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Fragrance related candidate gene specific SSR based PCR optimization in landraces and improved varieties of aromatic rice

Abhay Kumar and VK Sharma

Abstract
PCR has been extensively used for the amplification of DNA sequences. We conducted a study to obtain the best amplification condition for detection of fragrance related candidate gene specific SSR polymorphism in aromatic rice. Using leaf samples collected from young seedlings, DNA was isolated from 18 genotypes including landraces and improved varieties of aromatic rice. The extracted DNA sample of any five genotypes was randomly taken for PCR optimization. The reaction condition optimized through this study included a combination of 5 X PCR buffer (3.0 µl), 10 mM $MgCl_2$ (1.2 µl), 200 µM dNTP (3 µl), forward primer (1.2 µl), reverse primer (1.2 µl)), template DNA (1.5 µl) and 1 unit / ml Taq polymerase (0.5 µl). PCR was performed using initial denaturation for 5 minutes at 94 ^{0}C followed by 35 cycles of denaturation for 40 seconds at 94 ^{0}C, annealing for 1 min at 55-65 ^{0}C and extension for 2 minutes at 72 ^{0}C and final extension for 10 minutes at 72 ^{0}C. Utilizing 38 designed candidate gene-based SSR primer pairs, the optimized PCR condition produced sharp bands for molecular characterization of aromatic rice genotypes.

Keywords: aromatic rice, candidate genes, PCR optimization, SSR markers

1. Introduction
Rice is the most conspicuous crop of the country as it provides staple nourishment for the vast majority of the individuals (Mahajan *et al.*, 2017) [11]. As the human population continues to grow, agricultural production in general and rice production in particular must also keep pace. Most research and developmental effort in agriculture focuses on increasing crop production and improving crop quality (Frona *et al.*, 2019) [6]. Increasing emphasis on crop diversification and land diversification towards more remunerative horticultural crops as well as high yielding varieties has seriously contributed to the erosion of rice biological diversity. Consequently, there is an urgent need to identify and characterize the rice germplasm of specialized resources using biochemistry and molecular biology tools for their conservation and need based utilization in breeding programs.

PCR has become an essential technique in the field of molecular biology research for the amplification of a specific segment of DNA. Since the discovery of PCR (Saiki *et al*. 1988) [15], it has become a fundamental technique in present-day molecular biology research, including population genetic analysis, food quality testing, varietal selection, genetic resource management and genomic assisted crop breeding programs. PCR is most reliable due to its sensitivity, accuracy and speed. However, the PCR conditions need to be optimized in order to meet the specific requirements such as to amplify templates, which are AT or GC-rich or have a high number of tandem repeats. A prerequisite for achieving selective amplification through PCR, particularly in analytical applications is PCR optimization. While setting up PCR for the first time with new template DNA, new primers or a new preparation of Taq polymerase enzyme, less than optimal amplification is generally obtained. Fine-tuning of the reaction is usually required to suppress non-specific amplification and/or enhance the sensitivity of reaction. Depending on the success of the amplification, it therefore becomes necessary to optimize the PCR technique.

Very limited number of studies have focused on optimization of candidate-gene-specific SSR based PCR system as part of the biological indicator for accessing molecular genetic information in rice. The protocol optimized for candidate gene specific SSR will provide an inexpensive targeted amplification procedure especially in terms of use of $MgCl_2$, template DNA, primer, Taq polymerase and reaction volume. In this paper, we optimized the concentration of $MgCl_2$, the amount of DNA template, the concentration of primer pairs, the concentration of Taq polymerase and the annealing temperature to amplify the fragrance specific candidate gene-based SSR in aromatic rice.

2. Material and Methods

The DNA was extracted from 18 genotypes of aromatic rice, namely, Pusa Sughandha-1, Pusa Sughandha-2, Pusa Sughandha-3, Pusa Sughandha-5, Pusa-1121, Pusa-1509, Rajendra Kasturi, Rajendra Suwasini, Rajendra Saraswati, Basmati 370, Ranbir Basmati, Sanwal Basmati, Taraori Basmati, BurmaBhusi, Birsamati, Jasmine, Lalmati, and Marcha. The extracted DNA sample from any five genotypes was taken randomly for PCR optimization. The experiment was conducted in the Molecular Biology Laboratory, Department of Agricultural Biotechnology and Molecular Biology, RPCAU, Pusa, Samastipur, Bihar, India.

