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Optimization of SCoT-PCR reaction system in *Dactylis glomerata* by orthogonal design

B. Zeng[1]*, X. Huang[2]*, L.K. Huang[2], J. Zhang[3], H.D. Yan[1], D. Luo[1], H. Liang[1] and Y. Yuan[1]

[1]Department of Animal Science, Southwest University, Rongchang, Chongqing Province, China
[2]Department of Grassland Science, College of Animal Science and Technology College, Sichuan Agricultural University, Ya'an, Sichuan Province, China
[3]Chongqing Academy of Animal Sciences, Yuzhong, Chongqing Province, China

*These authors contributed equally to this study.
Corresponding authors: L.K. Huang / J. Zhang
E-mail: huanglinkai@sicau.edu.cn / zjturf@163.com

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ABSTRACT. The effects of 5 factors (template DNA, Mg^{2+}, dNTPs, *Taq* DNA polymerase, and primer) on the polymerase chain reaction (PCR) were investigated to optimize the start codon targeted polymorphism (SCoT)-PCR system of *Dactylis glomerata* L., using an orthogonal design L_{16} (4^5). A suitable SCoT-PCR system for *D. glomerata* was established; the 20 µL reaction volume contained 3.0 mM Mg^{2+}, 0.2 mM dNTPs, 1.0 U *Taq* DNA polymerase, 0.2 µM primer, 20 ng template DNA, and 2 µL 10X buffer. Each factor had a different effect on the amplification reaction, and the concentration of dNTPs had the largest effect on the SCoT-PCR system. We tested 10 orchardgrass samples to determine and verify the stability of the reaction system. The results showed that amplified bands from diverse materials were clear, stable,

and rich in polymorphisms, indicating that the optimized system was very stable.

Key words: *Dactylis glomerata* L.; Reaction system; Optimization; Start codon target polymorphism-polymerase chain reaction; Orchardgrass; Orthogonal design

INTRODUCTION

Orchardgrass, or cocksfoot (*Dactylis glomerata* L.), is a common species of Poaceae in the genus *Dactylis* and is one of the most important cool-season perennial C_3 bunchgrasses. This plant is commonly used for forage and hay production worldwide because of its remarkable local adaptation, good tolerance to shadow (and thus is also known as orchardgrass), high yield, and high sugar content (Horn et al., 1988; Bushman et al., 2011; Xie et al., 2012). It plays an important role as forage material in North America, Europe (Casler et al., 2000), and Oceania, as well as in grassland animal husbandry and ecological construction of southern subtropical mountains of China. Because of the importance of orchardgrass as a forage and hay grass, in recent years, a number of studies have been performed to examine orchardgrass morphology (Felfoldi, 1975; Turner et al., 2012), anatomy (Ashenden, 1978), cytology (Lentz et al., 1983; Tosun et al., 1999), physiology (Davidson and Milthorpe, 1966; Yoshida and Uemura, 1984; Volaire, 1995; Volaire and Lelièvre, 2001), ecology (Eagles, 1983; Fan, 1997; Kyriazopoulos et al., 2013), and breeding (Denchev et al., 1997; Casler et al., 2000; Hopkins and Bhamidimarri, 2009). Molecular-level studies of orchardgrass have also been conducted. Numerous types of molecular genetic marker systems have been developed for use in germplasm resources studies of orchardgrass, including amplified fragment length polymorphism markers (Reeves et al., 1998; Peng et al., 2008), random amplified polymorphic DNA markers (Kölliker et al., 1999; Tuna et al., 2004), sequence-related amplified polymorphism markers (Zeng et al., 2008; Scoles et al., 2010), inter-simple sequence repeat markers (Zeng et al., 2006), simple sequence repeat (SSR) markers (Xie et al., 2010, 2012; Song et al., 2011; Last et al., 2013), and expressed sequence tag-SSR markers (Bushman et al., 2011). These studies have revealed varying levels of molecular genetic diversity depending on the type of molecular marker and the population examined (Mulpuri et al., 2013). However, start codon targeted (SCoT) polymorphism has not been applied in orchardgrass.

