

## POLYACRYLAMIDE GEL ELECTROPHORESIS: A FASTER, EFFICIENT AND AFFORDABLE METHOD FOR DETECTING SSR MARKER POLYMORPHISM IN CASTOR

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### ABSTRACT

Within a decade, many microsatellite or Simple Sequence Repeats (SSR) markers have been developed for major crops. These markers have been predicted to lead to greater innovations in breeding programs and genetic markers development. However, in situations where there is lower variability among markers, genotyping becomes a hurdle. In an experiment to screen for polymorphic primers in castor (*Ricinus communis*), two methods were employed; 3-5% Agarose gel electrophoresis run at 100-120V for 90 to 120 minutes with Ethidium Bromide staining and 6% Non-denaturing Polyacrylamide gel electrophoresis run at 110V for 90 minutes and silver stained. Gel fingerprints of agarose gel showed unseparated bands, with a lower throughput while gel fingerprints of polyacrylamide gel, showed clear differences between bands even in the lower base pairs with a higher throughput. Polyacrylamide gel electrophoresis proved to give a clearer distinction between bands and separated the bands clearly at a relatively quicker time frame. Health concerns about the carcinogenicity of the bromide stain in agarose gels were also avoided since polyacrylamide gels are silver stained. Polyacrylamide gel electrophoresis can be therefore utilized in further experiments in identifying SSR products with relative ease, lower cost and quicker duration devoid of health concerns.

**Key words:** Castor (*Ricinus communis*); Agarose gel; Polyacrylamide gel; SSR markers.

### INTRODUCTION

Castor (*Ricinus communis*) is a crop widely grown in India, China, and Brazil predominantly for its oil. Although initially thought to have originated from Ethiopia in East Africa, castor is widespread throughout the world (Bajay *et al.*, 2011; Govaerts *et al.*, 2000). The widespread nature is due to the castor plant's deep rooting system making it resistant to drought. Castor is not cold hardy making it susceptible to frost but survives in tropical, sub-tropical and temperate climates. A moderate temperature from 20-26 °C is favorable during the crop period to obtain a higher yield. Castor oil contains ricinoleic acid which confers its unique qualities (Beltrão, 2003). Due to this, castor is applied in the pharmaceutical, cosmetic, aviation and paint industries (Bajay *et al.*, 2009; Jeong and Park 2009). Nonetheless, the castor seed contains ricin, a highly toxic protein capable of killing by ingesting just one seed (Zimmerman *et al.*, 1958). However, the protein is insoluble in the oil and research scientist are working on technologies to reduce this protein content in castor.

Since the introduction of marker-assisted selection (MAS), many markers have been used in selecting crops with the desired agro-morphological traits. Microsatellite markers or Simple Sequence Repeats (SSRs) have been extensively used due to their high polymorphism, locus-specificity and abundance. For

SSRs analysis, only small amounts of DNA are needed and SSRs are widespread in the genome (Maniruzzaman *et al.*, 2014). SSRs have been developed in many crops, including castor. Molecular genetic maps of crop species are not only for breeding purposes but are also used in genomic research. SSRs have been successfully used in studying genetic diversity (Korzun *et al.*, 2001), genome mapping (Korzun *et al.*, 1997; Liu *et al.*, 2016; Russell *et al.*, 1997) and genotype differentiation (Virk *et al.*, 1999). The advantages of SSR markers in genetic studies exceeds other markers.

Despite the suitability of SSR markers, an efficient methodology is required for mass genotyping (Mitchell *et al.*, 1997). Agarose or polyacrylamide gel electrophoresis have been mostly used for the separation of SSR PCR products. Agarose gels are visualized under UV light after being stained with ethidium bromide while polyacrylamide gels are visualized by silver staining. Despite the efficiency of radioactive or fluorescent labeled nucleotides in visualizing single stranded DNA in polyacrylamide and agarose gels (Kumar *et al.*, 2015), they are not cost-effective, time-consuming and need special facilities which make it impracticable for most tropical countries without proper infrastructure (Lagoda *et al.*, 1998). Compared to other methods used in staining DNA fragments, silver staining in polyacrylamide gels is much sensitive (Bassam *et al.*, 1991; Rabilloud, 1990) hence its wide usage in detecting DNA fragments like

SSR markers (Liang *et al.*, 2014). However, in the last decade, the silver staining methods described have been criticized because it is time-consuming and requires the continuous changing of solutions unlike the agarose gel (Meril *et al.*, 1981). Thus, an efficient method and less cumbersome method is needed. Nonetheless, polyacrylamide gels produce higher resolution gel images and make genotyping much easier. Gel fingerprints of agarose gels, on the other hand, have been reported to have lower resolutions and separations between bands of smaller sizes much difficult.

