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Research article

PCR Optimization Prior to Genetic Diversity Assessment of Sesame (*Sesamum indicum L.*) Genotypes Using Inter-Primer Binding Site (IPBS) Markers

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Abstract

This study aimed to accurately and reliably determine the genetic diversity among sesame (*Sesamum indicum L.*) genotypes, which is an important oil crop. To achieve this, Polymerase Chain Reaction (PCR) conditions based on Inter-Primer Binding Site (IPBS) molecular markers were optimized to reveal genetic variation, which forms the basis of plant breeding programs. For the methodology, DNA was isolated from fresh sesame leaves grown under controlled conditions using the CTAB method and analyzed from 50 local sesame lines. Since PCR success is directly dependent on the specificity of the primers and reaction parameters like temperature, the gradient temperature PCR method was applied using 22 different iPBS primers to determine the optimal annealing temperatures. According to the findings, 20 out of the 22 primers successfully generated polymorphic bands, revealing genetic diversity. Determining the optimal PCR conditions was critical for identifying the binding temperature at which iPBS primers exhibited the highest polymorphism. For example, Primer 2277 showed high amplification and activity at 47.6 and 50.9°C, while Primer 2218 was highly active at 50.9°C. This optimization establishes a precise molecular foundation that will contribute to future sesame breeding programs.

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Keywords:
local sesame lines, IPBS, optimal annealing temperatures, molecular marker .

I. Introduction

Sesame (*Sesamum indicu*m L.) is an important oil crop worldwide, with seeds containing high amounts of oil and protein (Saxena and Bisen, 2017). Its tolerance to drought and other abiotic stresses increases sesame's potential for cultivation in marginal lands (Lakhanpaul *et al*., 2012). The use of sesame plants, particularly local lines, in breeding programs and the genetic diversity they offer is important. Identifying genetic diversity, which forms the basis of plant breeding programs, enables the development of new varieties with superior traits. Molecular markers, one of the powerful tools used for this purpose, play an important role in assessing genetic diversity.

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Inter-Primer Binding Site (IPBS) markers, which are molecular markers, are linked to retrotransposon regions to reveal genetic variation (Özer *et al*., 2017). High levels of polymorphism (genetic diversity) can be detected because IPBS markers target recurrent DNA sequences in the plant genome. Polymerase chain reaction (PCR) is a fundamental molecular biology method for amplifying specific DNA regions (Al-Hadeithi and Jasim, 2021). The specificity of the primers used and the reaction parameters, particularly temperature, directly affect PCR success. Therefore, PCR optimization is a critical step to ensure the accurate and efficient amplification of target DNA regions.

Molecular markers are one of the powerful tools used for this purpose and play an important role in assessing genetic diversity. Inter-Primer Binding Site (IPBS) markers, which are molecular markers associated with retrotransposon regions and reveal genetic variation, enable the detection of high levels of polymorphism (genetic diversity) because they target repetitive DNA sequences in the plant genome.

The aim of this study was to determine the ideal annealing temperatures for various iPBS primers and to optimize PCR analysis based on iPBS markers in sesame genotypes. The resulting optimization is expected to form a basis for a precise and reliable assessment of the genetic diversity of sesame genotypes. The resulting optimization is expected to form a basis for the precise and reliable assessment of the genetic diversity of sesame genotypes.

Material and Methods

In this study, DNA samples from 50 different local sesame lines were used for temperature detection. For this study, fresh leaves of sesame were used in a plant growth chamber under controlled conditions.

DNA Isolation

Freshly harvested 3-5 leaves (100-150 mg) from plants grown in a growth chamber were ground into powder using liquid nitrogen. DNA extraction was performed according to the CTAB method developed by Doyle and Doyle (1990).

The sample was mixed with CTAB buffer (2% CTAB) 20 g CTAB/L, 20 mM EDTA, 100 mM Tris-HCl pH 8.0, 1.4 M NaCl, 0.2% Mercaptoethanol) and was vortexed after adding one unit of proteinase K to each sample. The samples were incubated for 60 min at 60°C; after the incubation, 1 U (20 mg/ml) proteinase K was added to the mixture. Added equal volume of chloroform/isoamylalcohol (24:1), mixed for about 15 min, then the samples were subjected to centrifugation at 16,000 xg for 10 min, and supernatants were transferred to new centrifuge tubes. Precipitated DNA with 2/3 volume of cold isopropanol centrifuged at 10,000 xg for 20 min. Supernatants were discarded, pellets were dried at room temperature and added to 1X TE buffer, and then left overnight at 4°C. The samples were incubated for 3 h at 65°C and 20 µL of RNase A (10 mg/mL) was added and then incubated for 1 h again. The upper phase was transferred to a new tube that contained 100 l of 1.2 M NaCl. After adding 0.6 ml of isopropanol, it was left to stand for 1 hour at -20°C. Samples taken from -20°C were centrifuged at 6000 rpm for 10 minutes at +4°C, and the liquid portion was removed by allowing the DNA to settle at the bottom of the tubes. The pellet was dried and then carefully washed with 500 ml of 70% ethanol without damaging the pellet. The DNA was then dissolved in 100 µl of water.