The start codon targeted (SCoT) polymorphism is simple, novel, and reliable method for generating gene-targeted markers developed by Collard and Mackill (2009) and validated in rice (*Oryza sativa*). It uses 18-mer single primers with polymerase chain reaction (PCR); the primers are simple to design and used to amplify the genomic region based on conservation of the ATG translation start site and flanking sequences in plant genes (Joshi et al., 1997; Collard and Mackill, 2009). SCoT is similar to random amplified polymorphic DNA or inter-simple sequence repeat, but has some advantages such as being closely linked with the target gene, provides more information correlated with biological traits, and universality in plants compared with random DNA markers. These advantages have been verified in rice (*Oryza sativa*) (Collard and Mackill, 2009), mango (*Mangifera indica*) (Luo et al., 2011), peanut (*Arachis hypogaea*) (Xiong et al., 2011), grape (*Vitis vinifera*) (Guo et al., 2012), dendrobe (*Dendrobium nobile*) (Bhattacharyya et al., 2013), and limpograss (*Hemarthria altissima*) (Huang et al., 2013) for cultivar identification and genetic diversity analysis.

Despite the advantages of the SCoT method, there are also limitations, such as the lack of polymorphisms detected because of the low stringency PCR conditions. SCoT relies on PCR-based methods, and the stability of the PCR is affected by numerous factors, including Mg^{2+}, dNTPs, primer, and *Taq* DNA polymerase concentrations, and diverse species required different PCR conditions. Hence, it is important to establish a stable and optimized reaction system for use with SCoT molecular markers. PCR reaction conditions are traditionally optimized using the one-factor-at-a-time method, neglecting the interactions between the various factors. We employed an orthogonal design referred to as L_{16} (4^5) (4 levels of 5 factors: *Taq* DNA polymerase, Mg^{2+}, DNA template, dNTPs, and primer) to optimize the SCoT-PCR system for orchardgrass and to provide a method for studying genetic diversity, genetic relationships, the construction of molecular linkage genetic maps, variety identification, gene localization, quantitative trait loci analysis, and molecular marker-assisted breeding in orchardgrass.

MATERIAL AND METHODS

Tested materials

The materials used in this study were collected from the Teaching and Research Center at Southwest University (Rongchang), Chongqing in China in May 2013 (Table 1). Fresh young leaves were sampled directly from soil, placed in a refrigerator, and stored at -80°C. The sequence of the SCoT primer was as described by Luo et al. (2011), SCoT 1: 5'-CAACAATGGCTACCACGC-3' and SCoT 2: 5'-ACAATGGCTACCACTGCC-3', and were synthesized by Shanghai Shenggong Biological Engineering Technology Services Ltd. (Shanghai, China). Mg^{2+}, dNTPs, *Taq* DNA polymerase, 10X buffer, 6X buffer, and DL2000 marker were purchased from Takara Biotechnology (Dalian) Co., Ltd. (Shiga, Japan).

Table 1. Names and types of orchardgrass samples used in this study.

No.	Material name	Type
1	Bao Xin	Cultivar
2	Parthlong	Wild Material
3	PG337	Wild Material
4	90-130	Wild Material
5	Smithii	Cultivar
6	Bueno chile	Wild Material
7	Judiaca	Wild Material
8	General Belgrano	Cultivar
9	PG318	Wild Material
10	Crown	Cultivar
11	Porto	Cultivar
12	Akimidori Japan	Cultivar
13	PG76xPorto	Hybrid Material

DNA extraction and SCoT-PCR program

DNA was extracted from fresh young leaf samples using a genomic DNA extraction kit (ComWin Biotechnology Co., Ltd., Beijing, China) following the manufacturer protocol. The quality and concentration of genomic DNA were determined using 1% (wt/vol) agarose gel electrophoresis and spectrophotometric analysis with the NanoDrop 2000 nucleic acid/

protein analyzer (Nanodrop Technologies, Wilmington, DE, USA). Isolated genomic DNA was diluted to 20 ng/mL and stored at -20°C.

PCR amplification reaction was performed in an Eppendorf Mastercycler (Hamburg, Germany) using the following program: 3 min at 94°C, followed by 36 cycles of denaturing at 94°C for 30 s, annealing at 50°C for 1 min, extension at 72°C for 2 min, followed by a final extension at 72°C for 10 min and storage at 4°C. After amplification, 2 µL 6X buffer was added to the PCR amplification products. Next, 8-10 µL PCR products for each treatment were separated on 1.5% agarose gels in 1X Tris-borate EDTA buffer and stained with GoldView™ dye (Beijing Bioteke Biotechnology Co., Beijing, China). DNA fragments were visualized under UV light and photographed using a gel documentation system (Bio-Rad, Hercules, CA, USA).