A. DNA Isolation

The genomic DNA was isolated from 18 genotypes comprising landraces and improved varieties of aromatic rice using the immature leaves of two to three weeks older seedlings. CTAB method was used to isolate genomic DNA from each genotype with some minor modifications. About 300 mg of fresh leaves were weighed, cut into small pieces and finely crushed with help of a glass rod in the 2 ml tube using 1000 µl of 3X DNA extraction buffer. While grinding, 40 µl of β-mercaptoethanol was added and incubated at 65°C in a water bath for 30 minutes. The extracted samples were then subjected to centrifugation at 14000 rpm for 10 minutes at room temperature. The supernatant was subsequently transferred to a fresh 2 ml Eppendorf tube and emulsified by adding 300 µl of C:I (Chloroform: isoamyl alcohol, 24:1) followed by centrifugation at 13500 rpm for 10 minutes. The aqueous phase was transferred to a new fresh Eppendorf tube (2 ml) and 600 µl of C:I (Chloroform: isoamyl alcohol) was added to it, followed by centrifugation at 13500 rpm for 10 minutes. The process was repeated for getting a transparent aqueous phase, if required. The transparent aqueous phase was again transferred to a new fresh 1.5 ml centrifuge tube, and 600 µl of chilled isopropanol was added. It was then incubated at -20 ^{0}C for 15 to 20 minutes. After incubation, tubes were centrifuged at 13500 rpm for 10 minutes for the DNA pellet to settle down. The supernatant was discarded, followed by addition of 300 µl of 70% ethanol to the pellet and centrifugation was done at 10000 rpm for 5 minutes. After centrifugation, ethanol was discarded and the pellet was allowed to air dry for 1 hour. The dried pellet was dissolved in 40 µl of TE (Tris-EDTA) buffer. To remove RNA contamination, 2 µl of RNase treatment was given. Finally, DNA samples were stored at -20 ˚C. Now, DNA was then ready to be utilized in agarose gel electrophoresis. The DNA was now prepared for qualitative and quantitative analysis using agarose gel electrophoresis and Nanodrop Spectrophotometer, respectively.

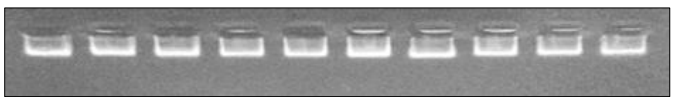

Fig 1: Image of the extracted DNA from the aromatic rice genotypes using CTAB method at 0.8 % agarose, 90 volts, and 30 minutes

B. Qualitative and Quantitative Analysis

The quality of isolated DNA samples was determined by agarose gel electrophoresis using 0.8 % agarose. Determination of the location of DNA within the gel was done by staining with a low concentration of ethidium bromide that acted as a fluorescent intercalating dye. The DNA concentrations were determined using the Nanodrop Spectrophotometer by loading 1 µl of each DNA sample on Nanodrop for quantification. The purity of recovered total DNA was checked by measuring the absorbance ratio at (260-280) nm. The sample with absorbance (A260/A280) ratio in the range of 1.85 - 1.95 was considered the best quality DNA sample. The final DNA concentration was diluted by using TE buffer.

C. PCR Master Mix Optimization

The concentrations of PCR components such as $MgCl_2$, Taq polymerase, DNA template and primer were optimized (Table 2) by giving total volume of 15 µl for each reaction.