For the qualitative analysis of DNA samples, a 0.8% agarose gel was prepared using 1X SB buffer. To load the samples into the gel wells, 5 µl of DNA sample, 5 µl of ddH_2O, and 3 µl of 6X loading buffer were mixed to prepare a total mixture of 13 µl. This mixture was carefully placed into the wells formed in the gel. Gel electrophoresis was performed at 70 V for 90 minutes, and after the process was completed, the DNA bands were visualized using a UV device (Vilber Lourmat, France).

The concentrations of the obtained DNA samples were measured using a Quantus Fluorometer (Invitrogen, USA) by taking 2 µl from each sample. The measurements were performed according to the QuantiFluor™ ONE dsDNA System Kit protocol.

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PCR Components and Conditions

Twenty-two iPBS markers were used to determine primer temperatures. These primers were selected because they demonstrated high polymorphism and efficiency in various plant species in previous studies (Özer *et al*., 2017; Erkoç *et al*., 2024). Only 20 of the 22 primers produced amplification products at the appropriate temperature. The PCR procedure was performed using a MultiGene Gradient Thermal Cycler (TC9600-G-230V, Labnet International, Inc.). For the gradient PCR test, reagents were added to 0.2 ml PCR tubes at the ratios shown in Table (1).

Table (1): Mixing ratios and final reaction volume of PCR components.

Component	Amount
10X PCR Buffer	2 µL
10 mM Dntp	2 µL
25 mM MgCl2	0.5 µL
Taq DNA polymerase	0.5 µL
100 pmol primer	1 µL

Table (2): Names and 5'→3' sequences of the 22 different iPBS primers used in gradient PCR analyses.

Primer No	IPBS Primer	Sequence 5-3’
1	2077	CTCACGATGCCA
2	2218	CTCCAGCTCCGATTACCA
3	2226	CGGTGACCTTTGATACCA
4	2228	CATTGGCTCTTGATACCA
5	2230	TCTAGGCGTCTGATACCA
6	2232	AGAGAGGCTCGGATACCA
7	2237	CCCCTACCTGGCGTGCCA
8	2243	AGTCAGGCTCTGTTACCA
9	2244	GGAAGGCTCTGATTACCA
10	2246	ACTAGGCTCTGTATACCA
11	2249	AACCGACCTCTGATACCA
12	2251	GAACAGGCGATGATACCA
13	2252	TCATGGCTCATGATACCA
14	2253	TCGAGGCTCTAGATACCA
15	2272	GGCTCAGATGCCA
16	2277	GGCGATGATACCA
17	2379	TCCAGAGATCCA
18	2380	CAACCTGATCCA
19	2389	ACATCCTTCCCA
20	2393	TACGGTACGCCA
21	2401	AGTTAAGCTTTGATACCA
22	2402	TCTAAGCTCTTGATACCA

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Results

In this study, PCR optimisation based on iPBS markers was performed to determine the genetic diversity of local sesame lines. Twenty of the 22 primers used in the study produced polymorphic bands. The primers generally showed effective amplification within a specific temperature range. Amplification efficiency decreased at temperatures outside this range. Some primers produced strong and distinct bands, while others produced weak or no amplification products. Some primers showed high polymorphism at a specific temperature in both DNA samples. Primer 2277 at 47.6°C and 50.9°C; Primer 2218 at 50.9°C; Primer 2246 at 45°C; Primer 2393 at 52.8°C; and Primer 2251 at 56.5°C showed the highest amplification and activity. Primers 2266, 2244, 2252, 2243, and 2401 showed high activity in the 47-48.3°C range; Primers 2402 and 2077 at 49.4°C; and Primers 2237 and 2249 in the 51.1-51.4°C range. Primers 2379 and 2389, however, yielded weak or no amplification products. Gel images of the amplification products of the primers are presented in Figure (1).

