Journal of Tropical Crop Science Vol. 13 No. 1, February 2026
www.j-tropical-crops.com

Received 06/10/2025; Revised 06/12/2025; Accepted 17/12/2025
https://doi.org/10.29244/jtcs.13.01.103-113

Improved Performance of *SiDREB2*-SNAP Marker in Foxtail Millet by Optimum Primer Concentration, PCR Cycle, and DNA Polymerase Specificity

Andrean Heskiel Wospakrik[1, 2], Ramadaniarto Rizqullah[2], M Reza Pahlevi[2], Yudiansyah[3], Bambang Sapta Purwoko[3], Willy Bayuardi Suwarno[3], and Sintho Wahyuning Ardie*[3]

[1] Crop Production Study Program, Faculty of Agriculture, Papua University, Indonesia
[2] Plant Breeding and Biotechnology Study Program, Graduate School, IPB University, Indonesia
[3] Department of Agronomy and Horticulture, Faculty of Agriculture, IPB University, Indonesia

*Corresponding author; email: sintho_wa@apps.ipb.ac.id

Abstract

Foxtail millet (*Setaria italica* L. Beauv.) is an emerging carbohydrate-producing crop. It is considered a climate-resilient crop due to its tolerance to abiotic stresses. Breeding for abiotic-tolerant crops requires powerful tools such as molecular markers. The single-nucleotide amplified polymorphism (SNAP) marker, developed from a single-nucleotide DNA variation at a specific location in the plant genome, is a simple yet powerful PCR-based marker widely used in phenotype-related selection. A *SiDREB2*-based SNAP marker was previously developed based on a base variation at the 558th base pair (an A/G transition) in the *SiDREB2* gene and used to estimate the drought tolerance in foxtail millet. However, the specificity of the marker depends on technical aspects, including the type of DNA polymerase used, primer concentration, and the number of PCR cycles. Here, we reported that non-specific and false positive amplicons can be eliminated by utilizing DNA polymerase with no 3' to 5' proofreading activity and reducing the final primer concentration to 1.25 µM. PCR cycle number 25 yielded the optimum specificity, while increasing the cycle to 30 resulted in false positive results. Altogether, our results showed that technical optimization is necessary for improving the specificity of the SNAP marker.

Keywords: abiotic stress, allele-specific marker, cereals, drought, molecular marker, protocol

Introduction

Climate change impacts global agriculture, as climate-induced abiotic stresses negatively affect crop productivity (Hasegawa et al., 2022; Liaqat et al., 2022). Utilizing climate-resilient crops and smart breeding strategies is therefore critical for adaptation (Grigorieva et al., 2023). Foxtail millet (*Setaria italica* L. Beauv.), a minor cereal, has gained attention as a climate-resilient crop and as a functional food for its adaptability and nutritional qualities (Ardie et al., 2025; Arora et al., 2023). Despite its many advantages, foxtail millet is not a widely grown food crop in Indonesia, where breeding efforts are still in the early stages, resulting in no superior variety available in the country to date (Muzzayyanah et al., 2024; Nugroho et al., 2020; Sintia et al., 2023a).

Molecular markers offer a powerful tool to accelerate breeding, particularly for traits such as drought and salinity tolerance that are difficult to assess reliably in early generations. The DNA-based molecular markers are not environmentally regulated and are observable in any stage of plant growth (Hasan et al., 2021). Among developed molecular markers, allele-specific PCR (AS-PCR) is a simple method to discriminate single-nucleotide polymorphism

Received 06/10/2025; Revised 06/12/2025; Accepted 17/12/2025
https://doi.org/10.29244/jtcs.13.01.103-113

(SNP) by a typical reverse primer and two forward allele-specific primers with standard PCR conditions (Gaudet et al., 2009). This method is also known as single-nucleotide amplified polymorphism (SNAP) markers and is widely used in marker-assisted selection (MAS) of many crops (Hatta et al., 2023; Jang & Lee, 2021; Scheuermann & Pereira, 2023). In foxtail millet, an A/G transition at the 558th base pair in the *Setaria italica dehydration-responsive element binding-2* (*SiDREB2*) gene was associated with tolerance to drought (Lata et al., 2011). A *SiDREB2*-based SNAP marker was later developed to assist in confirming successful hybridization and selecting foxtail millet's drought- or salt-tolerance traits (Butarbutar et al., 2024; Nugroho et al., 2024; Widyawan et al., 2018a).

However, SNAP marker specificity was reported to depend on some technical aspects, including the type of DNA polymerase (Liu et al., 2012), template concentration (Drenkard et al., 2000), and PCR cycle number (Kim et al., 2005). Such errors are problematic in breeding programs, as they risk misclassifying tolerant and sensitive genotypes, leading to inefficient resource utilization and slowing down the breeding program. While SNAP markers are widely used across crops, systematic optimization of these technical parameters has not yet been conducted for foxtail millet. To address this gap, the present study is the first to systematically evaluate and optimize primer concentration, PCR cycle number, and DNA polymerase specificity for the *SiDREB2*-based SNAP marker in foxtail millet. By improving marker fidelity, this work aims to improve the *SiDREB2*-based SNAP marker performance and prevent false positive or negative results.

