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Designing of fragrance related candidate gene specific microsatellite primers for molecular characterization of aromatic rice

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Abstract
Completely sequenced rice genome provides scope for designing candidate gene specific microsatellite markers for their effective utilization in genetic analysis and breeding programs. An effort was made to develop novel microsatellite markers from candidate genes related to fragrance in aromatic rice, which can be utilized for fragrance specific molecular characterization and biological individuation of the aromatic rice germplasm. Aroma specific gene sequences were retrieved from the rice genome database available in the public domain. From a total of 11 genes/QTLs sequences, 47 microsatellites (simple sequence repeats) were detected with the BatchPrimer3 v1.0 web application. Altogether thirty-eight simple sequence repeats (SSRs) specific primer pairs were designed and tested by commonly used SSR reaction condition using 18 landraces and improved varieties of aromatic rice genomic DNA as templates. Analysis of amplification profiles revealed that all primer pairs generated recognizable amplification patterns. These SSR primers could be utilized for molecular characterization, identification and genetic divergence analysis in relation to fragrance of landraces and improved varieties of aromatic rice.

Keywords: Aromatic rice, candidate gene, fragrance, SSR marker

Introduction
Among the various qualities, the aroma is considered to be the most important among the features possessed by aromatic rice, which comprises a little but impressive group of rice. It is considered as best in quality and preferred among the consumers in several countries world-wide. Aromatic rice receives a premium price and is profitable for the growers as well as the traders. Most of the traditional aromatic rice varieties are low yielding. Low yielding aromatic rice has become a significant survivor of the primary emphasis on yield rather than quality. An enormous number of fragrant rice landraces are already extinct and most are near extinction (Singh *et al*., 2000) [11]. Despite the high value and demand of aromatic rice, there has not been much progress in developing aromatic varieties so far.

A 'popcorn' like aroma component 2 acetyl-l-pyrroline has been reported as a primary flavour component of several aromatic varieties. Aromatic rice has been reported to contain 15 times more 2-acetyl-1-pyrroline content than non-aromatic rice (Rohilla *et al.* 2000) [10]. Microarray-based transcriptome profiling has revealed one down-regulated gene co-located in QTL region aro3.1 on chromosome 3 (Os03g0327600), eight genes co-located in the aro4.1 region on chromosome 4 (Os04g0352400, Os04g0434800, Os04g0401700, Os04g0438300, Os04g0445700, Os04g0468600, Os04g0474800, and, Os04g0469700) and the badh2 gene (Os08g0424500) on the chromosome (Pachuri *et al*., 2014) [8]. Gene expression analysis has revealed that reduced expression of betaine aldehyde dehydrogenase 2 (badh2) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and elevated level of triosephosphate isomerase (TPI) and Δ1-Pyrolline-5-carboxylic acid synthetase (P5CS) transcript enhances 2AP accumulation (Hinge *et al*., 2016) [4].

Conventional methods of plant selection for aroma are not easy because of the extensive effects of the environment and the low narrow-sense heritability of aroma. Recently, molecular markers, such as single nucleotide polymorphism (SNPs) and simple sequence repeats (SSRs) genetically linked to fragrance have been identified. These genomic markers, which have been developed for the selection of fragrant rice, offer the advantage of being inexpensive, simple, rapid and only requiring small amounts of tissue. Microsatellites or SSRs are the most informative and widespread molecular markers for diverse purposes, particularly in plants:

genetic diversity study, marker-assisted selection, breeding, mapping, phylogenetics and systematics. (Ghaffari *et al*., 2013) [3].

Primer designing is a crucial step in the optimization of the polymerase chain reaction simply because a poorly designed primer can lead to significantly less amplification or no amplification of the targeted genome fragment (Ruslan *et al*., 2014) [12]. The gene-based marker association studies have proven a potent tool in studying the genetic architecture of complex traits (Chirag *et al.*, 2019) [1]. Keeping all above into consideration, an effort was made to develop fragrance specific candidate gene based novel SSR markers to provide SSR-based analytical system for molecular characterization and biological individuation of the aromatic rice germplasm.

Material and Methods

a. DNA Isolation

A total of 18 landraces and improved varieties of aromatic rice were utilized in the present study (Table 1). Genomic DNA was isolated from leaves of all selected aromatic rice landraces and improved varieties. Seeds of the selected eighteen entries were planted in aluminium containers filled with soil. Genomic DNA was extracted from three-week-old immature leaves by using the standardized DNA isolation procedure (Doyle and Doyle, 1987) [2].

b. Candidate gene-specific microsatellite-based primer designing

Primer designing exercise was completed in four stages: (a) sorting out the candidate genes reported to be associated with the fragrance in rice; (b) retrieving the nucleotide sequences of the identified gene using NCBI database and Rice Genome Project; (c) searching for candidate gene-specific microsatellite primers using Batch Primer3 tool for finding specific primers on the explored nucleotides sequence of the candidate gene under consideration; (d) selecting the appropriate primer sequences for their utilization in targeted amplification of the genomic region during molecular profiling.

