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Optimizing SCoT-PCR Parameters for Reproducible Genetic Diversity Analysis in Cassava, Rice, Maize, and Foxtail Millet

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Abstract

Technical optimization of PCR parameters is essential to ensure efficiency and accuracy in genetic analyses. The performance and reproducibility of start codon targeted (SCoT) markers are strongly influenced by annealing temperature (Ta) and primer concentration. Raising Ta from 50 °C to 55 °C reduced both amplicon yield and reproducibility, whereas higher primer concentrations increased amplicon counts without substantially affecting reproducibility. Optimal amplification was achieved at Ta 50 °C with a primer concentration of 2.5 µM, where most primers produced high amplicon numbers with 100% reproducibility. Evaluation of 36 SCoT primers in foxtail millet identified 20 primers with full reproducibility, of which 10 were further tested across cassava, rice, and maize. Primer reproducibility varied among species, and only SCoT-35 and SCoT-36 consistently achieved 100% reproducibility across all three crops. These results highlight that primer suitability is species-dependent and emphasize the need for extensive primer screening in each plant species. Overall, optimizing Ta and primer concentrations, combined with careful primer selection, is essential for the reliable, efficient, and cost-effective application of SCoT markers in plant genetic diversity analysis.

Keywords: marker informativeness, molecular marker, polymerase chain reaction, protocol, reproducibility

Introduction

Molecular markers are essential in precision plant breeding because they overcome the limitations of phenotypic observations, which are often influenced by environmental conditions (Hudak & Dybdahl, 2023). Molecular markers can be broadly classified by their analytical methods into hybridization-based markers, such as restriction fragment length polymorphism (RFLP); PCR-based markers, which constitute the majority of currently used techniques; and sequence-based markers derived from nucleotide sequencing, such as single-nucleotide polymorphisms (SNPs) (Amiteye, 2021). Ease of application, level of polymorphism, and cost-efficiency are the primary considerations when selecting appropriate markers. Hybridization-based markers are relatively complex and time-consuming, while sequence-based markers require specialized instruments and are generally costly. Consequently, PCR-based markers combined with agarose gel electrophoresis have become the most widely adopted approach (Hasan et al., 2021).

Universal markers that can be applied across diverse plant species and do not require prior genomic sequence information of the target plant—such as random amplified polymorphic DNA (RAPD), inter simple sequence repeat (ISSR), and start codon targeted (SCoT) (Amiteye, 2021)—are particularly advantageous because they can be implemented using standard laboratory equipment. RAPD amplifies random genomic regions, ISSR targets repeats,

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while SCoT markers are designed from conserved regions flanking the ATG start codon. Each SCoT amplicon represents a gene locus that may regulate important traits (Rai, 2023), making gene-based markers highly valuable in precision breeding (Salgotra & Stewart, 2020).

SCoT markers represent a promising alternative to other PCR-based molecular markers such as RAPD, which often suffer from poor reproducibility due to short 10-mer primers (Babu et al., 2021), or simple sequence repeats (SSR), which require polyacrylamide gels to achieve high-resolution results (Amiteye, 2021). SCoT markers employ 18-mer primers, which have been reported to improve reproducibility (Rai, 2023). SCoT markers have been widely used in genetic diversity studies across a wide range of crops, including rice (Safitri et al., 2026), maize (Chňapek et al., 2024), and wheat (Shehata et al., 2025). Despite the advantages, SCoT markers remain PCR-based and are therefore subject to factors that generally influence PCR performance. Among these, annealing temperature (Ta) and primer concentration are key parameters that can significantly affect amplification success (Mubarok et al., 2025; Wospakrik et al., 2026). Although most studies employing SCoT markers have used an annealing temperature of approximately 50 °C (Ragab et al., 2025; Shehata et al., 2025; Safitri et al., 2026), systematic information on the optimal Ta and primer concentration required to achieve reproducible amplification with SCoT markers is still lacking. Therefore, we aimed to optimize the technical PCR conditions to enhance the performance of the SCoT markers, while also examining species-specific differences in reproducibility across staple crops such as rice, maize, and cassava, which were selected to represent major food crops in Indonesia and to demonstrate the applicability of our study across different crop types.