Materials and Methods

Genetic Materials and DNA Isolation

Genetic materials used in this study included four parental foxtail millet genotypes with known *SiDREB2* alleles: ICERI-5 (I5, drought/salinity tolerant, A/A allele at the 558th position of the exon-2 of the *SiDREB2* gene; Indonesian Cereals Research Institute, ICERI), ICERI-6 (I6, A/A allele), Botok-4 (B4, drought/salinity sensitive, G/G allele; local genotype from East Nusa Tenggara, Indonesia), and Botok-10 (B10, G/G allele) (Ratnawati et al., 2024; Widyawan et al., 2018a). Four segregating lines carrying unknown *SiDREB2* alleles were included in the analyses. These comprised three F2 progenies derived from the crossing of Botok-4 and ICERI-5, or its reciprocal (#160: B4×I5; #259: I5×B4; #303: B4×I5), and one F3 progeny derived from the crossing of Botok-10 and ICERI-6 (#143: B10×I6). These F2 and F3 lines were used for PCR optimization, with possible allele combinations of A/A, G/G, or A/G. To validate the optimized protocol, we analyzed 28 F5 lines derived from the ICERI-6 × Botok-4 cross: 27 lines represented by three individuals each and one line represented by two individuals, yielding a total of 83 F5 genotypes. Two parental genotypes, ICERI-6 and Botok-4, were included as controls (Supplementary Table 4). Total genomic DNA from the shoot parts of 14-day-old seedlings was isolated using the CTAB method (Aboul-Maaty & Oraby, 2019) and slightly modified by excluding 0.2% (v/v) 2-mercaptoethanol in the lysis buffer. The isolated DNA was then diluted in nuclease-free water to a 12 ng/µl concentration for further application.

General PCR Optimization Schemes

We tested three PCR components to obtain the most specific amplification of the *SiDREB2*-based SNAP marker, namely (i) type of DNA polymerase, (ii) primer concentration, and (iii) number of PCR cycles. The PCR was performed in a 10 µl total volume using Esco's Swift Maxi Thermal Cycler (Esco Technologies, Singapore). Except if it is explicitly described in the following sub-sections, the final reaction mixture consisted of 2.5 µl of DNA template (12 ng/µL), 2.5 µl of forward (SD2-558-SNP-A or SD2-558-SNP-G) and reverse primer (SD2-558-SNP-Rev) (the concentration depended on the experimental set), and 5.0 µl of 2× PCR

Received 06/10/2025; Revised 06/12/2025; Accepted 17/12/2025
https://doi.org/10.29244/jtcs.13.01.103-113

mix (the DNA polymerase type depended on the experimental set). The PCR profile was arranged to be: denaturation for 5 min (94 ºC), 25 cycles (except for the PCR cycle comparison) of denaturation for 5 sec (94 ºC), annealing for 1 min (55 ºC), and extension for 30 sec (72 ºC). The final extension was performed for 10 min at 72 ºC after the end of the cycles. An additional reaction without a DNA template was set as a negative control (NC) in all experimental sets. The list of primers used in this study is shown in Table 1. The gene *Seita.5G115000*, annotated as *SiDREB2* in the Phytozome database, comprises two exons separated by a single intron. Forward primers were designed to target a single-nucleotide polymorphism (SNP) located at position 558, defined by numbering from the first base of exon 2, where an A/G transition distinguishes two genotypes. This variation is illustrated in Figure 1 using sequences from ICERI-5 (GenBank accession no. KY404097.1) and ICERI-10 (GenBank accession no. KY404101.1). Amplicons were visualized by electrophoresis at 90 volts for 45 minutes in 1x TAE buffer on 1.5% (w/v) agarose gel. PCR results were stained in 0.5 µg/ml ethidium bromide solution and were visualized using a UV transilluminator (AlphaImager® Mini).

DNA Polymerase Comparison

All PCR conditions were set as described above, except for the type of DNA polymerase. Two types of DNA polymerase used in this study represented different types of polymerases. KAPA2G Fast HotStart ReadyMix (Sigma-Aldrich, Germany) is used for routine PCR with no 3'-to-5' exonuclease proofreading activity. Meanwhile, MyTaq HS Red Mix (Bioline, US) is suitable for cloning due to its high-yielding and proofreading activity of an efficient 3'-to-5' exonuclease. Two sets of PCR reactions were prepared, each using a different DNA polymerase. The final concentration of each primer used in this scheme was 2.5 µM. The specificity of the PCR conditions was evaluated based on the appearance of specific amplicons following visualization in the agarose gel.

Primer Concentration Comparison

All PCR conditions were set as described in the general PCR optimization scheme, except the final concentration of each primer used in this