D. Primers design and PCR optimization

Altogether 38 primers were designed from nine fragrance related candidate genes, namely, Os03g0327600, Os04g0352400, Os04g0434800, Os04g0401700, Os04g0438300, Os04g0468600, Os04g046970, Os08g0424500 and OsNPB_0504555500 (Pachauri *et al.*, 2014; Kaikavoosi *et al.*, 2015) [14, 7] by using BatchPrimer3 web-based application. The DNA template from five genotypes was randomly taken to determine the optimal PCR amplification temperature. All primers had ten different annealing temperatures ranging from 55 to 65 ^{0}C. Selective amplification of targeted region in genomic templates was performed by adopting a standardized protocol of polymerase chain reaction optimized to laboratory condition. The candidate gene-specific microsatellite primer directed amplification reaction was successfully conducted in a thermal cycler by utilizing 15 µl of the reaction mixture. The standardized PCR reaction mixture for 15 µl volume consisted of 3.4 µl nuclease-free water, 3.0 µl 5 X PCR buffers, 1.2 µl 2mM $Mgcl_2$, 3 µl of 10mM dNTP mixed, 1.2 µl of 10µM forward primer, 1.2 µl of 10µM reverse primer, 1.5 µl of DNA template (40-80 ng/ µl) and 0.5 µl of *Taq polymerase*. Polymerase chain reaction-based amplification was performed by adopting a usable program of thermal cycler standardized for this purpose, which included initial denaturation for 5 minutes at 94 ^{0}C followed by 35 cycles of denaturation for 40 seconds at 94 ^{0}C, annealing for 1 min at 55-65 ^{0}C and extension for 2 minutes at 72 ^{0}C and final extension for 10 minutes at 72 ^{0}C.

E. Agarose gel electrophoresis for amplified products

Agarose gel (2%) was prepared by adding 2g of agarose in 100 ml of TBE (0.5X) in a volumetric flask. The solution was placed in the microwave oven to boil with intermittent mixing until complete melting of agarose was achieved, and the solution became crystal clear. The liquid gel was allowed to obtain 60 ^{0}C to add ethidium bromide (5µl). Subsequently, the liquid gel was carefully decanted in the gel casting plate of the gel casting unit; the formation of any bubble was avoided and leave it for 20-30 minutes at room temperature for the gel solidification. After solidification, comb was gently taken out to form wells. The gel was then placed in the migration chamber connected to the power pack. The running buffer (0.5X TBE) was filled to submerge the gel. The gel loading dye (40 % sucrose and 0.25 % bromophenol blue) was added to the amplified products to track the DNA movement. Samples, along with a 50 bp DNA ladder to determine the molecular sizes were loaded carefully into the wells with micropipette and micro-tips. The separation was carried out by applying 110V for 1hr 30min, and then the amplified products were indirectly visualized under UV (332 nm) light in the gel documentation system (Bio-Rad).

Table 1: Optimization of PCR by using different volumes of reagents in combination with primer CHR3.1_2 at 60 ^{0}C

Reagent	$MgCl_2$						Reagent	Taq					
H_2O	4.6	3.6	3.4	3.1	2.6	1.6	H_2O	3.9	3.8	3.7	3.6	3.3	2.9
$MgCl_2$	00	1.0	1.2	1.5	2.0	3.0	$MgCl_2$	1.2	1.2	1.2	1.2	1.2	1.2
PCR Buffer	3.0	3.0	3.0	3.0	3.0	3.0	PCR Buffer	3.0	3.0	3.0	3.0	3.0	3.0
dNTP	3.0	3.0	3.0	3.0	3.0	3.0	dNTP	3.0	3.0	3.0	3.0	3.0	3.0
F-Primer	1.2	1.2	1.2	1.2	1.2	1.2	F-Primer	1.2	1.2	1.2	1.2	1.2	1.2
R-Primer	1.2	1.2	1.2	1.2	1.2	1.2	R-Primer	1.2	1.2	1.2	1.2	1.2	1.2
DNA Template	1.5	1.5	1.5	1.5	1.5	1.5	DNA Template	1.5	1.5	1.5	1.5	1.5	1.5
Taq	0.5	0.5	0.5	0.5	0.5	0.5	Taq	00	0.1	0.2	0.3	0.6	1.0
Reagent	DNA Template						Reagent Primers						
H_2O	4.9	4.4	3.9	3.4	2.9	1.9	H_2O	5.8	5.4	4.8	3.4	2.8	1.8
$MgCl_2$	1.2	1.2	1.2	1.2	1.2	1.2	$MgCl_2$	1.2	1.2	1.2	1.2	1.2	1.2
PCR Buffer	3.0	3.0	3.0	3.0	3.0	3.0	PCR Buffer	3.0	3.0	3.0	3.0	3.0	3.0
dNTP	3.0	3.0	3.0	3.0	3.0	3.0	dNTP	3.0	3.0	3.0	3.0	3.0	3.0
F-Primer	1.2	1.2	1.2	1.2	1.2	1.2	F-Primer	00	0.2	0.5	1.2	1.5	2.0
R-Primer	1.2	1.2	1.2	1.2	1.2	1.2	R-Primer	00	0.2	0.5	1.2	1.5	2.0
DNA Template	00	0.5	1.0	1.5	2.0	3.0	DNA Template	1.5	1.5	1.5	1.5	1.5	1.5
Taq	0.5	0.5	0.5	0.5	0.5	0.5	Taq	0.5	0.5	0.5	0.5	0.5	0.5