Stage I: Based on the information available in the literature (Pachauri *et al.*, 2014; Kaikavoosi *et al.*, 2015) [8, 6], a total of eleven genes reported to be responsible for aroma in aromatic rice were searched through the internet (Table 3.2). The fragrance related these 11 candidate genes are known to be located on four different chromosomes (3, 4, 8 and 5) existing in the rice genome. The gene OsNPB_0504555500 (Δ1-Pyrroline-5-Carboxylate Synthetase (P5CS)) is not directly related to the fragrance, but it is responsible for the synthesis of the proline, which is the precursor of 2-Acetyl-1-Pyrroline (Kaikavoosi *et al.*, 2015) [6].

Stage II: The publicly available gene sequences related to fragrance responsible genes were searched from the NCBI (https://www.ncbi.nlm.nih.gov/gene/) and Rice Genome Project (http://rice.plantbiology.msu.edu/) websites.

Stage III: Fragrance related candidate gene sequences were subjected to the SSRs detection and primer designing. The retrieved DNA sequence of an identified candidate gene was copied and pasted in Batch Primer3 (https://probes.pw.usda.gov/batchprimer3/), which gave SSR sequences and primers as a result.

Stage IV: Primer selection was done by considering the following criteria

- Appropriate primer length (18 to 22 bp) with 50 to 55%.GC content
- A lock (GC) on the 3'end of the primer and the melting temperature in the range of 50-550C.
- Absence of poly base regions and avoidance of four or more bases that complement either direction of the primers.
- Approximately similar melting temperature (+/-20) for forward and reverse primers used during the PCR amplification
- Primer pairs without regions containing four or more complementary bases.
- Inclusion of tail in the calculation of Tm values for tailed primers.

c. Polymerase Chain Reaction (PCR)

Thirty-eight SSR primer pairs (Table. 3) were shortlisted and tested to amplify the targeted genomic regions in the genomic DNA templates of 18 landraces and improved varieties of aromatic rice under the polymerase chain reaction condition. The SSR primer pairs were designed using BatchPrimer3 v1.0 software. The primer id nomenclature was adopted according to Singh *et al.* (2007) [14].

SSR amplification was conducted in 15 µl of reaction mixture containing 10X PCR buffer (3.0 µl), ten mmol MgCl2 (1.3 µl), 1.0 mmol dNTP (3 µl), 20 ng template DNA (1.5 µl), and 1.0U Taq DNA polymerase (0.5 µl), five µM forward and reverse primers (1.2 µl + 1.2µl respectively) and final volume was maintained by addition of nuclease-free water (3.3 µl). The amplification reaction was performed by using different thermal profile in a thermal cycler. After denaturation at 94 ^{0}C for 5 min, the reaction mixture was subjected to amplification for 35 cycles consisting of 1 min at 94 ^{0}C (denaturation), 45 seconds at 55-63 ^{0}C (depending on the different primers for annealing) and 2 min for the extension at ^{0}C with a final extension at 72 ^{0}C for 10 min. The amplified products were separated by electrophoresis using 1.5% agarose gel, stained with ethidium bromide and visualized under gel documentation system.

Results

Search for the SSRs within the candidate genes

A total of eleven genes responsible for aroma in rice were searched and sequences were retrieved from the genome sequence database. Altogether 47 SSRs were detected by using BatchPrimer3 v1.0 software. Using primer designing web-based tool (BatchPrimer3 v1.0), candidate gene-specific microsatellite primers were developed. In the present investigation, a total of 47 simple sequence repeats (SSRs) were detected for nine genes, namely, Os03g0327600, Os04g0352400, Os04g0434800, Os04g0401700, Os04g0438300, Os04g0468600, Os04g046970, Os08g0424500, and OsNPB_0504555500, whereas any simple sequence repeats was not detected for two genes, namely, Os04g0445700 and Os04g0474800. In the case of the gene Os08g0424500, the highest number of simple sequence repeats was detected. Most of the SSRs possessed tri-nucleotide followed by di-nucleotide repeat motifs. Only few SSRs had tetra- nucleotide repeat motifs. As a result, altogether 38 microsatellites contained 10, 22 and 6 microsatellites with di-nucleotide, tri-nucleotide and tetra-nucleotide repeat motifs, respectively (Table 2). All these accounted for 31, 55 and 12% di-, tri-, and tetra-nucleotides repeat motifs, respectively, in all fragrance related genes under consideration, It was also found that dinucleotide repeats consisted predominantly of A/T repeats (Table.3).