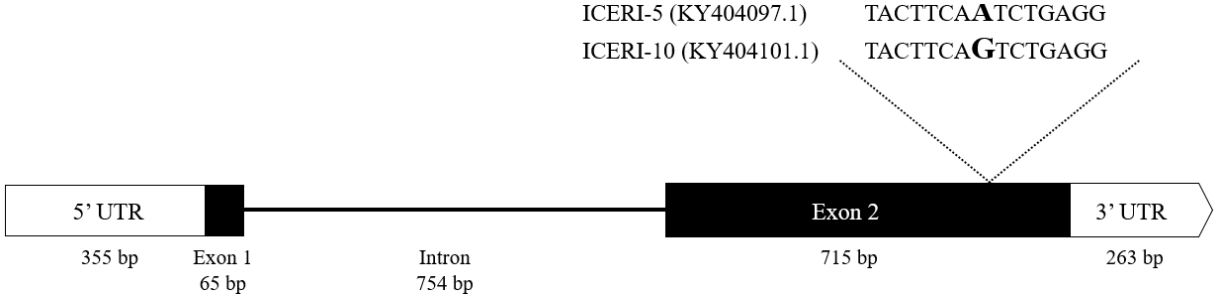
Table 1

Forward- and Reverse Primers of SiDREB2-Based SNAP Marker Used in This Study

Primer name	Nucleotide sequence (5'-3')	Tm (°C)	Primer type
SD2-558-SNP-G	GCAAGTCCGTGGAGGTACTACAG	58.8	Forward
SD2-558-SNP-A	AAGTCCGTGGAGGTACTGCAA	58.3	Forward
SD2-558-SNP-Rev	AGGAACTCAACACACAGGACAACT	57.9	Reverse

Figure 1

Schematic Representation of the SiDREB2 Gene Structure Illustrating the Position of the SNP



Received 06/10/2025; Revised 06/12/2025; Accepted 17/12/2025
https://doi.org/10.29244/jtcs.13.01.103-113

scheme was reduced to 1.25 µM. The specificity of the PCR conditions was evaluated based on the appearance of specific amplicons following visualization in the agarose gel.

PCR Cycle Comparison

All PCR conditions were set as described in the general PCR optimization scheme, except for the number of PCR cycles. Two sets of PCR reactions were prepared, using KAPA2G Fast HotStart ReadyMix (Sigma-Aldrich, Germany) as the DNA polymerase, but each used a different PCR cycle number, i.e., 25 and 30 cycles. The final concentration of each primer used in this scheme was 1.25 µM. The specificity of the PCR conditions was evaluated based on the appearance of specific amplicons following visualization in the agarose gel.

Gel Image Quantification

Gel results were quantified using GelAnalyzer software (version 32.1.1). Prior to analysis, gel images were adjusted for brightness and contrast and subsequently uploaded into the program. Band intensities were then analyzed and quantified following the standard software workflow, and the resulting peak values were reported as band intensity measurements. Band quantification was performed only for the PCR optimization experiments and was not applied to the validation analyses.

Results and Discussion

DNA Polymerase Specificity

The accuracy of PCR amplification was crucial for obtaining reliable results in allele-specific PCR (Gaudet et al., 2009). The polymerase chain reaction was conducted in a reaction mixture containing the template DNA, DNA polymerase, primers, and the four deoxyribonucleoside triphosphates (dNTPs), all dissolved in a buffer solution (Nazir & Mahmood, 2020). DNA polymerases, widely employed for in vitro DNA manipulation with a fundamental role in deoxyribonucleotide chain synthesis, exhibited variations in properties, including thermostability, fidelity, processivity, and specificity (Laura & Rukmanidevi, 2023). In this study, we evaluated two types of DNA polymerase, namely KAPA2G and MyTaq HS Red Mix, on the specificity of the *SiDREB2*-based SNAP marker. Differences were observed in the amplicons generated by the type of DNA polymerase used (Figure 2).

The appearance of a 300 bp amplicon produced by the SD2-558-SNP-A and SD2-558-SNP-rev primer pair in ICERI-5 (A lane or A allele) or a 300 bp amplicon produced by the SD2-558-SNP-G and SD2-558-SNP-rev primer pair in Botok-4 (G lane or G allele) served as the control of successful specific amplification based on previous studies (Ratnawati et al., 2024; Sintia et al., 2023a). The negative control (NC) ensured that only template-dependent amplicons were observable. KAPA2G DNA polymerase produced more suitable amplicons for ICERI-5 and Botok-4 since the 300 bp amplicon only appeared on the A allele for ICERI-5 and the G allele for Botok-4, with band intensities of 124 and 133, respectively. Based on the amplicon appearance generated by KAPA2G, the *SiDREB2*-allele of line numbers #143, #160, #259, and #303 were A/G, A/A, A/A, and A/A, respectively (with band intensities ranging from 130-163). However, non-specific amplicons of around 2,000 bp were detected on line numbers #160 and #259 (band intensity of 77-98, supplementary Table 1), as indicated by the white arrow in Figure 2.

Contrasting outcomes were noted with MyTaq HS DNA polymerase, where a weak 300 bp amplicon (band intensity 99) appeared on the G allele (in addition to the A allele, band intensity 116) of the ICERI-5 genotype. Weak amplicons of around 300 bp also appeared on the G allele of line numbers #160, #259, and #303 (band intensities ranging from 103 to 110), as indicated by the white arrows in the lower panel of Figure 2. Based on the amplicon appearance generated by MyTaq HS, the *SiDREB2*-allele of ICERI-5 genotype and line numbers #160, #259, and #303 are all A/G. Primer extension highly depends on the type of DNA polymerases, i.e., with and without proofreading function. Previous

Received 06/10/2025; Revised 06/12/2025; Accepted 17/12/2025
https://doi.org/10.29244/jtcs.13.01.103-113

studies showed that the primer's 3′ terminal mismatched nucleotide was removed by the proofreading function when DNA polymerase with the proofreading function was used (Zhang et al., 2003a; Zhang et al., 2003b). Our previous experiments utilizing three different DNA polymerases to assess the specificity of the *SiDREB2*-based SNAP marker showed similar results. R-Taq DNA polymerase (B-bridge, Japan) with no proofreading activity resulted in specific amplicons. In contrast, with proofreading activity, Ex-Taq and PrimeStar GxL DNA polymerases (TaKaRa Bio, US) resulted in false positive amplicons (Widyawan et al., 2018b). MyTaq HS is a DNA polymerase with 3'-to-5' exonuclease proofreading activity; thus, the weak appearance of 300 bp amplicons on the G allele in ICERI-5 genotype and in the line numbers #160, #259, and #303 was suspected to be false positive amplicons due to the proofreading function of MyTaq HS DNA polymerase.