Orthogonal optimization design of SCoT-PCR system

We examined the optimum concentrations of template DNA (Bao Xin), Mg^{2+}, dNTPs, *Taq* DNA polymerase, and primer (SCoT 1: 5'- CAACAATGGCTACCACGC-3') using an orthogonal design L_{16} (4^5). The 4 concentrations for each of the 5 factors were chosen based on references and experience, and the levels and detailed experimental concentrations for each factor are listed in Table 2. The L_{16} (4^5) orthogonal experimental design is shown in Table 3. Amplification conditions were as follows: total volume of 20 µL, 2.0 µL 10X buffer and other components, and ddH_2O to reach the total volume.

Table 2. Factors and volume levels of SCoT-PCR amplification for *Dactylis glomerata*.

Levels	Factors				
	Mg^{2+} (mM)	dNTPs (mM)	*Taq* DNA polymerase (U)	Primers (µM)	Template DNA (ng)
1	1.5	0.15	0.75	0.2	20
2	2.0	0.20	1.00	0.3	30
3	2.5	0.25	1.25	0.4	40
4	3.0	0.30	1.50	0.5	50

Table 3. L_{16} (4^5) orthogonal design of SCoT-PCR amplification for *Dactylis glomerata*.

Treatment No.	Mg^{2+} (mM)	dNTPs (mM)	*Taq* DNA polymerase (U)	Primers (µM)	Template DNA (ng)	Score
1	1.5 (1)	0.15 (1)	0.75 (1)	0.2 (1)	20 (1)	11
2	1.5 (1)	0.20 (2)	1.00 (2)	0.3 (2)	30 (2)	9
3	1.5 (1)	0.25 (3)	1.25 (3)	0.4 (3)	40 (3)	14
4	1.5 (1)	0.30 (4)	1.50 (4)	0.5 (4)	50 (4)	3
5	2.0 (2)	0.15 (1)	1.00 (2)	0.4 (3)	50 (4)	6
6	2.0 (2)	0.20 (2)	0.75 (1)	0.5 (4)	40 (3)	12
7	2.0 (2)	0.25 (3)	1.50 (4)	0.2 (1)	30 (2)	10
8	2.0 (2)	0.30 (4)	1.25 (3)	0.3 (2)	20 (1)	1
9	2.5 (3)	0.15 (1)	1.25 (3)	0.5 (4)	30 (2)	7
10	2.5 (3)	0.20 (2)	1.50 (4)	0.4 (3)	20 (1)	13
11	2.5 (3)	0.25 (3)	0.75 (1)	0.3 (2)	50 (4)	4
12	2.5 (3)	0.30 (4)	1.00 (2)	0.2 (1)	40 (3)	8
13	3.0 (4)	0.15 (1)	1.50 (4)	0.3 (2)	40 (3)	5
14	3.0 (4)	0.20 (2)	1.25 (3)	0.2 (1)	50 (4)	15
15	3.0 (4)	0.25 (3)	1.00 (2)	0.5 (4)	20 (1)	16
16	3.0 (4)	0.30 (4)	0.75 (1)	0.4 (3)	30 (2)	2

Stability of optimal reaction system

We used the DNA samples to examine the stability of the highest scored orthogonal experiment treatment and the statistical optimal treatment. We also selected the best treatment to verify the stability of the reaction system using the primer SCoT 2 and 10 orchardgrass samples.

RESULTS

Visual analysis of PCR orthogonal design

The PCR amplification products of the orthogonal experiment treatments showed variable fingerprinting patterns, treatment 8 showed no fragments, and treatment 15 showed the largest number of fragments (Figure 1). A scoring system was applied to determine variance between the SCoT-PCR fingerprint patterns using different treatments. According to He et al. (1998), DNA amplification patterns generated were scored on the scale of the best (16 points) to worst (1 point) using scoring criteria, including amplified fragment number and clear degree of PCR amplification results (Table 3). According to the score for each treatment, the experimental value K_i of the score for each factor at the same level, the experimental value ki of mean of score in each factor under levels, and the range R of the mean of score for the same factors between different levels are shown in Table 4.