Penta and hexanucleotide SSRs were excluded from consideration by BatchPrimer3 v1.0 web application settings.

Shortlisting and testing of SSR primers

Not all SSRs were appropriate for primer synthesis. According to the sequences of fragrance related candidate genes, only thirty-eight primer pairs were shortlisted and finally synthesized. These primer pairs were tested individually by carrying out SSR analyses using the genomic DNAs of 18 improved varieties and landraces of aromatic rice as template (Figure 1). Primer annealing temperature varied from 55 to 65^{0}C depending on the base composition of the primers (Table. 4). The lengths of amplified products ranged from 141 to 711 base pairs (bp). The results indicated that all 38 SSR primer pairs gave good amplification patterns and could be successfully utilized during molecular characterization and fragrance gene specific genetic divergence analysis of improved varieties and landraces belonging to aromatic rice germplasm.

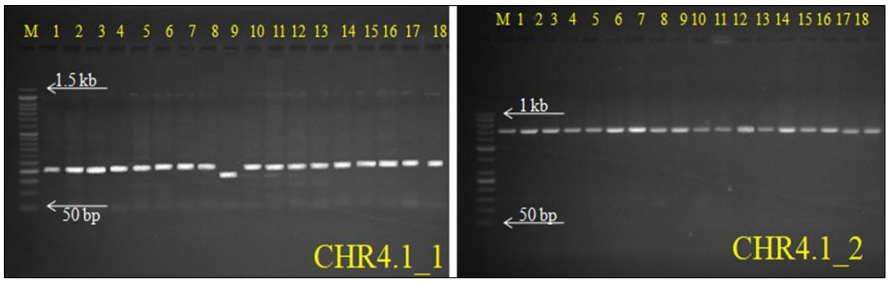

Fig 1: Amplification pattern of targeted genomic region in eighteen landraces and improved varieties of aromatic rice obtained with the primers CHR4.1_1 and CHR4.1_2

Table 1: List of improved varieties and landraces of aromatic rice utilized in the present study

S. No	Genotypes	Type	S. No	Genotypes	Type
1.	Pusa Sugndha-1	Improved variety	10.	Basmati 370	Landrace
2.	Pusa Sugndha-2	Improved variety	11.	Ranbir Basmati	Landrace
3.	Pusa Sugndha-3	Improved variety	12.	Sanwal Basmati	Landrace
4.	Pusa Sugndha-5	Improved variety	13.	Taraori basmati	Landrace
5.	Pusa 1121	Improved variety	14.	BurmaBhusi	Landrace
6.	Pusa 1509	Improved variety	15.	Birsamati	Landrace
7.	Rajendra Kasturi	Improved variety	16.	Jasmine	Landrace
8.	Rajendra Subhasini	Improved variety	17.	Lalmati	Landrace
9.	Rajendra Saraswati	Improved variety	18.	Marcha	Landrace

Table 2: Details of detected SSR within the fragrance related candidate genes

Sl. No.	Gene ID	SSR detected		
		Di-nucleotide	Tri-nucleotide	Tetra-nucleotide
1	Os03g0327600	00	02	00
2	Os04g0352400	00	10	01
3	Os04g0434800	01	01	00
4	Os04g0401700	00	04	01
5	Os04g0438300	00	02	00
6	Os04g0445700	00	00	00
7	Os04g0468600	00	03	00
8	Os04g0474800	00	00	00
9	Os04g046970	00	01	00
10	Os08g0424500	14	11	04
11	OsNPB_0504555500	00	01	00

Table 3: List of microsatellites targeted within the candidate gene using 38 designed primers

Sl. No	Primer	SSR motif	Motif length	Repeat number	SSR length
1	CHR3.1_1	(CGG)6	3	6	18
2	CHR3.1_2	(GTG)4	3	4	12
3	CHR4.1_1	(TGC)4	3	4	12
4	CHR4.1_2	(TGC)4	3	4	12
5	CHR4.1_3	(ACGC)4	4	4	16
6	CHR4.1_4	(TCC)4	3	4	12
7	CHR4.1_5	(TGC)5	3	5	15
8	CHR4.1_6	(CGA)5	3	5	15
9	CHR4.1_7	(AGCC)5	4	5	20
10	CHR4.1_8	(TG)6	3	6	12
11	CHR4.1_9	(GAG)4	3	4	12
12	CHR4.1_10	(GAT)4	3	4	12
13	CHR4.1.11	(GCG)6	3	6	18