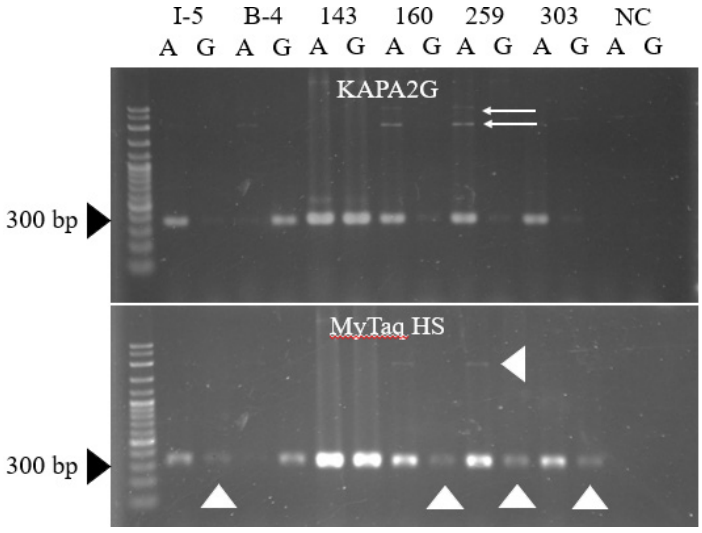
Primer Concentration

The non-specific amplicons can be attributed to high primer concentrations, causing non-specific primer binding (Bhardwaj et al., 2020; Ghashghaei et al., 2023). Therefore, we evaluated the final primer concentration (forward and reverse, respectively) by reducing it to 1.25 µM. The specificity of the PCR conditions was assessed based on the appearance of specific amplicons following visualization in the agarose gel (Figure 3, Supplementary Table 2). Amplification using KAPA2G DNA polymerase resulted in specific amplicons at 300 bp, with the A/A allele (band intensity 125) observed in genotype ICERI-5, and the G/G allele (band intensity 129) was found in the Botok-4 genotype. Similar results were obtained in previous studies (Ratnawati et al., 2024; Sintia et al., 2023a), confirming alleles of the *SiDREB-2* gene for genotypes ICERI-5 and Botok-4. The amplicon appearance generated by KAPA2G in Figure 3 was consistent with that in Figure 2, where the *SiDREB2*-allele of line numbers #143, #160, #259, and #303 were A/G, A/A, A/A, and A/A, respectively. Reducing the final primer concentration to 1.25 µM diminished the non-specific amplicons of around 2,000 bp previously detected on line numbers #160 and #259.

In contrast, MyTaq HS Red Mix still produced false positive amplicons at the G allele (band intensity 99) in the ICERI-5 genotype,

Figure 2

Visualization of SiDREB2-based Amplicons Resulted from Different Types of DNA Polymerase

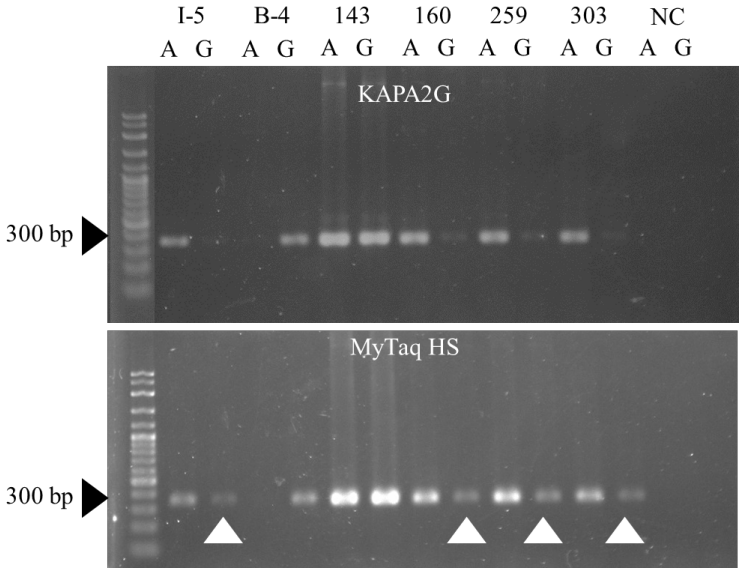


Notes. I-5 = ICERI-5 genotype; B-4 = Botok-4 genotype; line number #143, #160, #259, #303; NC = negative control; A = A allele; G = G allele. White arrows indicate non-specific amplicons.