3. Results and Discussion

The purity of DNA for all samples had a stable absorption ratio according to the absorption range. The outcomes were in agreement with the earlier report (Ferdous *et al*., 2012) [5] that the purity or quality of DNA is more than sufficient if absorption ratio (A260 / A280) varies from 1.85 to 1.95. The recommended values for the A260/A280 ratio range from 1.8-2.0, and the absorption ratio at A260/230 is 2.0-2.22 for impurity-free DNA (Arruda *et al.,* 2017) [2].

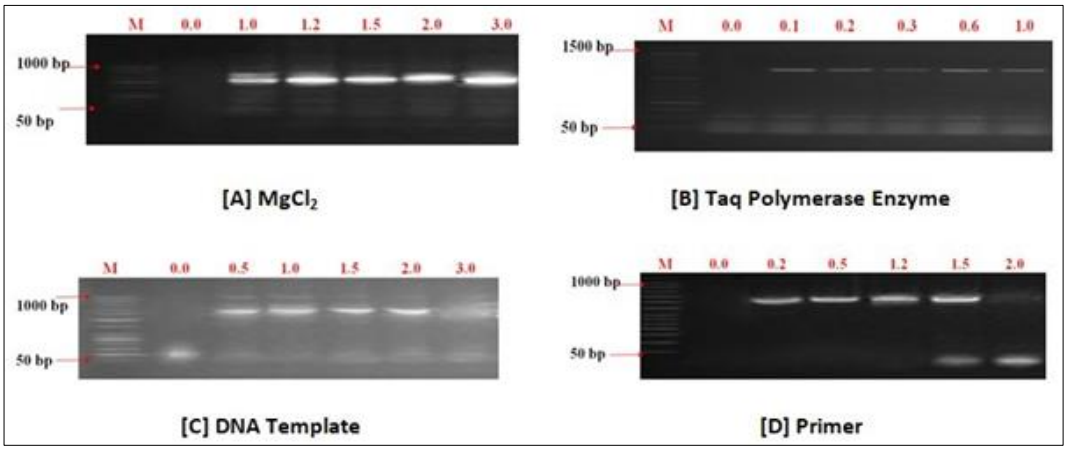

Fig 2: Optimization of the PCR parameters at different volumes (µl) using primer CHR3.1_2 at 2 % agarose

In order to obtain successful PCR amplification, 5X PCR buffer, 10 mM $MgCl_2$, 10 mM dNTP, 10 µM primer, 10 µM reverse primer, DNA template and 1 unit/ml Taq polymerase were required at explicit concentrations for the selection of relatively polymorphic bands, which can be reproduced and produced as clear bands. We optimized $MgCl_2$ concentration, Taq polymerase concentration, DNA template concentration, and primer concentrations and excluded buffer concentration and dNTPs from consideration. Based on previously reported standardized PCR protocols (Padmalatha and Prasad, 2006; Anerao *et al.,* 2016; Kumar *et al.,* 2018; Subedee *et al.,* 2020) [13, 1, 8, 17], the buffer concentration and dNTPs concentration were given in the fixed amount (3 µl) in all optimized conditions (Table 2). Magnesium ions work as a cofactor for the activity of the Taq polymerase. They also interact with other components in the reaction mixture, form complexes with dNTPs and stabilize dsDNA, increasing its melting temperature. Each new PCR experiment, therefore, requires the optimization of the magnesium salt concentration. Sub optimal $MgCl_2$ concentration may lead to low amplification efficiency or fainted bands may be seen. Conversely, too high concentration results in the appearance of non-specific amplified products (Batra *et al.,* 2006) [3]. For the optimization purpose, $MgCl_2$ was used at different concentrations (0, 1.0, 1.2, 1.5, 2.0 and 3 µl) (Table. 1) presented at 1.2 µl (Table.2) $MgCl_2$ per PCR Master Mix (15 µl) where amplification showed precise amplification of PCR products that were reproducible and produced clear bands (Figure 2.A).