Between the two main methods used in genotyping SSR products, continuous enhancements are being made to polyacrylamide and agarose gel procedures. However, to date, no ideal genotyping method has been identified in genotyping SSR products, taking into account the sizes of PCR products. Therefore this experiment was designed to visualize SSR products of castor using enhanced or modified polyacrylamide gel electrophoresis (PAGE) and agarose gel electrophoresis (AGE) methods and determine the ideal among the two with respect to resolution, specificity, clarity and cost.

## MATERIALS AND METHODS

**Plant Materials:** Castor plants used for the experiment were provided by the tropical crop breeding and genetics laboratory at the College of Agricultural Sciences in Guangdong Ocean University.

**SSR Primers:** Already existing primers designed by our lab using the genomic DNA Sequence released by the

institute for genomic research (TIGR) (<http://castorbean.jcvi.org/downloads.php>), which contains 25, 828 assemblies of castor genome. The designed primers were synthesized by Sangon Biotech and were used for the experiment (Table 1).

### Extraction of Genomic DNA from Castor and PCR:

The Genomic DNA was extracted from fresh leaves using the CTAB method (Cullings, 1992) and DNA was quantified by spectrophotometer (Simplinano) by reading at 260 nm absorbance. The absorbance ratio of 260nm/280nm was also read to assess the purity of the DNA. The standard of pure DNA was from 1.7 – 2.0 and all not within this range were extracted de novo. 1µl of template DNA mixed with 5µl of 2X loading buffer, standard DNA of known size were loaded on a 1% agarose gel and were electrophoresed at 80-100V for 30min. The quantity and purity of the DNA were analyzed based on the brightness, clarity and the presence of smears on the DNA bands.

The PCR reaction system was 20µl consisting of 1µl of genomic DNA, 8µl sterile ddH<sub>2</sub>O, 0.5µl for each pair of primers and 10µl of 2xTaq PCR Starmix (Genstar). The PCR program consisted of initial denaturation at 94°C for 5mins followed by 35 cycles of amplification [94°C of denaturation (30 secs), 55-60°C of annealing (45 secs) and extension for 72°C (45 secs)] and final extension at 72°C for 5mins.

**Table 1. SSR Primer sequences and their annealing temperatures.**

PRIMER NO.	FORWARD PRIMER (5'-3')	REVERSE PRIMER (5'-3')	T <sub>m</sub> (°C)
RCM703	TGGGGCAATGGTTACCTG	CACCCAAATGCAGGCTTC	60.20
RCM704	CAAGAAGGCCAACTGTCCA	TGAGCATATGCCTTGCCCTT	59.39
RCM705	CGGTGCGATGCAATGATA	TGAATTTCTCGCCAAACA	60.18
RCM711	ATGGAGCAATTTGGGCAG	CCCATCCAGGAATGCTGT	59.42
RCM835	TTCTGACGCCAACAAGAGG	CGATCCTTTTCGGGTGTG	60.04
RCM836	TGGCATGCCCTGTAAGAT	CGTCACGTTTGTATTCATTTTCG	60.40
RCM953	GGTGACACCCATGTGCCT	TTTTTGGGTTGACAGTGGC	59.53
RCM1393	TTGGCCAAACGGGTAAAAT	TTGGTTGTTTGAATTCCTGA	59.09
RCM1554	TAGGGATTAAGGCGCAAGC	CAAACCACAAGCAATCCAAA	59.56
RCM1574	AAAACCTTTCATTTCAATCAGACAAA	CAGAGTTGCTGCTATGTCCC	58.47

Agarose Gel Electrophoresis: 4% Agarose gel electrophoresis was prepared using 4g of Agarose mixed into precooled 0.5x TBE buffer.