Received 06/10/2025; Revised 06/12/2025; Accepted 17/12/2025
https://doi.org/10.29244/jtcs.13.01.103-113

Figure 3

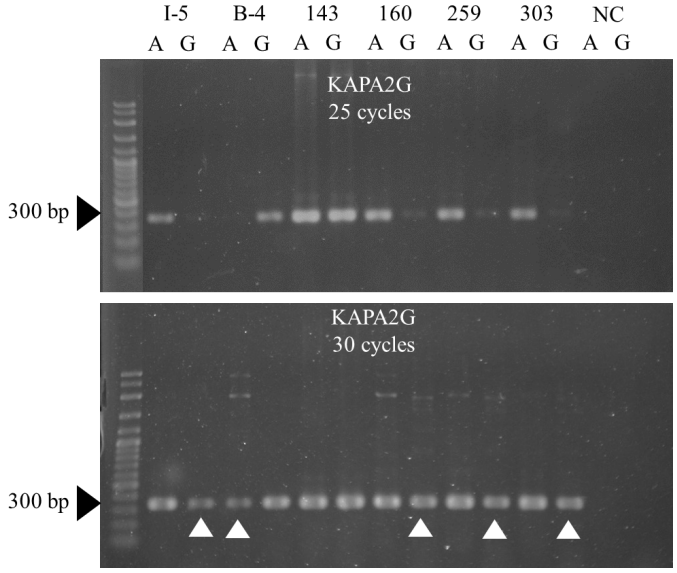
Visualization of SiDREB2-based Amplicons with an Optimized Primer Concentration of 1.25 µM



Notes. I-5 = ICERI-5 genotype; B-4 = Botok-4 genotype; line number #143, #160, #259, #303; NC = negative control; A = A allele; G = G allele. White arrows indicate non-specific amplicons.

Figure 4

Visualization of SiDREB2-based Amplicons Resulted from Different PCR Cycle Numbers



Notes. I-5 = ICERI-5 genotype; B-4 = Botok-4 genotype; line number #143, #160, #259, #303; NC = negative control; A = A allele; G = G allele. White arrows indicate non-specific amplicons.

Received 06/10/2025; Revised 06/12/2025; Accepted 17/12/2025
https://doi.org/10.29244/jtcs.13.01.103-113

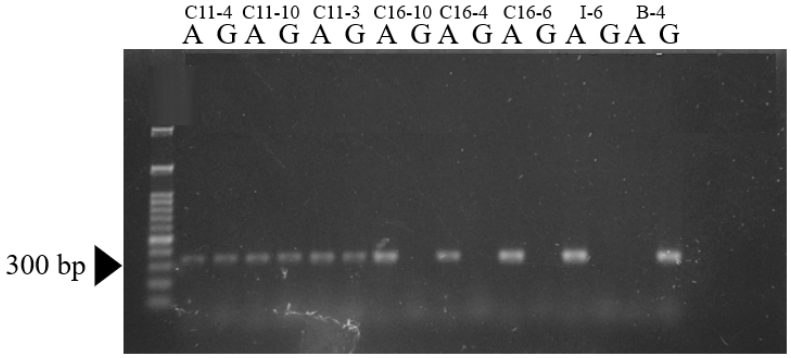
thereby yielding less valid results, shifting allele ICERI-5 from A/A to A/G. Also, allele A/G was formed in the amplification results of line numbers #160, #259, and #303 using MyTaq HS Red Mix. These findings showed that reducing the final primer concentration in amplification using KAPA2G DNA polymerase can eliminate non-specific amplicons and produce target amplicons with a single, appropriate amplicon (300 bp). These results also emphasize the importance of choosing a DNA polymerase without 3'-to-5' exonuclease proofreading activity for specific amplification in the *SiDREB2*-based SNAP marker.

PCR Cycle Number

In addition to the primer concentration, the number of cycles in the PCR setup also affects the success of obtaining the amplicon (Lorenz, 2012). Since DNA polymerase KAPA2G produced more specific amplicons, we evaluated the performance of KAPA2G under different cycling conditions, 25 and 30 cycles, respectively. The resulting amplicons with their band intensities are presented in Figure 4 and Supplementary Table 3, respectively. Despite using a DNA polymerase without proofreading activity and with an optimized final primer concentration (1.25 µM), increasing the PCR

Figure 5

Visualization of SiDREB2-based Amplicons Carrying Alleles A/G and A



Notes. I6 = ICERI-6 genotype; B4 = Botok-4 genotype; F5 lines numbered #C11-4, #C11-10, #C11-3, #C16-10, #C16-4, and #C16-6. A = allele A; G = allele G.