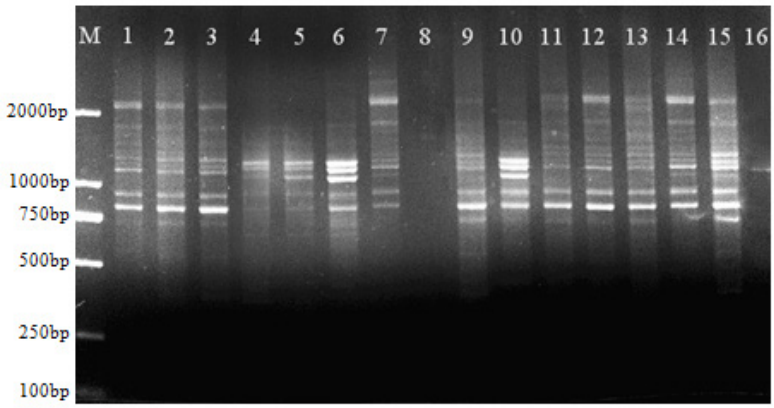

Figure 1. Electrophoresis using orthogonal design to obtain PCR products. *Lane M* = DNA marker DL2000; *lanes 1-16*: Treatment numbers are same as those in Table 1.

Table 4. Analysis of orthogonal design.

Results	Mg^{2+} (mM)	dNTPs (mM)	*Taq* DNA polymerase (U)	Primers (μM)	Template DNA (ng)
K_1	37.00	29.00	29.00	44.00	41.00
K_2	29.00	49.00	39.00	19.00	28.00
K_3	32.00	44.00	37.00	35.00	39.00
K_4	38.00	14.00	31.00	38.00	28.00
k1	9.25	7.25	7.25	11.00	10.25
k2	7.25	12.25	9.75	4.75	7.00
k3	8.00	11.00	9.25	8.75	9.75
k4	9.50	3.50	7.75	9.50	7.00
R	2.25	8.75	2.50	6.25	3.25

Range R reflects the influence template DNA, Mg^{2+}, dNTPs, *Taq* DNA polymerase, and primer concentrations on the reaction system. A larger range for R indicated a more significant influence of the factor. We found that the factors affected fingerprinting patterns in the order of dNTPs, primers, template DNA, *Taq* DNA polymerase, and Mg^{2+}, from largest to smallest effect.

The values ki reflects the influence of each factor under each level on the reaction system; a larger ki value indicates a better reaction level. The ki value can indicate the optimal concentration of each factor. By transforming the ki value of mean of score in each factor under levels into line charts, the relationship between the 5 factors and the mean of result of PCR orthogonal design observed (Figure 2). An Mg^{2+} concentration of 2 mM showed poor performance in the amplification reaction, while other tested Mg^{2+} concentrations showed good amplification results; the best performance was observed at 3.0 mM Mg^{2+} (Figure 2A).