Materials and Methods

Genetic Materials and Genomic DNA Isolation

Genetic materials used for genomic DNA isolation in this research consisted of two Indonesian foxtail millet genotypes ('ICERI5' and 'Botok10'), four local Indonesian rice genotypes ('Pare Bau', 'Mansyur Buri', 'Ambo Rambut Pendek', and 'Pare Kaloko Jawa'), four commercial maize genotypes ('BISI-18', 'NK-78', 'Arinta', and 'BISI-22'), and four cassava national genotypes ('Mentega', 'Gajah', 'Ratim', and 'Malang-1'). The two foxtail millet genotypes ('ICERI5' and 'Botok10') were selected for their reported genetic dissimilarity (Jannah et al., 2024). Seeds of foxtail millet, rice, and maize were sown in the planting tray containing planting media consisting of soil, manure, and coconut coir (1:1:1 v/v) for ±10 days. For cassava, young leaves from each genotype were collected from about 6-month-old plants in the field. Young leaves from each sample were harvested, and genomic DNA was isolated using the modified CTAB method (Aboul-Maaty & Oraby, 2019). DNA integrity was evaluated by electrophoresis of 5 µl of genomic DNA on a 1.5% (w/v) agarose gel at 90 V for 45 min in 1× TAE (Tris-Acetate-EDTA) buffer. DNA concentration and purity ($A_{260}/_{280}$ and $A_{260}/_{230}$ ratios) were assessed using a nano-spectrophotometer (MaestroNano MN-917, Taiwan).

Annealing Temperature (Ta) and SCoT Primer Concentration Optimization using Genomic DNA of Foxtail Millet

PCR optimization was performed using a Bio-Rad T100™ Thermal Cycler with a total reaction volume of 10 µl. Each reaction contained 5 µl of DNA polymerase (MyTaq HS Red Mix 2×), 1.5 µl of foxtail millet genomic DNA template (12 ng/µl), and 2.5 µl of SCoT primer at tested concentrations (2.5 µM, 1.5 µM, and 0.5 µM). The thermal cycling protocol consisted of an initial denaturation step at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 5 sec, annealing at the evaluated temperature (50 °C and 55 °C) for 1 min, and extension at 72 °C for 30 sec. A final extension step was performed at 72 °C for 10 min to complete the amplification process. The SCoT primer used in this experiment consisted of 36 primers, as described by Collard and Mackill (2009).

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Each PCR reaction was performed in three independent runs for each evaluated annealing temperature. The PCR products were analyzed using electrophoresis on 1.5% (w/v) agarose gel in 1× TAE buffer at 115 V for 110 min. The gel was stained with ethidium bromide (0.5 µg/ml) and visualized using a UV transilluminator (Gel Doc EZ™, Bio-Rad, USA).

PCR data were analyzed by evaluating DNA banding patterns obtained from gel electrophoresis. Each amplicon, representing a locus, was converted into binary data using a scoring system (0 = absence, 1 = presence). Amplicon scoring was performed using GelAnalyzer 23.1 software, applying an amplicon intensity threshold of 30 and requiring a minimum of three agarose gel images per primer to ensure marker reproducibility. Amplicon sizes were determined by comparison with 1 kb and 100 bp DNA ladders. The primary variables assessed included the total number of amplified amplicon, the number of consistent amplicon, and the number of inconsistent amplicon. Reproducibility percentage was calculated as (number of consistent amplicons ÷ total number of amplicons) × 100%. No additional statistical comparisons were conducted beyond reproducibility percentage calculations.

Validation of Optimized Ta and SCoT Primer Concentration

This experiment used the genomic DNA samples from rice, maize, and cassava genotypes to validate the previously optimized protocol (Ta and primer concentration). PCR was performed as described above, with the selected Ta and primer concentration. The SCoT primer used in this experiment consisted of 10 primers with the highest amplicon count and the highest percentage of reproducible amplicons (100%), as determined by a PCR optimization experiment using foxtail millet DNA. Each PCR reaction was performed in triplicate to obtain reproducible data for the DNA amplicons. The PCR products were analyzed using electrophoresis on 1.5% (w/v) agarose gel in 1× TAE buffer at 115 V for 110 min. The gel from electrophoresis was stained with ethidium bromide (0.5 µg/ml) and visualized using a UV transilluminator (Gel Doc EZ™, Bio-Rad, USA). PCR data were analyzed as described above. In addition to the amplicon count and reproducibility percentage, subsequent analysis was conducted only on reproducible amplicon to determine the percentage of polymorphism.