Table 2

Genotypic Outcomes of SiDREB2-based SNAP Marker Under Varying PCR Conditions

PCR conditions		Genotype					
		I5	B4	#143	#160	#259	#303
				Experiment-1			
KAPA 2G	2.5 µM	AA	GG	AG	AA	AA	AA
MyTaq HS	25 cycles	AG	GG	AG	AG	AG	AG
				Experiment-2			
KAPA 2G	1.25 µM	AA	GG	AG	AA	AA	AA
MyTaq HS	25 cycles	AG	GG	AG	AG	AG	AG
				Experiment-3			
KAPA 2G	25 cycles	AA	GG	AG	AA	AA	AA
1.25 µM	30 cycles	AG	AG	AG	AG	AG	AG

Received 06/10/2025; Revised 06/12/2025; Accepted 17/12/2025
https://doi.org/10.29244/jtcs.13.01.103-113

cycle to 30 resulted in false-positive amplicons. The 300 bp amplicons appeared at the G allele of the ICERI-5 genotype (band intensity 141) and line numbers #160, #259, and #303 (band intensities ranging from 153 to 164), previously identified as having A/A alleles. The 300 bp amplicon also appeared at the A allele of the Botok-4 genotype (band intensity 142) previously identified as having G/G alleles. PCR specificity depends on the combination of template amount, primers, and the PCR cycle number (Asif et al., 2021). There would be few or no amplified products for a particular amount of DNA template if the cycle number is too low. In contrast, an excessive PCR cycle number might produce non-specific amplicons and high molecular weight smears (Asif et al., 2021; Mubarak et al., 2020).

Validation of the Optimized PCR Protocol

The PCR protocol for the *SiDREB2*-based SNAP marker was successfully optimized, with the most reliable amplification obtained using 3 ng/µl DNA template, 1.25 µM of each primer (SD2-558-SNP-A or SD2-558-SNP-G and SD2-558-SNP-Rev), and a 1× PCR mix containing DNA polymerase lacking 3'-to-5' proofreading activity. The optimized cycling profile consisted of an initial denaturation at 94 °C for 5 min, followed by 25 cycles of denaturation at 94 °C for 5 sec, annealing at 55 °C for 1 min, and extension at 72 °C for 30 sec, with a final extension at 72 °C for 10 min. Validation of this protocol using 83 F5 genotypes derived from the ICERI-6 × Botok-4 cross demonstrated high accuracy and reproducibility, as evidenced by clear banding patterns in gel electrophoresis (Figure 5, Supplementary Figure 1). The majority of genotypes carried the homozygous A/A allele of *SiDREB2*, while three individuals from the F5 line I6B4-7-357-175-3.19 exhibited the heterozygous A/G allele (Supplementary Table 4). Interestingly, these same lines were previously identified as A/A during the F2 generation (Sintia et al., 2023b), suggesting possible mis-genotyping under the earlier unoptimized protocol. Additionally, although foxtail millet is predominantly a self-pollinated species, it has an estimated 4% probability of outcrossing (Nugroho et al., 2020; Nugroho et al., 2024: Wang et al., 2010), which could explain the heterozygous A/G genotype observed in the F5 lines I6B4-7-357-175-3.19. Nevertheless, this observation highlights the importance of re-genotyping at later breeding generations, such as F5, to ensure accuracy in allele identification and to detect unexpected genetic variation.

To summarize, this study reports a systematic evaluation and optimization of primer concentration, PCR cycle number, and DNA polymerase specificity for the *SiDREB2*-based SNAP marker in foxtail millet. The optimized protocol demonstrates its applicability in breeding populations. As summarized in Table 2, different *SiDREB2* genotypes can be misattributed when inappropriate PCR protocols are applied. This result highlights the necessity of an optimized protocol for accurate genotyping. However, our study represents an initial effort that warrants further refinement. Future direction for optimization should include validating marker accuracy under field conditions for drought and salinity tolerance traits, expanding the evaluation to cover broader genetic materials, and integrating multiplex PCR approaches to improve genotyping accuracy. The relevance of multiplex PCR in SNAP-genotyping has been demonstrated in rice, where internal control primers were integrated to enhance assay precision and distinguish allele absence from PCR failure (Mubarok et al., 2025). These improvements will strengthen the utility of the *SiDREB2*-based SNAP marker in marker-assisted selection and accelerate genetic improvement in foxtail millet. Finally, our results not only refine the protocol for the *SiDREB2*-based SNAP marker in foxtail millet but also provide a valuable framework for improving the accuracy of other SNAP markers used in different genes or plant species.

Received 06/10/2025; Revised 06/12/2025; Accepted 17/12/2025
https://doi.org/10.29244/jtcs.13.01.103-113

Conclusions

To conclude, DNA polymerase with no 3' to 5' proofreading activity is more suitable for producing specific amplicons using the *SiDREB2*-based SNAP marker. PCR fidelity could be enhanced by decreasing the final primer concentration to 1.25 µM and limiting the PCR cycle number to a maximum of 25.

Acknowledgement

The authors wish to acknowledge financial support from the Ministry of Finance of the Republic of Indonesia through the Indonesia Endowment Fund for Education Agency (LPDP Scholarship Program) to AHW.

Supplementary Data Availability

Supplementary materials supporting this study are available from the corresponding author upon reasonable request.