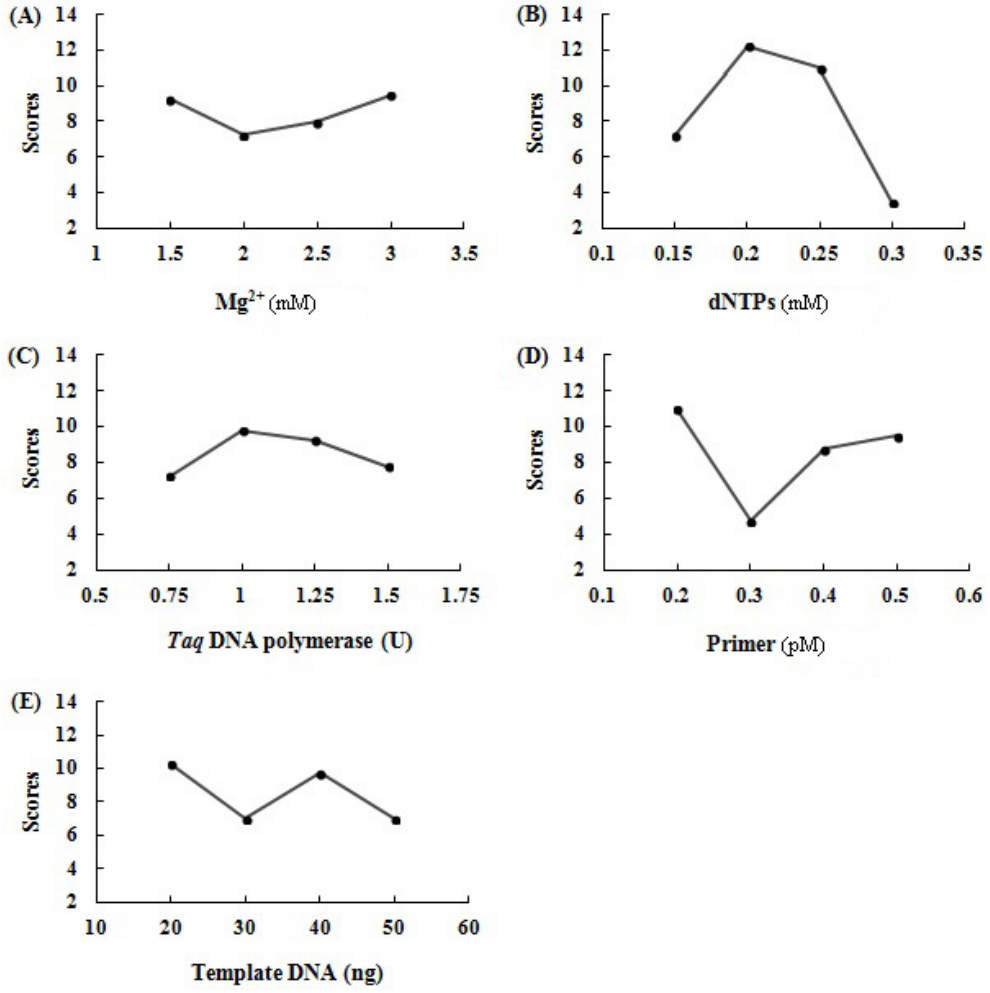

Figure 2. Relationship between 5 factors of (**A**) Mg^{2+}, (**B**) dNTPs, (**C**) *Taq* DNA polymerase, (**D**) Primer, (**E**) Template DNA, as well as the mean of score of PCR orthogonal design.

dNTPs are the substrates of *Taq* DNA polymerase, and their concentrations directly affect the results of the PCR. In the study, changes in dNTP concentration had a large impact on the orchardgrass SCoT-PCR reaction. With increasing dNTP concentration, the mean of result of PCR orthogonal design increased and then decreased. A dNTP concentration of 0.2 mM was associated with the best amplification results (Figure 2B). *Taq* DNA polymerase is a key factor in the PCR. The fingerprint patterns showed large differences at varying *Taq* DNA polymerase concentrations of 0.75-1.5 U, and the best fingerprint patterns were obtained at 1.0 U *Taq* polymerase (Figure 2C). Primer concentration also plays an important role in fingerprinting band patterns, and high primer concentration can increase the formation of primer dimers. Hence, the concentration with the highest score, 0.2 μM, should be used (Figure 2D). Appropriate DNA concentration is a prerequisite for amplification; high DNA concentration will increase nonspecific amplification products. As shown in Figure 2E, template DNA concentrations of 20 and 40 ng showed good performance in the amplification reaction, and the best performance was observed at a concentration of 20 ng.

Based on our results, we found that the most suitable concentration of the 5 factors in this study were as follows: 3.0 mM Mg^{2+}, 0.2 mM dNTPs, 1.0 U *Taq* DNA polymerase, 0.2 μM primer, and 20 ng template DNA. These concentrations showed some differences in amplification efficiency compared to the highest scored treatment (3.0 mM Mg^{2+}, 0.25 mM dNTPs, 1 U *Taq* DNA polymerase, 0.5 μM primer, and 20 ng template DNA). To verify the efficiency of the conditions showing the highest score, different template DNA was amplified. As shown in Figure 2, the amplification results of the 2 systems were good, and the fingerprinting patterns were very similar, but the amplified fragments for the statistically optimal treatment were brighter than those for the highest scored treatment (Figure 3). Therefore, the most suitable SCoT-PCR reaction system was 20 μL and contained 3.0 mM Mg^{2+}, 0.2 mM dNTPs, 1.0 U *Taq* DNA polymerase, 0.2 μM primer, 20 ng template DNA, and 2 μL 10X buffer.