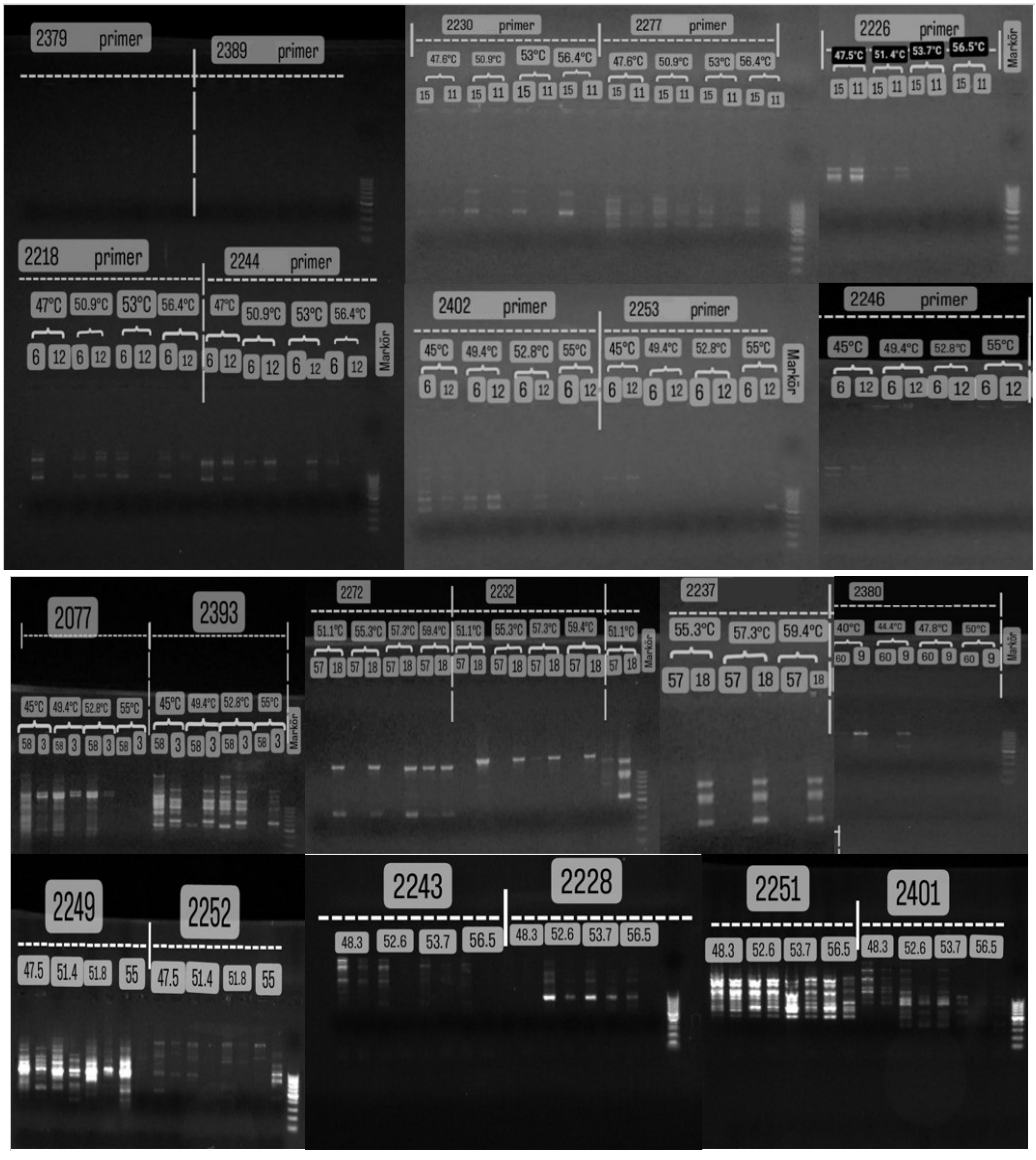

Figure (1): Agarose gel electrophoresis images showing PCR band profiles produced by 22 different iPBS primers at various optimized annealing temperatures, revealing genetic diversity in DNA samples.

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Discussion

The primary objective of this study was to optimize Polymerase Chain Reaction (PCR) conditions based on iPBS molecular markers to accurately and reliably determine the genetic diversity of local sesame (*Sesamum indicum* L.) lines. PCR success is directly dependent on the specificity of the primers used and the reaction parameters, particularly temperature. Therefore, PCR optimization is a critical step to ensure accurate and efficient amplification of target DNA regions. Determining the annealing temperature at which iPBS primers exhibit the highest polymorphism is a crucial step in determining optimal PCR conditions. The results clearly demonstrate that different iPBS primers have different annealing temperatures. This supports the notion that each primer requires a unique optimum temperature to effectively anneal to a specific target region, as emphasized in similar studies such as Al-Obaidi (2015), Erkol *et al*. (2024) and Mahyar and Fatemeh (2021). The fact that 20 of the 22 primers used in our study produced polymorphic bands demonstrates the high efficiency of iPBS markers in sesame genetic studies. This high efficiency stems from the fact that iPBS markers target repetitive retrotransposon regions in the plant genome. Determining the optimal annealing temperatures using the gradient PCR method increased amplification efficiency and specificity. Primer 2277 exhibited the highest activity at 47.6°C and 50.9°C, confirming that the primer binds best to its target at these two temperatures. In contrast, primers 2379 and 2389 yielded weak or no amplification products, suggesting that these primers lack suitable binding sequences for the sesame genome or that their annealing temperatures are still outside the optimal range. This optimization study provides a sensitive and reliable molecular basis for the reliable assessment of sesame genetic diversity and the classification of landraces. This molecular basis will make an important contribution to future sesame breeding programs.

Conclusion

This study demonstrates the effectiveness of PCR analyses based on iPBS markers in determining genetic diversity in sesame genotypes. Determining optimal annealing temperatures using gradient PCR has increased amplification efficiency and specificity. The results obtained indicate that iPBS primers can be used to assess genetic diversity in sesame and classify local lines. This optimization provides an important molecular basis that will contribute to sesame breeding programs.

Author Contributions

S.E. and B.P.: Conceptualization, Methodology, Writing, Formal Analysis, Visualization

F.K., A.T., M.Y.: Formal Analysis, Investigation, Supervision, Writing – Review and Editing.

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Conflicts of Interest / Competing Interests

The authors declare no competing interests.

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