References

Aboul-Maaty, N. A. F., & Oraby, H. A. S. (2019). Extraction of high-quality genomic DNA from different plant orders applying a modified CTAB-based method. *Bulletin of the National Research Centre, 43*, 25. https://doi.org/10.1186/s42269-019-0066-1

Ardie, S. W., Nugroho, R. B., Dirpan, A., & Anshori, M. F. (2025). Foxtail millet research in supporting climate change resilience efforts: Bibliometric analysis and focused literature review. *Heliyon, 11*(3), e42348. https://doi.org/10.1016/j.heliyon.2025.e42348

Arora, L., Aggarwal, R., Dhaliwal, I., Gupta, O. P., & Kaushik, P. (2023). Assessment of sensory and nutritional attributes of foxtail millet-based food products. *Frontiers Nutrition, 10*, 1146545. https://doi.org/10.3389/fnut.2023.1146545

Asif, S., Khan, M., Arshad, M. W., & Shabbir, M. I. (2021). PCR optimization for beginners: A step by step guide. *Research in Molecular Medicine, 9*(2), 81–102. http://dx.doi.org/10.32598/rmm.9.2.1189.1

Bhardwaj, T., Rathore, A. S., & Jha, S. K. (2020). The selection of highly specific and selective aptamers using modified SELEX and their use in process analytical techniques for Lucentis bioproduction. *RSC Advances, 10*(48), 28906–28917. https://doi.org/10.1039/D0RA03542D

Butarbutar, L. K. S., Syawaluddin, D. D., Suwarno, W. B., & Ardie, S. W. (2024). *SiDREB2*-based SNAP marker-assisted and multi-trait selection in the early generation of foxtail millet (*Setaria italica* L. Beauv.). *Journal of Tropical Biodiversity and Biotechnology, 9*(3), jtbb90407. https://doi.org/10.22146/jtbb.90407

Drenkard, E., Richter, B. G., Rozen, S., Stutius, L. M., Angell, N. A., Mindrinos, M., Cho, R.J ., Oefner, P. J., Davis, R. W., & Ausubel, F. M. (2000). A simple procedure for the analysis of single nucleotide polymorphisms facilitates map-based cloning in *Arabidopsis*. *Plant Physiology, 124*(4),1483-1492. https://doi.org/10.1104/pp.124.4.1483

Gaudet M., Fara A. G., Beritognolo I., & Sabatti M. (2009). Allele-Specific PCR in SNP Genotyping. In A. Komar (Ed.), *Single Nucleotide Polymorphisms vol 578* Methods in Molecular Biology™ (pp 415–424). Humana Press. https://doi.org/10.1007/978-1-60327-411-1_26

Ghashghaei, S., Etemadifar, Z., Tavassoli, M., & Mofid, M. R. (2023). Optimization of degenerate PCR conditions for reducing error rates in detection of PKS and NRPS gene groups in actinomycetes. *Avicenna Journal of Medical Biotechnology, 15*(1), 28. https://doi.org/10.18502/ajmb.v15i1.11422

Grigorieva, E., Livenets, A., & Stelmakh, E. (2023). Adaptation of agriculture to climate change: A scoping review. *Climate, 11*(10), 202. https://doi.org/10.3390/cli11100202

Hasan, N., Choudhary, S., Naaz, N., Sharma, N., & Laskar, R. A. (2021). Recent

Received 06/10/2025; Revised 06/12/2025; Accepted 17/12/2025
https://doi.org/10.29244/jtcs.13.01.103-113

advancements in molecular marker-assisted selection and applications in plant breeding programmes. *Journal of Genetic Engineering and Biotechnology, 19*(1), 128. https://doi.org/10.1186/s43141-021-00231-1

Hasegawa, T., Wakatsuki, H., Ju, H., Vyas, S., Nelson, G. C., Farrell, A., Deryng, D., Meza, F., & Makowski, D. (2022). A global dataset for the projected impacts of climate change on four major crops. *Scientific Data. 9*(1), 58. https://doi.org/10.1038/s41597-022-01150-7

Hatta, A. N. N. L., Sukma, D., Maskromo, I., & Sudarsono, S. (2023). Validated SNAP markers based on the CYP P450 87 A3 gene in coconut (*Cocos nucifera*) are associated with yearly stem height increase. *Biodiversitas, 24*(5), 2503–2512. https://doi.org/10.13057/biodiv/d240501

Jang, Y. E. & Lee, S. (2021). Gene-based allele specific marker for resistance to Phytophthora sojae in soybean (*Glycine max* L.). *Plant Breeding and Biotechnology, 9*(2), 164–169. https://doi.org/10.9787/PBB.2021.9.2.164

Kim, M. Y., Van, K., Lestari, P., Moon, J. K., & Lee, S. H. (2005). SNP identification and SNAP marker development for a *GmNARK* gene controlling supernodulation in soybean. *Theoretical and Applied Genetics, 110*,1003–1010. https://doi.org/10.1007/s00122-004-1887-2

Lata, C., Bhutty, S., Bahadur, R. P., Majee, M., & Prasad, M. (2011). Association of an SNP in a novel DREB2-like gene *SiDREB2* with stress tolerance in foxtail millet [*Setaria italica* (L.)]. *Journal of Experimental Botany, 62*(10), 3387–3401. https://doi.org/10.1093/jxb/err016

Laura, S. L., & Rukmanidevi, D. (2023). A review of PCR, principle, and its applications. *International Journal of Pharmaceutical Research and Applications, 8*(3), 3502–3513.