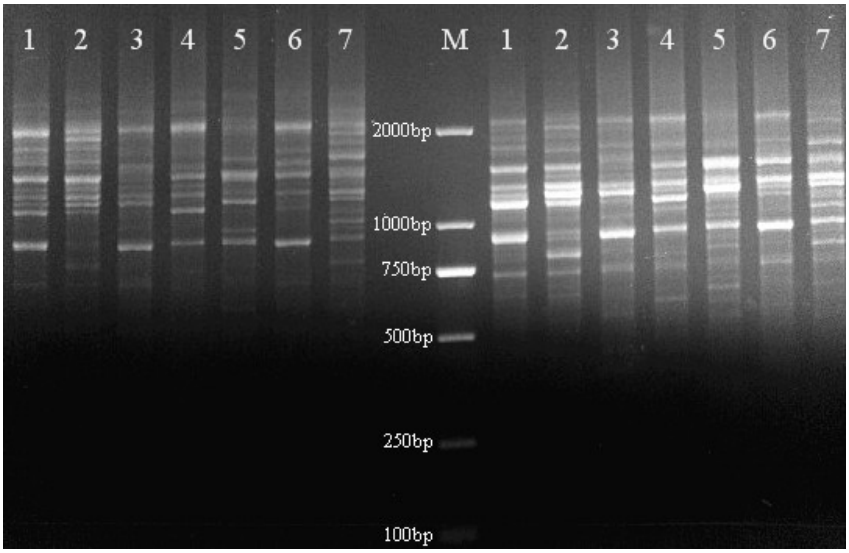

Figure 3. Electrophoresis of the statistical optimal reaction system (right) and the highest scored orthogonal reaction system (left). *Lane M*: DNA marker DL2000; *lanes 1-7*: Parthlong, PG337, Bueno Chile, PG318, Porto, Akimidori Japan, PG76 x Porto.

Stability of the optimized reaction system

The primer SCoT 2 and 10 orchardgrass samples were used to verify the stability of the optimal reaction system. Figure 4 shows that the amplification bands of diverse samples were clear, stable, and rich in polymorphisms. The fingerprinting patterns of the 10 orchardgrass samples not only reflected the genetic stability of each sample, but also revealed the genetic differences between samples, indicating that the optimized reaction system can be applied for SCoT-PCR analysis of orchardgrass.

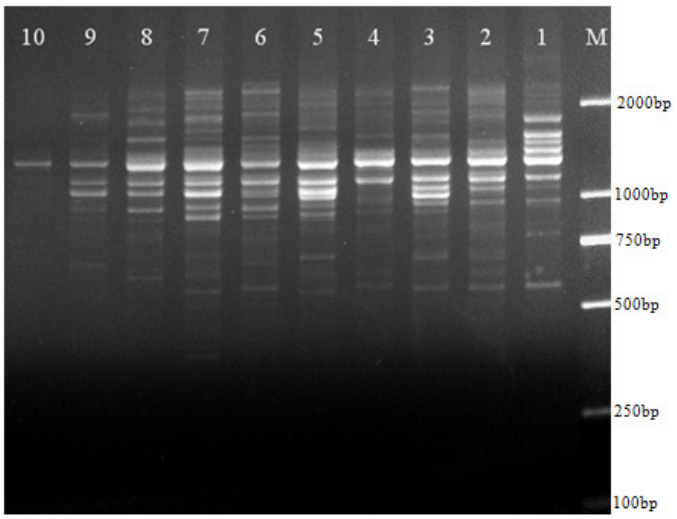

Figure 4. Electrophoresis of materials of orchardgrass using the optimized reaction system. *Lane M*: DNA marker DL2000; lanes 1-10: 90-130, Smithii, Bueno Chile, Judiaca, General Belgrano, PG318, Crown, Porto, Akimidori Japan, PG76 x Porto.

DISCUSSION

Variations in the 5 factors tested changed the fingerprinting patterns significantly, and dNTP concentration was considered to be the most important factor affecting the PCR, which is consistent with the results obtained by Wang et al. (2007). Low dNTP concentration produce relatively poor amplification fingerprints, while high concentration compete with *Taq* DNA polymerase for Mg^{2+}, reducing Mg^{2+} concentration and the activity of *Taq* DNA polymerase, eventually leading to the low PCR amplification levels. Therefore, the most effective dNTP concentration was a median of 0.2 mM. Mg^{2+} concentration had a minimal impact on the PCR results in our study. However, this result was in contrast to those of Xie et al. (2008) who used SSR markers analysis. This may be because Mg^{2+} concentration was influenced by the interaction of various factors (*Taq* DNA polymerase, dNTPs, etc.) in the PCR system, thus showing varying levels of performance; this may be associated with the orthogonal design and the use of subjective scoring.

It is important to optimize the SCoT-PCR system for orchardgrass. Using an orthogonal design L_{16} (4^5) to optimize the SCoT-PCR system in this study, we developed a rapid and simple method for investigating the mutual effects of factors and obtain satisfactory results.

Our SCoT-PCR system can be used for follow-up studies to further optimize the SCoT-PCR conditions in orchardgrass, particularly for genetic diversity analysis and molecular marker-assisted breeding of orchardgrass using SCoT.

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