitable for research laboratories with limited budgets but adequate technical expertise. Commercial kits, while more expensive per sample, provide significant advantages in terms of speed, reproducibility, and workflow standardization. These characteristics make them particularly valuable for high-throughput applications or routine service laboratories where consistency and time efficiency are prioritized over cost considerations (Shinde et al., 2021; Daniel et al., 2012). The magnetic-bead or spin-column technologies employed in many commercial kits can deliver high concentration and purity while being more workflow-friendly for processing multiple samples. The SDS-based method represents a middle ground, potentially offering cost savings once optimized, though protocol development time and validation requirements may offset initial cost advantages (Xiao et al., 2021). Our successful implementation of SDS extraction for maize SSR analysis adds to the limited literature on this approach for cereal crops.



Notes: Comparison of DNA extraction methods using SDS, CTAB, Kit Methods (Vazyme Fastpure DNA Extraction).

Figure 1. Simplified Schematic Flowchart of 3 DNA Extraction Methods

Table 2. Concentration and purity of DNA extracted using 3 methods SDS, CTAB, and Kit Methods (Vazyme Fastpure DNA Extraction)

Treatment	Conc. (ng/ul)	A260/A280	A260/A230
SDS	1735.05	1.9815	2.466
CTAB	149.65	1.899	2.482
Kit	114.55	1.886	2.2635

PCR Analysis using SSR markers

The ability of all three extraction methods to produce DNA suitable for SSR amplification directly supports their utility in hybrid purity testing and genetic relationship analysis. This is particularly significant given the critical role of DNA quality in molecular marker-based applications. Previous studies have demonstrated that CTAB-extracted DNA enables clear SSR genotyping in maize, with reproducible banding patterns suitable for genetic diversity analysis and variety discrimination (Kovincic et al., 2023; Xihai et al., 2010). Our current study reveals that all methods of DNA extraction were capable of produced DNA extracts from maize seedlings that are adequate both quantitatively and qualitatively for PCR amplification. Figure 2 showed that all DNA extraction methods work well in amplification of *rbcL* gene, a standard constitutive gene for verifying normal PCR works.

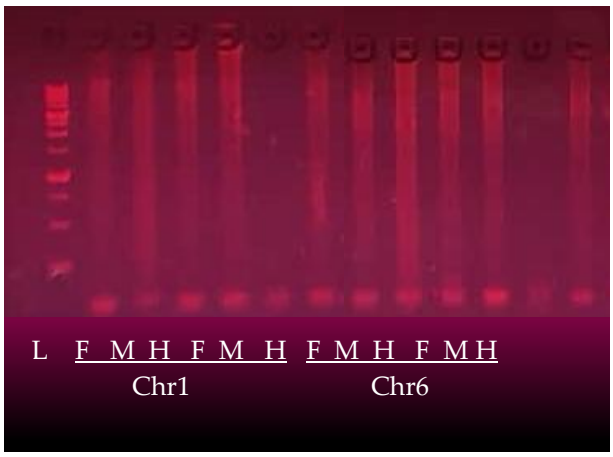
Similarly, commercial kit-extracted DNA has been successfully employed in waxy corn studies, where SSR markers unambiguously distinguished hybrids from parents and confirmed hybrid purity (Kumar et al., 2022). The consistency of SSR amplification across all three extraction methods in our study suggests that the choice of extraction protocol should not compromise the reliability of downstream applications. The successful performance of all three extraction methods has important implications for the implementation of molecular marker-based hybrid purity testing in maize breeding programs. Traditional grow-out tests (GOT), while considered the gold standard, are

time-consuming, resource-intensive, and require extensive field space and maintenance (Hasan et al., 2021; Abhijit et al, 2022). The validation of multiple DNA extraction approaches provides flexibility for different laboratory settings and operational scales. For large-scale commercial breeding programs, commercial kits may offer the best balance of throughput and consistency, despite higher per-sample costs. Research institutions with budget constraints may find CTAB extraction more suitable, particularly when processing moderate numbers of samples. The SDS method could serve as an alternative approach for laboratories seeking a balance between cost and efficiency. The successful application of SSR markers using DNA from all extraction methods also validates the reliability of simple sequence repeat technology for maize genetic analysis, consistent with previous reports highlighting the effectiveness of SSR markers for genetic diversity assessment and population structure analysis in maize (Varshney et al., 2005; Bocianowski et al., 2021).