1. The solution was microwaved for the solution to dissolve completely leaving behind no agarose.
2. 5µl of Ethidium Bromide (EB) was added to the solution, and the solution was poured into the casting tray, and the comb was fixed to form the wells.
3. The gel was left to dry for about over 30 minutes. After the gel was dry, it was placed in 4°C refrigerator for about 20 minutes.
4. After 20 minutes, 4µl of PCR products were loaded in the wells while the gel was on ice.
5. The loaded gel was placed in the electrophoresis apparatus, and electrophoresis was run at 100-120V for 90-120 minutes. (Note: ice packs were placed on

the electrophoresis apparatus to avoid heating and denaturation of PCR products).

Silver staining as described by (Huang *et al.*, 2018) was adopted for our experiment. A 6% non-denaturing acrylamide gel solution was prepared using 30% Acrylamide solution [(29:1)Acrylamide: Bisacrylamide], 10ml of 5×TBE, 30ml of distilled water, 250µl of 10% ammonium persulfate and 20µl TEMED. Acrylamide gel solution was poured into glass plates (with notched glasses cleaned with repel silane and glass with spaces cleaned with bind silane). Clamped glass plates were left for thirty minutes for the gel to polymerize. Glass plates were placed in electrophoresis chamber with all buffer solutions poured inside over the gels. Electrophoresis was done at a constant voltage of 110V for 70 – 90 mins. Glass plates were removed after electrophoresis, notched glass was taken off and the gel facing upwards was placed in silver nitrate solution for silver staining.

## RESULTS AND DISCUSSION

**Extraction of Genomic DNA from Castor:** DNA of the castor plants extracted were in good quality showing bright and clear bands with no smears (Figure 1). Gel images of the SSR products after being loaded in agarose gels were electrophoresed for 90 to over 120 minutes (Figures 2 and 3). In figure 2, the electrophoresis apparatus was placed on ice due to the longer duration taken during electrophoresis (over 120 minutes) whilst the electrophoresis of SSR products in figure 3 lasted for 90 minutes with no ice. A clear difference is observed between Figures 2 and 3. Bands in Figure 2, although unclear and diffused look better than bands in figure 3.

On the contrary, gel images of SSR products viewed on the silver stained 6% non-denaturing polyacrylamide are shown in Figures 4, 5 and 6. The polyacrylamide gel electrophoresis lasted for 90 minutes with the staining period lasting for about 10 minutes. Gel fingerprints are specific and clear, revealing polymorphism in some markers (RCM 704 and RCM 705).

In this experiment, SSR markers were used to identify polymorphism between castor plants. In order to identify polymorphism, the SSR products have to be clearly visualized. PAGE and AGE methods have been

used throughout the years for visualizing PCR products (Barril and Nates, 2012; Wang *et al.*, 2009). Hence, these two were compared to identify the method with high specificity, resolution, clarity, cost and time. The silver staining process of PAGE which used to last for about 30 minutes in the past now takes about 10 minutes due to the introduction of new technologies (An *et al.*, 2009; Bassam *et al.*, 1991; Byun *et al.*, 2009; Ji *et al.*, 2007; Kumar *et al.*, 2015; Liang *et al.*, 2014; Liu *et al.*, 2017; Sanguinetti *et al.*, 1994; Qu *et al.*, 2005). In this experiment, PAGE lasted for about 130 minutes whilst AGE lasted for over 150 minutes, about 20 minutes more than PAGE.

Despite taking longer than PAGE, AGE gel bands cannot distinguish between smaller size bands, but have lower resolution and diffused bands as shown in Figures 2 and 3 (Stellwagen, 1998). SSR markers are specially known for having smaller sizes and some of these smaller sized bands are the polymorphic bands. The ability to clearly differentiate between these sizes leads to better analysis. As shown in figures 4 to 6, PAGE bands are clear, showing enough information about the markers. This work confirms Kumar *et al.*, 2018, who identified PAGE with the best resolution of SSR products of *Aloe vera* plants, after comparing the PAGE to AGE.

The silver stain method, employed in the PAGE is seen as a highly sensitive method of staining with no known health complications so far while many reservations have been made on the use of ethidium bromide as a stain in agarose gels. Ethidium bromide is a carcinogenic agent whose use has been widely discouraged (Singer *et al.*, 1999). In spite of the relative cheapness of reagents used in preparing PAGE gels (Wang *et al.*, 2003), the sensitivity of the silver stain is an added advantage, which does not require expensive machines like ultraviolet light chamber, molecular imager and gel documentation systems in viewing end products (Kumar, 2018). PAGE images can be viewed with the naked eye using light boxes and can be captured clearly using cameras or sometimes phones whilst ethidium bromide stained agarose gels have lower sensitivity and thus require sophisticated machines for visualization (Fountain *et al.*, 2011). This is an added cost, despite the other expenses.