Results and Discussion

An increase in annealing temperature (Ta) from 50 to 55 °C resulted in a reduction in both the mean number of amplicons and the proportion of amplicons producing reproducible results (Table 1). The selection of an appropriate Ta is critical to primer performance, as Ta influences banding patterns and primer reproducibility (Collard & Mackill, 2009; Santoso et al., 2024). Generally, higher Ta values enhance primer binding specificity, leading to more specific and reproducible amplification. However, excessively high Ta may reduce the number of amplicons or even lead to amplification failure (Collard & Mackill, 2009; Santoso et al., 2024). Studies using RAPD markers demonstrated that a Ta of 50 °C improved RAPD reproducibility compared with the lower Ta values commonly used (34–36 °C), without reducing the capacity to detect polymorphism, as higher Ta values reduced non-specific reactions (Atienzar et al., 2000). Research on ISSR markers has indicated that each primer has a significantly different optimal Ta, based on the optimization of four ISSR primers, and that, in general, the Ta of each primer is higher than its melting temperature (Tm) (Enan, 2008). Most studies employing SCoT markers have used a single Ta of 50 °C for all tested primers (Altaf et al., 2025; Collard & Mackill, 2009; Nitiworakarn et al., 2023; Vanijajiva, 2020). Initial optimization of SCoT primer Ta by Collard & Mackill (2009) showed that DNA amplification in rice with SCoT primers at Ta values above 55 °C yielded fewer reproducible amplicons, leading to the recommendation of an optimal Ta of 50 °C. Conversely, Santoso et al. (2024) reported that of 18 SCoT primers used to amplify seaweed DNA, only 10 successfully

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amplified at 50 °C, while the remaining eight primers amplified only at a lower Ta of 48 °C. Sharma and Thakur (2021), using Ta values five degrees lower than Tm, demonstrated that 13 SCoT primers with high Tm values (60.1–71 °C) successfully amplified ginger DNA at Ta values above 55 °C. Collectively, these studies confirm that Ta strongly influences the specificity and reproducibility of amplification with SCoT markers. Nevertheless, the use of a single Ta for efficient and reproducible DNA amplification with SCoT markers may simplify their application in plant genetic analysis.

Primer concentration is another critical PCR parameter influencing amplification success (Mubarok et al., 2025; Wospakrik et al., 2026). In general, the mean number of amplicons increased with higher primer concentrations, from 0.5 to 1.5 to 2.5 µM. Primer concentration had little effect on the percentage of amplicons yielding reproducible results (Table 2). Based on the number of amplicons and the reproducibility percentage, a primer concentration of 0.5 µM produced relatively poor primer performance. Raising Ta from 50 to 55 °C tended to reduce amplicon yield and reproducibility, while lowering primer concentration decreased the number of amplicons; however, primer performance varied among SCoT primers. Therefore, SCoT primer performance can be evaluated based on both the number of amplicons and the percentage of reproducible amplicons. Most SCoT primers produced relatively high amplicon numbers with 100% reproducibility at Ta 50 °C and a primer concentration of 2.5 µM (Table 3). In this study, reproducibility was confirmed through replication. Under these tested conditions, primers that consistently achieved 100% reproducibility may reduce the need for additional replication in similar diversity analyses, thereby lowering analytical costs. Nonetheless, replication remains essential in most genetic diversity studies to avoid bias and ensure reliability.

Based on the evaluation of 36 SCoT primers in two foxtail millet genotypes, 20 primers exhibited 100% reproducibility across three replicates. The ten primers with the highest amplicon numbers and reproducibility were further evaluated in three major food crops—cassava, rice, and maize—to determine whether their performance was generalizable across species. These ten primers were SCoT 14, SCoT 35, SCoT 7, SCoT 15, SCoT 22, SCoT 20, SCoT 23, SCoT 36, SCoT 21, and SCoT 24. Primer reproducibility varied across plant species (Table 4). The representative gel image is shown in Figure 1. Surprisingly, not all primers exhibited 100% reproducibility as observed in foxtail

Table 1

Effect of Annealing Temperature (Ta) on the Mean Number and Reproducibility Percentage of Amplicons Averaged Across 36 SCoT Primers in Two Foxtail Millet Genotypes

Annealing temperature (°C)	Mean number of amplicons	Percentage of reproducible amplicons (%)
50	11.5 ± 6.5	86.2 ± 24.5
55	8.6 ± 6.9	61.0 ± 61.0

Table 2

The Effect of Primer Concentration on the Mean Number of Amplicons and Reproducibility Percentage of Amplicons Averaged Across 36 SCoT Primers in Two Foxtail Millet Genotypes

Primer concentration (µM)	Mean number of amplicons	Percentage of reproducible amplicons (%)
0.5	4.5 ± 4.2	72.5 ± 35.3
1.5	12.0 ± 5.1	71.0 ± 32.9
2.5	13.6 ± 7.1	79.7 ± 31.4

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Table 3

SCoT Primers with the Highest Amplicon Counts and Reproducible Amplicon Percentage