While our study demonstrates the effectiveness of all three extraction methods, several considerations require attention for future applications. The optimization of extraction protocols may vary depending on the specific maize tissues, the developmental stage of the plant material, and the storage conditions of samples prior to extraction. Additionally, the specific SSR markers employed and the complexity of the genetic analysis may influence the minimum DNA quality requirements.

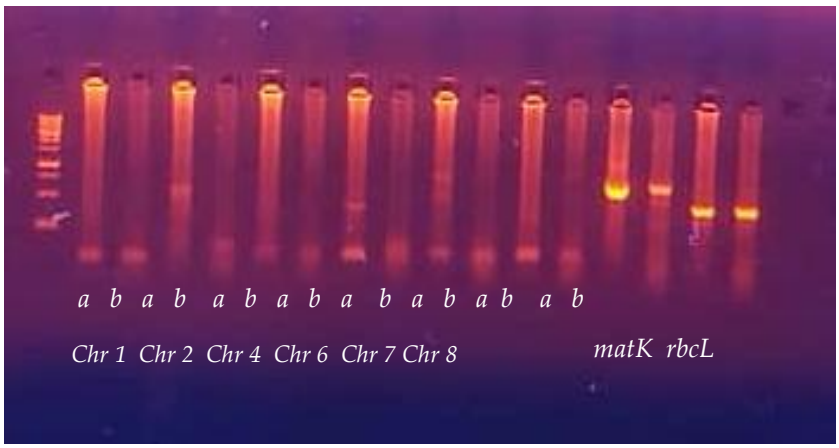
Notes: Three sources of DNA extracted from SDS-based, CTAB-based, and Kit-based methods, respectively. All DNA extraction result similar size of PCR band pattern.

Figure 2. Comparison of PCR results using standard constitutive gene *rbcL*



Notes: Primer chromosomes 1 and 6. M, F, and H were male parent, female parent, and hybrid of maize varieties from PT Jafran Indonesia, respectively. L is 1 kb DNA ladder (GeneDirex) for confirming the size of DNA.

Figure 3. Electrophoresis of PCR product using designed SSR primers



Notes: Primer chromosomes 1, 2, 4, 6, 7, and 8. matK and rbcL were internal control from *maturase K* and *rubisco large subunit*. A, B, M, F, and H were maize varieties from PT Jafran Indonesia. L is 1 kb DNA ladder (GeneDirex) for confirming the size of DNA.

Figure 4. Electrophoresis of PCR product using designed SSR primers

4. Conclusions

The current study delivers a short communication of DNA extraction methods to provide template for SSR-based seed hybridity test. Although the current study did not examine all positions within maize chromosomes throughout genomic DNA of maize, our reports suggest that all 3 methods were able to be utilized to distinguish one maize varieties to others. This study provides compelling evidence that multiple DNA extraction approaches—CTAB-based, SDS-based, and commercial kit methods—are equally suitable for SSR-based molecular marker analysis in maize. The successful amplification and analysis of SSR markers using DNA from all three extraction protocols validates their utility for hybrid purity testing and genetic relationship studies. This flexibility in methodological approaches enhances the accessibility of molecular marker technology for maize breeding programs with varying resource constraints and operational requirements. The findings support the broader adoption of molecular markers as a reliable, efficient alternative to traditional field-based methods for hybrid purity verification. By demonstrating that multiple extraction protocols can produce DNA of adequate quality for SSR analysis, this study removes a potential methodological barrier to the implementation of molecular marker-based approaches in maize breeding and quality control programs.

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