14	CHR4.1_12	(CGG)4	3	4	12
15	CHR8.1_1	(AT)19	2	19	38
16	CHR8.1_2	(AT)13	2	13	26
17	CHR8.1_3	(TA)9	2	9	18
18	CHR8.1_4	(TA)8	2	8	16
19	CHR8.1_5	(TA)6	2	6	12
20	CHR8.1_6	(CT)6	2	6	12
21	CHR8.1_7	(GA)6	2	6	12
22	CHR8.1_8	(TA)26	2	26	52
23	CHR8.1_9	(AT)7	2	7	14
24	CHR8.1_10	(CT)6	2	6	12
25	CHR8.1_11	(GAG)4	3	4	12
26	CHR8.1_12	(TCG)4	3	4	12
27	CHR8.1_13	(CGC)4	3	4	12
28	CHR8.1_14	(TTG)6	3	6	18
29	CHR8.1.15	(TTA)5	3	5	15
30	CHR8.1_16	(TCA)5	3	5	15
31	CHR8.1._17	(CGG)5	3	5	15
32	CHR8.1_18	(CCG)5	3	5	15
33	CHR8.1_19	(AGCT)3	4	3	12
34	CHR8.1_20	(AAAT)5	4	5	20
35	CHR8.1_21	(AATC)3	4	3	12
36	CHR8.1_22	(GTTT)3	4	3	12
37	CHR8.1_23	(GCG)4	3	4	12
38	CHR5.1_1	(ATA)4	3	4	12

Table 4: List of 38 designed SSR primers within candidate gene employed during amplification of genomic template isolated from 18 genotypes of rice

Sl. No	Primer ID	Forward Sequence	Reverse Sequence	EPS (bp)	Ta (^{0}C)
1	CHR3.1_1	TTTTTCCTTCACTCCAAGCAG	AGTTGAGGCGGATGTTGTTC	699	60
2	CHR3.1_2	CGTTGGTTTTGCTGACTCAC	CACCAGCTTCACCTGTTTGA	688	60
3	CHR4.1_1	GTCCGGGACAACCCAAAAG	GACGGAGTACCGATGGTT	203	58
4	CHR4.1_2	TTCAAAGGCTGTCAAGACGA	TTTGCAGGAACAACAGCAAG	701	58
5	CHR4.1_3	AAATACCCACCTCCACCACA	CCCCTTCTTCATCGTCTTGA	695	60
6	CHR4.1_4	AGGAGGCCAAGGAAATCAAT	CACCTGCAACGTCATCAGAG	693	60
7	CHR4.1_5	AGGCACAGAGGGAATGTTTG	ACCTGCCCCTCGTACTTCTT	710	61
8	CHR4.1_6	GGAGGTGGTGTACCTGTCGT	GCTTCTCCTCCTCCACGTC	701	63
9	CHR4.1_7	TCCTTAGCTTCTCCCTCCAG	TCCCTATGACGTCAGAACCAT	692	62
10	CHR4.1_8	GCCTTCTCCTCCACCTCCT	AGGCAAGCTGGTCATGAAAT	711	60
11	CHR4.1_9	CGCTTGGTTACTCGCATACA	TCAATTTTGGGACGATAGCC	701	59
12	CHR4.1_10	TCATATGGGCATGCATTCTG	ATCCCCAACCTCCAGTTACC	713	60
13	CHR4.1_11	TTCCCGCTGCTAATCGTTAC	TCGTCATCGGCGTGTAGTAG	703	61
14	CHR4.1_12	GATGGAGCAGGTGTGGAATC	ATCAAATCGGCCTCATCATC	705	60
15	CHR4.1_13	TTCTTCCTCGTCCCTGTGTT	CCCTTGGGTCATGTTACACC	749	59
16	CHR8.1_1	TCTCAGTTCTCCACATTTCTC	TGTAGTTGAACTGGGCATATT	155	59
17	CHR8.1_2	AATGCACATGCTCAATCATAC	GCCCCATATGAGTGTCCTA	172	58
18	CHR8.1_4	GGAGCACCAAACAAATTTAC	AAAGCAACGACGTTTAACAG	135	56
19	CHR8.1_5	TGACCAGAATAAAGGGATTTT	AGGAATCATTCATCACTGTGT	170	57
20	CHR8.1_6	CCAGGGATAGAACAATCTTTT	CCTTTGCAGTCAAGATAGAGA	133	59
21	CHR8.1_7	TTGTCAGCTCGTGCTTCT	CTACTCTTCTTCCCCTCTCAC	140	57
22	CHR8.1_8	AATCACCTAAGAGCGAATGTA	ACTTACAATAAAACGAAGGTAAC	167	59
23	CHR8.1_9	AGGCTCTCATGTAAGCAATTT	GAACAAATGAGCTGCATTAAC	141	58
24	CHR8.1_10	GGTCTCTCTTTCCCCTACTCT	AGCTCGTTGTCTTTGATCTC	168	61
25	CHR8.1_11	ACTGTCAGATGAAGTGGTCAT	CCTCTCTCTCCTCATTCTCTC	145	65
26	CHR8.1_12	AAATACCCCTACAAGCCTAAG	CGAACTGATCGTCTTCCTC	193	59
27	CHR8.1_13	ACATCATGGTCAGGTTCG	GTGTTGAGATGCACCATGT	166	55
28	CHR8.1_14	CTGCCTCTGATTAGCCTTT	GTAGTCACCACCCTACCTTG	224	59
29	CHR8.1_15	TTAATTTCTAGTGCCAAATGC	TATGGGTAATCTTGTTCTGGA	157	57
30	CHR8.1_16	TAAGTCAAACCCTGGTTACAG	CTCGACCTTTTTAATTCGTTT	144	58
31	CHR8.1_17	ATCTCCCTCCTCCTTCTC	GAACCGTGGGAGGAGGTC	171	58
32	CHR8.1_18	CACCCTCACCCATTCCTC	CAGAAACCAAGATATGGTGAA	158	58
33	CHR8.1_19	CCACGTAGCTAGCCCTATACT	ATGTTTGCGTTTCGTTGT	142	57
34	CHR8.1_20	TTGGGTGAGATTGAGATTCTA	CGTGATGAATTAAGGGTTGTA	165	58
35	CHR8.1_21	TGCTAAATGGTGTGTTTTATGT	CTATCTTCTCTCCAACCTTCC	201	60
36	CHR8.1_22	CACACATTTTAGCACAATGAA	TTTCCTCTATTCCACTTCACA	150	57
37	CHR8.1_23	TTCCTCCTCTCTCTCTCTCAC	GAGCTCGTTGTCTTTGATCT	145	61
38	CHR5.1_1	TACCGTTGCTTTTGAGTTTAG	CCGCACAGAGATAAAAACAT	155	57