No	Primer name	Primer concentration (μM)	Amplicon count	Percentage of reproducible amplicons (%)
1.	SCoT-14	2.5	24	100.0
2.	SCoT-35	2.5	24	100.0
3.	SCoT-7	2.5	23	100.0
4.	SCoT-15	2.5	23	100.0
5.	SCoT-22	2.5	23	100.0
6.	SCoT-20	2.5	21	100.0
7.	SCoT-23	2.5	21	100.0
8.	SCoT-36	2.5	21	100.0
9.	SCoT-21	2.5	20	100.0
10.	SCoT-24	2.5	20	100.0
11.	SCoT-30	2.5	20	100.0
12.	SCoT-13	2.5	19	100.0
13.	SCoT-17	2.5	19	100.0
14.	SCoT-12	2.5	18	100.0
15.	SCoT-18	1.5	17	100.0
16.	SCoT-8	2.5	16	100.0
17.	SCoT-11	2.5	16	100.0
18.	SCoT-10	2.5	15	100.0
19.	SCoT-28	1.5	15	100.0
20.	SCoT-33	2.5	9	100.0

millet. Four primers achieved 100% amplicon reproducibility in cassava, eight in rice, and six in maize. Of the ten primers tested across the three species, only two primers, SCoT-35 and SCoT-36, showed 100% reproducibility in all three crops. The relatively low number of universally reproducible primers may be attributable to the fact that optimization was initially performed using only two foxtail millet genotypes. Bidyananda et al. (2024) highlighted the need to use a sufficient number of genetically diverse materials to assess primer performance. Beyond technical parameters, species-dependent primer reproducibility may also reflect underlying genomic differences. For instance, foxtail millet has a relatively small genome (~515 Mb; 46% GC) (Bennetzen et al., 2012), rice has ~430 Mb with ~43% GC (International Rice Genome Sequencing Project & Sasaki, 2005), maize has a much larger genome (~2.3 Gb; 46% GC) (Schnable et al., 2009), and cassava has ~770 Mb with ~36% GC (Prochnik et al., 2012). These differences in genome size and GC composition, along with variation in sequence conservation around start codons, likely influence primer binding efficiency and reproducibility. These findings highlight the need for species-specific primer selection and careful optimization to achieve reproducible performance across crops. Future studies should broaden genotype coverage, refine PCR parameters such as Mg^{2+} concentration and polymerase choice, and extend validation to additional tropical species to enhance the general applicability of SCoT markers.

Conclusions

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Table 4

Amplicon Count, Percentage of Reproducible Amplicons, and Polymorphism Percentage of the 10 Selected SCoT Primers in Cassava, Rice, and Maize (Ta 50 °C, Primer Concentration 2.5 µM)

Primer name	Amplicon count	Percentage of reproducible amplicons (%)	Polymorphism percentage (%)
Cassava			
SCoT-7	13	38.5	7.7
SCoT-14	27	66.7	18.5
SCoT-15	35	45.7	34.2
SCoT-20	27	25.9	7.4
SCoT-21	17	100.0	70.5
SCoT-22	11	100.0	9.1
SCoT-23	22	90.9	22.7
SCoT-24	19	73.7	15.8
SCoT-35	24	100.0	70.8
SCoT-36	12	100.0	41.7
Rice			
SCoT-7	26	88.5	61.5
SCoT-14	19	100.0	57.9
SCoT-15	25	96.0	64.0
SCoT-20	24	100.0	54.2
SCoT-21	18	100.0	44.4
SCoT-22	21	100.0	42.9
SCoT-23	20	100.0	25.0
SCoT-24	22	100.0	36.4
SCoT-35	15	100.0	26.7
SCoT-36	17	100.0	41.2
Maize			
SCoT-7	21	71.4	28.6
SCoT-14	21	100.0	42.9
SCoT-15	33	100.0	69.7
SCoT-20	25	80.0	36.0
SCoT-21	30	13.3	13.3
SCoT-22	21	95.2	57.1
SCoT-23	24	100.0	33.3
SCoT-24	21	100.0	47.6
SCoT-35	12	100.0	41.7
SCoT-36	25	100.0	36.0