Table 2: Concentration of optimized PCR components ustilized in master mix

Reagent	Final Concentration	Volume (15µl)
Nuclease free water	_	3.4
5X PCR buffer	1X	3.0
2mM $Mgcl_2$	0.2 mM	1.2
10 mM dNTPs mix	0.2 mM	3.0
10µM forward primer	0.5 µM	1.2
10µM reverse primer	0.5 µM	1.2
Template DNA	40-80 ng/ µl	1.5
Taq Polymerase	0.1 unit/ µl	0.5

The optimization of *Taq polymerase* was done by using its different concentrations (0, 0.1, 0.2, 0.3, 0.6, and 1.0 µl). The results of the gel electrophoresis image showed all concentrations reproduced the amplification of PCR products, but the clear bands were observed with 0.1 µl of *Taq*

polymerase (Figure 2.B). DNA template concentrations (0, 0.5, 1, 1.5, 2.0 and 3.0 µl) were optimized and it was observed that all concentrations between 0.5 - 2 µl reproduced the amplified PCR products. However, the clarity of the bands decreased with 0 .5 µl template DNA in contrast to the higher concentration (3.0 µl), which did not amplify or resulted in almost invisible smear (Figure 2.C). Higher DNA template concentrations may result in non-specific amplification or may not be amplified, as sometimes the DNA duplex inhibits DNA polymerase from binding (Vestheim and Jarman, 2008) [18]. The forward and reverse primer concentrations (0, 0.2, 0.5, 1.2, 1.5, and 2.0 µl) were optimized separately in the present study. The concentration of 1.2 µl of forward and reverse primer showed desirable band clarity of PCR amplified products (Figure 2.D). The PCR reaction buffers are selected to provide buffering pH conditions and a monovalent salt environment for optimal polymerase activity (Shahzad *et al.*, 2020) [16]. In our case, 3 µl PCR buffers elicited precise PCR amplification. It is well conceived that dNTPs are substrates for the PCR reaction and they allow the synthesis of new DNA molecules. The optimal concentration of dNTPs depends on the length of the amplified product, Mg^{2+} and the primer concentration (Lorenz, 2012) [9]. In the present experiment, 3 µl of 200 µM dNTP was used. Finally, the optimized PCR reagents, which produced sharp band, were selected as 5 X PCR buffer (3.0 µl), 10 mM $MgCl_2$ (1.2 µl), 200 µM dNTP (3 µl), forward primer (1.2 µl), reverse primer (1.2 µl)), template DNA (1.5 µl) and 1 unit / ml Taq polymerase (0.5 µl) (Table 2 and Figure 2).

The optimum annealing temperature for a particular PCR set-up depends mainly on the primers, the DNA template (length of the targeted fragment of the template, GC-content), and the salts concentration in the PCR buffer (Ekman, 1999) [4]. The optimal annealing temperature is the most critical factor in the PCR to obtain high quality amplified products in the form of sharp bands (Moraga *et al.*, 2012) [12]. While higher annealing temperature leads to a lack of amplification, too low annealing temperature dramatically increases the chances of non-specific binding (McPherson and Moller, 2006; Lorenz, 2012) [10, 9]. The chances of the primer dimer formation could be reduced by optimizing the annealing temperature. Primer-dimers will form if the primers have one or more complementary bases so that base pairing between the 3' ends of the two primers can occur (Yang *et al.*, 2020) [19]. Optimal annealing temperature for the selected primers determined in this study clearly reflects that fragrance related candidate gene-based 38 SSR primers gave positive amplification at ten different optimal temperatures (Table 3 and Table 4).

4. Conclusion

This study shows that optimal concentration of the four components, namely, $MgCl_2$, Taq polymerase, DNA template and primer concentration is essentially required for getting reproducible and recognizable amplification in combination with candidate gene-based primer. The conditions described in the current work demonstrate the ability to use candidate gene-specific SSR primers as an influential determinant for need based genetic studies at molecular level in rice species. The present optimized protocol for candidate gene-specific SSR technique may serve as a strong starting point for future genetic improvement and molecular characterization works in aromatic rice.