Liaqat, W., Barutçular, C., Farooq, M. U., Ahmad, H., Jan, M. F., Ahmad, Z., Nawaz, H., & Li, M. (2022). Climate change in relation to agriculture: A review. *Spanish Journal of Agricultural Research, 20*(2), e03R01. https://doi.org/10.5424/sjar/2022202-17742

Liu, J., Huang, S., Sun, M., Liu, S., Liu, Y., Wang, W., Zhang, X., Wang, H., & Hua, W. (2012). An improved allele-specific PCR primer design method for SNP marker analysis and its application. *Plant Methods, 8*(34). https://doi.org/10.1186/1746-4811-8-34

Lorenz, T. C. (2012). Polymerase chain reaction: basic protocol plus troubleshooting and optimization strategies. *JoVE Journal, 63*, e3998. https://doi.org/10.3791/3998

Mubarak, S. M., Al-Koofee, D. A., Radhi, O. A., Ismael, J. M., & Al-Zubaidi, Z. F. (2020). An optimization and common troubleshooting solving in polymerase chain reaction technique. *Systematic Reviews in Pharmacy, 11*(2), 427-436. https://doi.org/10.5530/srp.2020.2.63

Mubarok, A. F., Purwantomo, S., Suwanto, A., Reflinur., & Ardie, S. W. (2025). SNAP markers for pyramided yield and BLB resistance genes in rice. *Crop Breeding and Applied Biotechnology, 25*(4), e534425411.

Muzzayyanah, P. N., Suwarno, W. B., & Ardie, S. W. (2024). Gene action and heritability estimates in F2 populations of foxtail millet (*Setaria italica* L.). *SABRAO Journal of Breeding and Genetics, 56*(1), 65–75. http://doi.org/10.54910/sabrao2024.56.1.6

Nazir, I., & Mahmood, H. Z. (2020). Polymerase chain reaction: A creative review. *Journal of Applied Biotechnology and Bioengineering, 7*(4),157–159. https://doi.org/10.15406/jabb.2020.07.00228

Nugroho, R. B., Suwarno, W. B., Khumaida, N., & Ardie, S. W. (2020). Male-sterile induction method in foxtail millet (*Setaria italica*). *Biodiversitas, 21*(9), 4325–4330. https://doi.org/10.13057/biodiv/d210951

Nugroho, R. B., Chusnah, U., Suwarno, W. B., & Ardie, S. W. (2024). Flowering phenology and *SiDREB2*-based SNAP marker-assisted hybridity confirmation for artificial hybridization of Indonesian foxtail

Received 06/10/2025; Revised 06/12/2025; Accepted 17/12/2025
https://doi.org/10.29244/jtcs.13.01.103-113

millet (*Setaria italica* L. Beauv) genotypes. *HAYATI Journal of Biosciences, 31*(6), 1082–1094. https://doi.org/10.4308/hjb.31.6.1082-1094

Ratnawati, S., Jannah, R. M., Dewi, Y. I., Rizqullah, R., Suwarno, W. B., & Ardie, S. W. (2024). The genetic variability of Indonesian local foxtail millet accession based on agro-morphological traits and early salinity tolerance evaluation utilizing *SiDREB2*-based SNAP marker. HAYATI *Journal of Biosciences, 31*(1), 82–93. https://doi.org/10.4308/hjb.31.1.82-93

Scheuermann, K. K. & Pereira, A. (2023). Development of a molecular marker for the *Pi1* gene based on the association of the SNAP protocol with the touch-up gradient amplification method. *Journal of Microbiological Methods, 214*, 106845.https://doi.org/10.1016/j.mimet.2023.106845

Sintia, M., Ardie, S. W., & Suwarno, W. B. (2023a). Genetic variability of F2 foxtail millet population derived from ICERI-5 and Botok-10 cross. *Biodiversitas, 24*(6), 3559–3567. https://doi.org/10.13057/biodiv/d240655

Sintia, M., Suwarno, W. B., & Ardie, S. W. (2023b). Genetic Study of Three F2 Populations of foxtail millet [*Setaria italica* (L.) Beauv.] Assisted by *SiDREB2*-based SNAP Marker for Drought and/ or Salinity Tolerance [Master thesis, IPB University]. IPB University Scientific Repository.

Wang, C., Chen, J., Zhi, H., Yang, L., Li, W., Wang, Y., Li, H., Zhao, B., Chen, M., & Diao, X. (2010). Population genetics of foxtail millet and its wild ancestor. *BMC Genetics, 11*(1), 90. https://doi.org/10.1186/1471-2156-11-90

Widyawan, M. H., Khumaida, N., Kitashiba, H., Nishio, T., & Ardie, S. W. (2018a). Optimization of dot-blot SNP analysis for the detection of drought or salinity stress associated marker in foxtail millet (*Setaria italica* L.). *SABRAO Journal of Breeding and Genetics, 50*(1), 72–84.

Widyawan, M. H., Khumaida, N., & Ardie, S. W. (2018b). *Development of transcription factor gene-based SNP marker for drought or salinity tolerance in foxtail millet* (*Setaria italica* (L.) Beauv) [Master thesis, IPB University]. IPB University Scientific Repository.

Zhang, J., Li, K., Liao, D., Pardinas, J. R., Chen, L., & Zhang, X. (2003a). Different applications of polymerases with and without proofreading activity in single-nucleotide polymorphism analysis. *Laboratory Investigation, 83*(8),1147–1154. https://doi.org/10.1097/01.LAB.0000081589.91390.DF

Zhang, J., Li, K., Deng, Z., Liao, D., Fang, W., & Zhang, X. (2003b). Efficient mutagenesis method for producing the template of single nucleotide polymorphisms. *Molecular Biotechnology, 24*, 105-10. https://doi.org/10.1385/MB:24:2:105