EPS: Expected product size; Ta: Annealing temperature

Discussion

Various PCR-based strategies can be utilized to recognize polymorphisms in plants amongst which RAPD, ISSR, AFLP and SSR are the most well-known PCR-based molecular markers for molecular characterization (Jones *et al.,* 1998) [5]. SSR markers fulfil all characteristics of an ideal marker *viz*. polymorphic, independent, highly reproducible and rapid with genuinely low expenses (Poczai *et al.,* 2013) [9]. It has been expressed that the most frequent SSR motifs in terrestrial plants are tri-nucleotide repeats, varying from 30 to 78% (Xiao *et al.,* 2020; Sharma *et al.,* 2020) [15, 13]. Among the fragrance related candidate gene-based 47 novel SSRs identified in the present study, 26 (55%) SSRs had tri-nucleotide repeat motifs. Only 15 SSRs had di-nucleotide repeat motifs, whereas 6 SSrs were character by tetra-nucleotide repeat motifs. Several studies have also acknowledged the presence of SSRs in the genomic regions containing protein-coding genes and expressed sequence tags (ESTs) (Nadeem *et al.,* 2018) [7]. Microsatellites are well known to signify the lesser repetition per locus with a higher polymorphism level (Zane *et al.,* 2002) [16]. Utilizing the freely accessible database, 11 gene sequences responsible for fragrance in rice were retrieved and 47 SSRs were detected within nine genes Among these 47 SSRs, primers specific to the flanking regions of 38 SSRs were successfully tested. While majority of the newly designed primers generated only one amplified product in each genotype tested, only few primers amplified two bands in a particular aromatic rice DNA sample. Some primer pairs failed to generate any amplified product in a specific combination with a particular landrace or improved variety of aromatic rice most probably due to nucleotide sequence alteration at primer binding site in the genomic template.

Conclusion

A web-based primer design program (BatchPrimer3) provided the facilities to design fragrance gene specific microsatellite detection and primer design in the present study conducted by utilization of the genomic sequence database of rice. The results provided the evidence to document that microsatellite markers can be successfully detected and targeted within the candidate genes for analysis of molecular level genetic polymorphism in rice by utilization of this program, validating its practical usefulness.

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