Table 3: Optimal annealing temperature for fragrance related candidate gene-specific 38 SSR primers

Sl. No.	Annealing Temperature (^{0}C)	Total primers	Name of the SSR primer
1	55	1	CHR8.1_13
2	56	1	CHR8.1_4,
3	57	6	CHR8.1_5, CHR8.1_7, CHR8.1_15, CHR8.1_19, CHR8.1_22 and CHR5.1_1
4	58	8	CHR4.1_1, CHR4.1_2, CHR8.1_2, CHR8.1_9, CHR8.1_16, CHR8.1_17, CHR8.1_18 and CHR8.1_20
5	59	7	CHR4.1_9, CHR4.1_13, CHR8.1_1, CHR8.1_6, CHR8.1_8, CHR8.1_12, and CHR8.1_14
6	60	8	CHR3.1_1, CHR3.1_2, CHR4.1_3, CHR4.1_4, CHR4.1_8, CHR4.1_10, CHR4.1_12 and CHR8.1_20
7	61	4	CHR4.1_5, CHR4.1_11, CHR8.1_10 and CHR8.1_23
8	62	1	CHR4.1_7,
9	63	1	CHR4.1_6
10	65	1	CHR8.1_11

Table 4: Summary of information for fragrance related candidate gene specific 38 SSR primers based PCR optimization

Sl. No	Primer ID	Primer Sequence (5′-3′)	Good amplification	Moderate amplification	No amplification	PS (bp)	Ta (^{0}C)
1	F- CHR3.1_1	TTTTTCCTTCACTCCAAGCAG	✓			699	60
	R- CHR3.1_1	AGTTGAGGCGGATGTTGTTC					
2	F- CHR3.1_2	CGTTGGTTTTGCTGACTCAC	✓			688	60
	R- CHR3.1_2	CACCAGCTTCACCTGTTTGA					
3	F- CHR4.1_1	GTCCGGGACAACCCAAAAG	✓			203	58
	R- CHR4.1_1	GACGGAGTACCGATGGTT					
4	F- CHR4.1_2	TTCAAAGGCTGTCAAGACGA	✓			701	58
	R- CHR4.1_2	TTTGCAGGAACAACAGCAAG					
5	F- CHR4.1_3	AAATACCCACCTCCACCACA	✓			695	60
	R- CHR4.1_3	CCCCTTCTTCATCGTCTTGA					
6	F- CHR4.1_4	AGGAGGCCAAGGAAATCAAT	✓			693	60
	R- CHR4.1_4	CACCTGCAACGTCATCAGAG					
7	F- CHR4.1_5	AGGCACAGAGGGAATGTTTG	✓			710	61
	R- CHR4.1_5	ACCTGCCCCTCGTACTTCTT					
8	F- CHR4.1_6	GGAGGTGGTGTACCTGTCGT	✓			701	63
	R- CHR4.1_6	GCTTCTCCTCCTCCACGTC					