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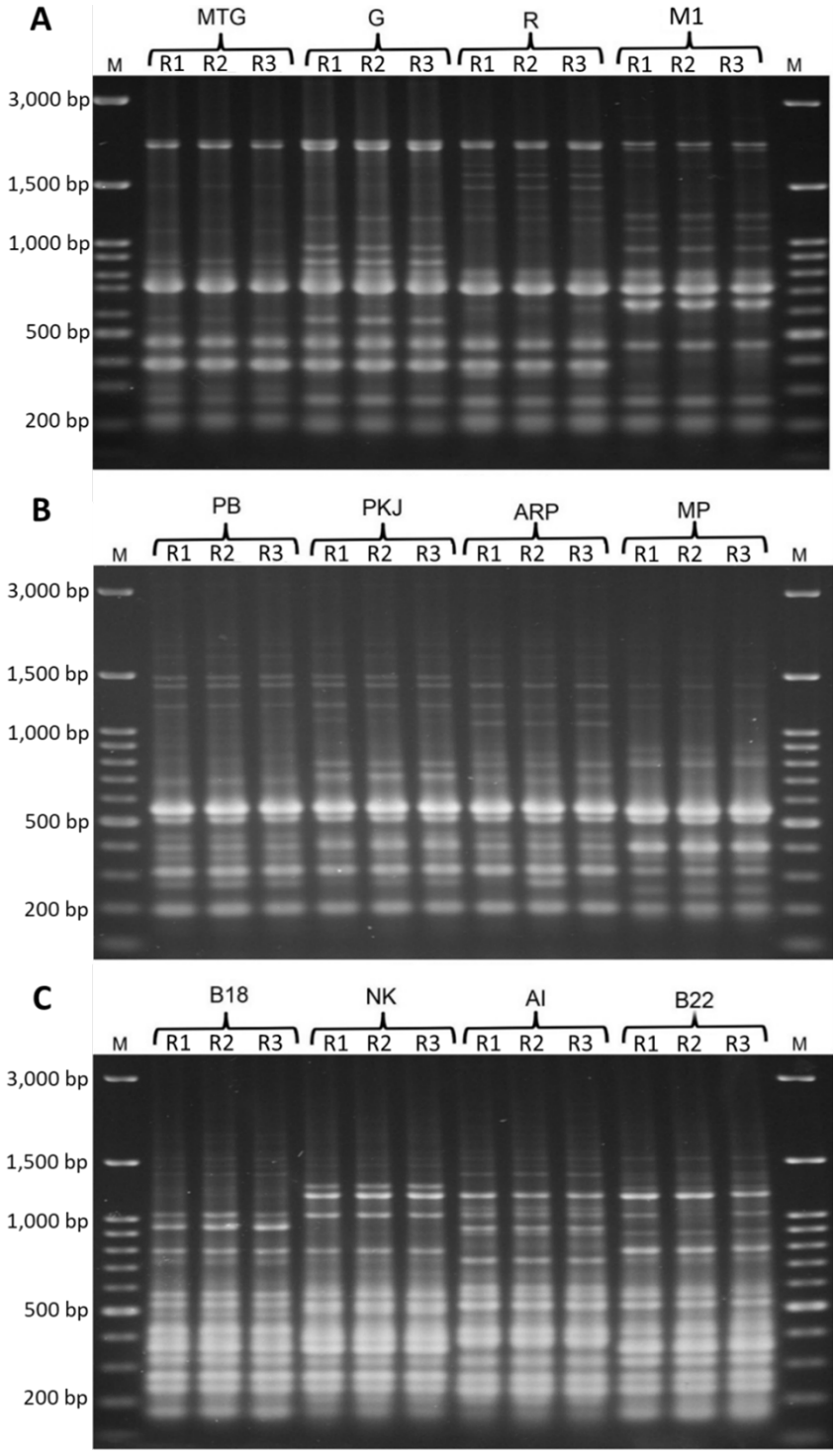
Figure 1

Visualization of SCoT Amplicons in Cassava using SCoT-35 (A), Rice using SCoT-20 (B), and Maize using SCoT-15 (C)



Notes. Cassava genotypes, MTG= 'Mentega', G= 'Gajah', R= 'Ratim', M1= 'Malang-1'. Rice genotypes, PB= 'Pare Bau', PKJ= 'Pare Kaloko Jawa', ARP= 'Ambo Rambut Pendek', MB= 'Mansyur Buri'. Maize genotypes, B18= 'BISI-18', NK= 'NK-78', AI= 'Arinta', B22= 'BISI-22'.

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This study demonstrates that annealing temperature (Ta) and primer concentration critically influence SCoT marker performance, with Ta at 50 °C and a primer concentration of 2.5 µM yielding the highest amplicon numbers and 100% reproducibility for most primers. While 20 of 36 primers showed full reproducibility in foxtail millet, cross-species evaluation in cassava, rice, and maize revealed variable performance, with only SCoT-35 and SCoT-36 consistently reproducible across all three crops. These findings underscore that primer suitability is species-dependent and highlight the need for extensive primer screening in each plant species to ensure the reliable, efficient, and cost-effective application of SCoT markers in genetic diversity analysis.

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