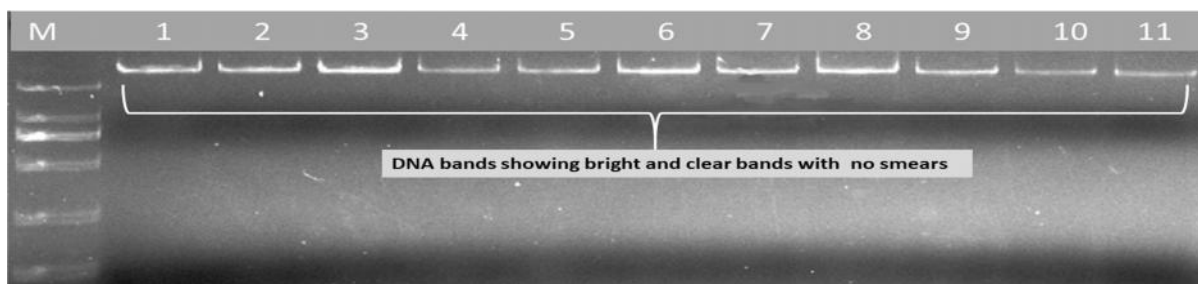


Figure 1. 1% agarose gel showing DNA fingerprints. (M: Standard marker, Lanes 1-11: randomly selected DNA)

Agarose Gel Electrophoresis

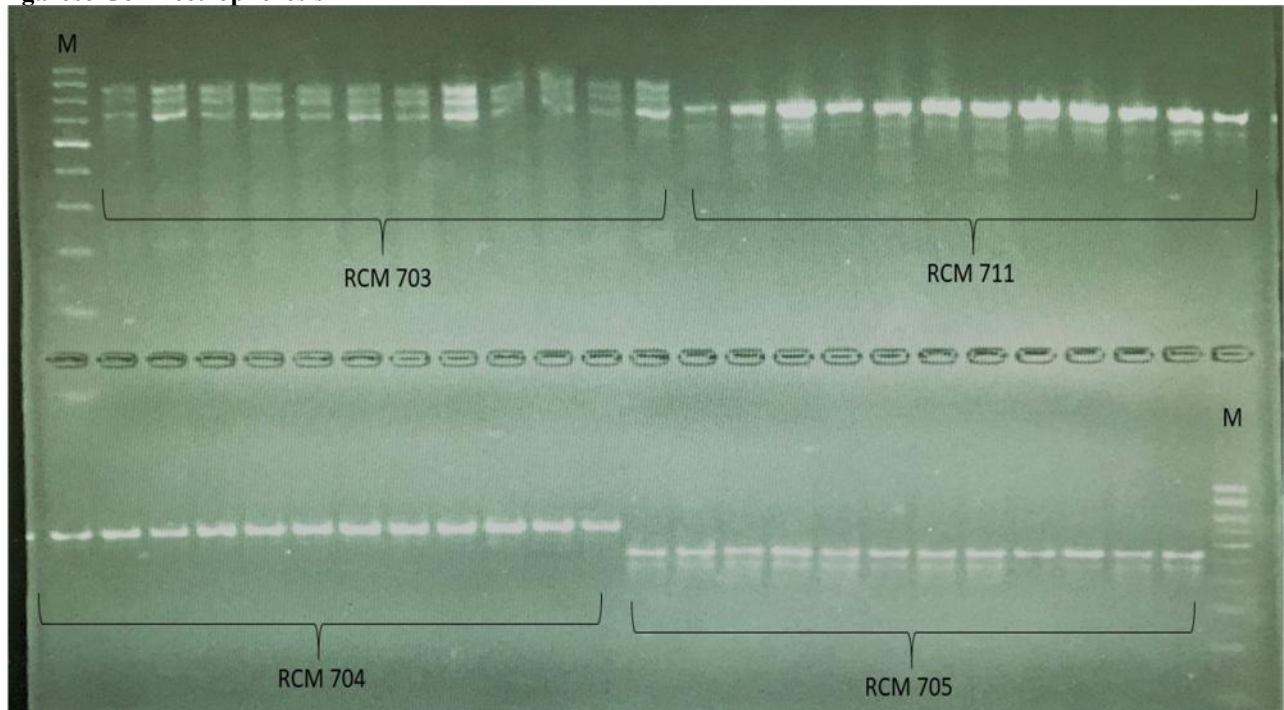


Figure 2. Amplified SSR images on a 4% agarose gel. (M: Standard DNA Marker; RCM 703, RCM 711, RCM 704, RCM 705: SSR markers).