9	F- CHR4.1_7	TCCTTAGCTTCTCCCTCCAG	✓			692	62
	R- CHR4.1_7	TCCCTATGACGTCAGAACCAT					
10	F- CHR4.1_8	GCCTTCTCCTCCACCTCCT	✓			711	60
	R- CHR4.1_8	AGGCAAGCTGGTCATGAAAT					
11	F- CHR4.1_9	CGCTTGGTTACTCGCATACA	✓			701	59
	R- CHR4.1_9	TCAATTTTGGGACGATAGCC					
12	F- CHR4.1_10	TCATATGGGCATGCATTCTG	✓			713	60
	R- CHR4.1_10	ATCCCCAACCTCCAGTTACC					
13	F- CHR4.1_11	TTCCCGCTGCTAATCGTTAC	✓			703	61
	R- CHR4.1_11	TCGTCATCGGCGTGTAGTAG					
14	F- CHR4.1_12	GATGGAGCAGGTGTGGAATC	✓			705	60
	R- CHR4.1_12	ATCAAATCGGCCTCATCATC					
15	F- CHR4.1_13	TTCTTCCTCGTCCCTGTGTT	✓			749	59
	R- CHR4.1_13	CCCTTGGGTCATGTTACACC					
16	F- CHR8.1_1	TCTCAGTTCTCCACATTTCTC	✓			155	59
	R- CHR8.1_1	TGTAGTTGAACTGGGCATATT					
17	F- CHR8.1_2	AATGCACATGCTCAATCATAC	✓			172	58
	R- CHR8.1_2	GCCCCATATGAGTGTCCTA					
18	F- CHR8.1_4	GGAGCACCAAACAAATTTAC		✓		135	56
	R- CHR8.1_4	AAAGCAACGACGTTTAACAG					
19	F- CHR8.1_5	TGACCAGAATAAAGGGATTTT	✓			170	57
	R- CHR8.1_5	AGGAATCATTCATCACTGTGT					
20	F- CHR8.1_6	CCAGGGATAGAACAATCTTTT	✓			133	59
	R- CHR8.1_6	CCTTTGCAGTCAAGATAGAGA					
21	F- CHR8.1_7	TTGTCAGCTCGTGCTTCT	✓			140	57
	R- CHR8.1_7	CTACTCTTCTTCCCCTCTCAC					
22	F- CHR8.1_8	AATCACCTAAGAGCGAATGTA	✓			167	59
	R- CHR8.1_8	ACTTACAATAAAACGAAGGTAAC					
23	F- CHR8.1_9	AGGCTCTCATGTAAGCAATTT	✓			141	58
	R- CHR8.1_9	GAACAAATGAGCTGCATTAAC					
24	F- CHR8.1_10	GGTCTCTCTTTCCCCTACTCT	✓			168	61
	R- CHR8.1_10	AGCTCGTTGTCTTTGATCTC					
25	F- CHR8.1_11	ACTGTCAGATGAAGTGGTCAT	✓			145	65
	R- CHR8.1_11	CCTCTCTCTCCTCATTCTCTC					
26	F- CHR8.1_12	AAATACCCCTACAAGCCTAAG	✓			193	59
	R- CHR8.1_12	CGAACTGATCGTCTTCCTC					
27	F- CHR8.1_13	ACATCATGGTCAGGTTCG		✓		166	55
	R- CHR8.1_13	GTGTTGAGATGCACCATGT					
28	F- CHR8.1_14	CTGCCTCTGATTAGCCTTT		✓		224	59
	R- CHR8.1_14	GTAGTCACCACCCTACCTTG					
29	F- CHR8.1_15	TTAATTTCTAGTGCCAAATGC	✓			157	57
	R- CHR8.1_15	TATGGGTAATCTTGTTCTGGA					
30	F- CHR8.1_16	TAAGTCAAACCCTGGTTACAG	✓			144	58
	R- CHR8.1_16	CTCGACCTTTTTAATTCGTTT					
31	F- CHR8.1_17	ATCTCCCTCCTCCTTCTC	✓			171	58
	R- CHR8.1_17	GAACCGTGGGAGGAGGTC					
32	F- CHR8.1_18	CACCCTCACCCATTCCTC	✓			158	58
	R- CHR8.1_18	CAGAAACCAAGATATGGTGAA					
33	F- CHR8.1_19	CCACGTAGCTAGCCCTATACT	✓			142	57
	R- CHR8.1_19	ATGTTTGCGTTTCGTTGT					
34	F- CHR8.1_20	TTGGGTGAGATTGAGATTCTA	✓			165	58
	R- CHR8.1_20	CGTGATGAATTAAGGGTTGTA					
35	F- CHR8.1_21	TGCTAAATGGTGTGTTTTATGT	✓			201	60
	R- CHR8.1_21	CTATCTTCTCTCCAACCTTCC					
36	F- CHR8.1_22	CACACATTTTAGCACAATGAA		✓		150	57
	R- CHR8.1_22	TTTCCTCTATTCCACTTCACA					
37	F- CHR8.1_23	TTCCTCCTCTCTCTCTCTCAC	✓			145	61
	R- CHR8.1_23	GAGCTCGTTGTCTTTGATCT					
38	F- CHR5.1_1	TACCGTTGCTTTTGAGTTTAG	✓			155	57
	R- CHR5.1_1	CCGCACAGAGATAAAAACAT					

F- Forward Primer R- Reverse Primer Ta- Annealing Temperature, PS-Amplified Product Size

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