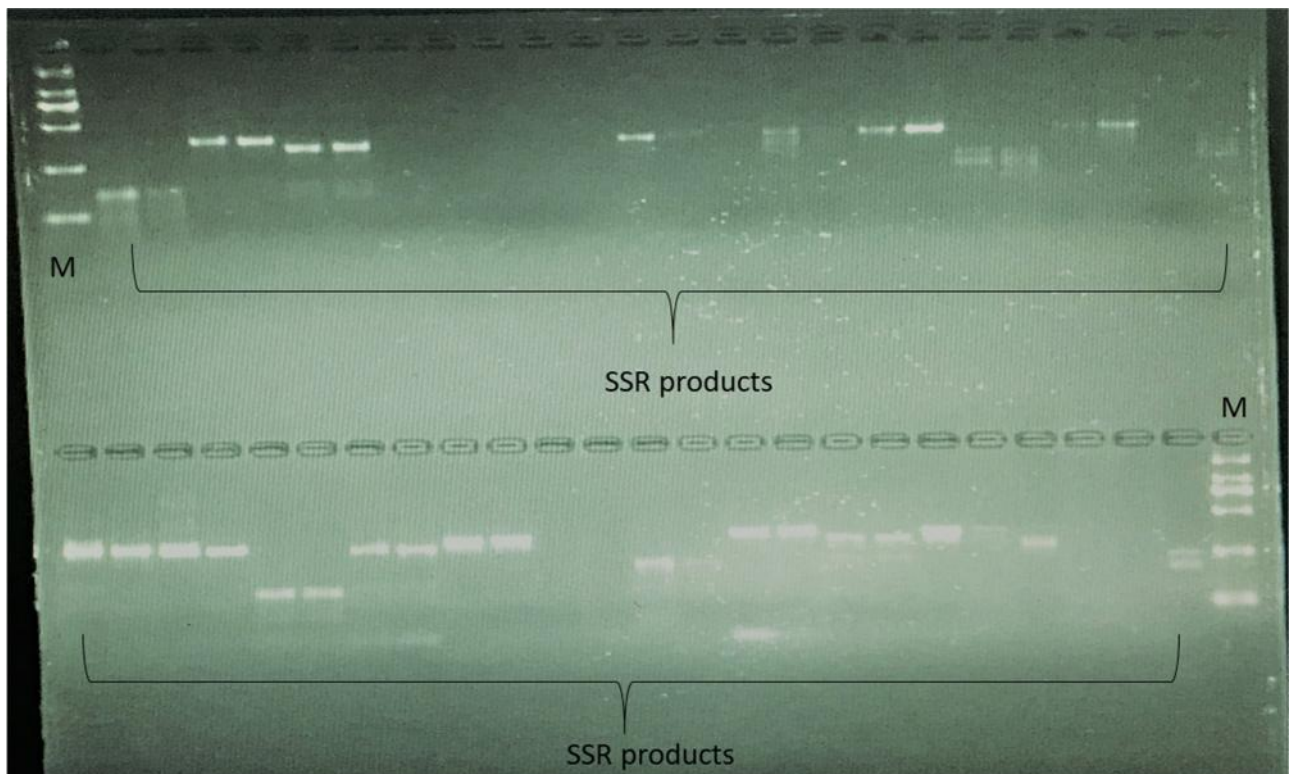
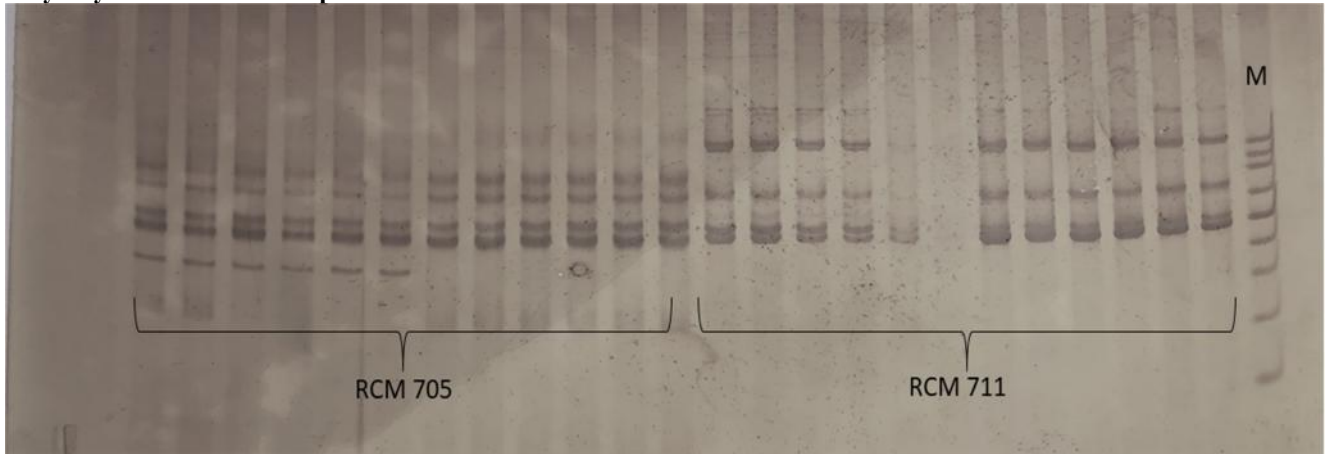
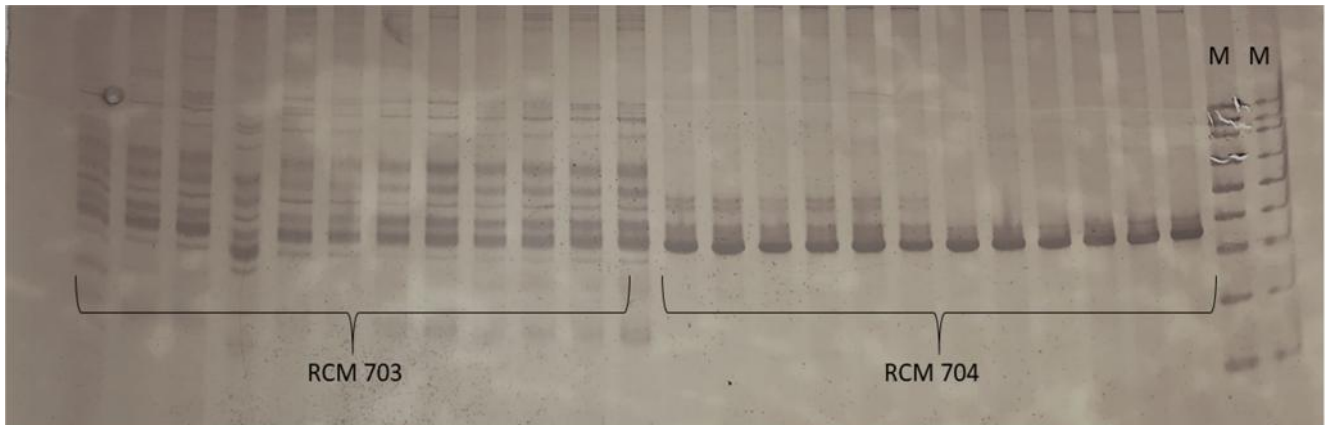


Figure 3. Amplified SSR products on a 4% agarose gel.

**Polyacrylamide Gel Electrophoresis**



**Figure 4. Amplified SSR products on a silver-stained Polyacrylamide gel. (M: Standard DNA marker; RCM 705, RCM 711: SSR markers)**



**Figure 5. Amplified SSR profiles on silver-stained polyacrylamide gel (M: Standard DNA marker; RCM 703, RCM 704: SSR Markers)**



**Figure 6. Amplified SSR products on silver-stained polyacrylamide gel. (M: Standard DNA marker).**

SSR markers are great tools in molecular genetics and the ability to view clearly and analyze their PCR products is essential. From this experiment, it has been proven that the PAGE method is fast and cost effective and produces

bands with higher resolution and clarity, and thus can be useful in genotyping SSR markers.

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