Original Research Article

Evaluation for parental polymorphism and identification of SSRs linked to drought tolerance in rice (*Oryza sativa* L.)

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ABSTRACT

Aims: To identify the ready-to-use polymorphic microsatellite markers associated with drought tolerance for marker-assisted backcross breeding through polymorphism survey between rice varieties Jyothi and Chuvannamodan

Place and Duration of Study: Centre for plant biotechnology and molecular biology, Thrissur, Kerala, India, between January 2023 and May 2023.

Methodology: Genomic DNA of Jyothi and Chuvannamodan was isolated by following CTAB method with modification. Isolated DNA from both varieties was subjected to PCR amplification using 208 Simple Sequence Repeats (SSRs) primer pairs distributed in 12 chromosomes. The amplified PCR products were electrophoresed in 3 % agarose gel and separated fragments were visualized and documented in Gel Documentation System. Different allele size produced by same SSR primer between two varieties identified as a polymorphic marker. Polymorphism per cent was calculated, frequency distribution and chromosome distribution of polymorphic markers was analyzed.

Results:A total of 208 SSRs primers surveyed for the parental polymorphism. Out of which, 85 SSR primers exhibited clear polymorphism between Jyothi and Chuvannamodanand the remaining 123 were monomorphic primers. The amplicon size ranged from 83bp (RM430) to 495bp (RM18919) among the different primers. The survey revealed maximum parental polymorphism on chromosomes 4 (69.23%), followed by chromosome 5 (64.28%), and minimum polymorphism on chromosome 8 (21.73%). The average per cent of polymorphism between the parents was 40.86%. Among the 85 polymorphic markers, 66 had dinucleotide repeats, 17 had trinucleotide repeats, and 1 had tetranucleotide repeats.

A group of 28 polymorphic markers were identified to be linked with traits including root related traits, grain yield, leaf rolling and leaf drying under drought conditions.

Conclusion: The polymorphic markers identified in the present study form the basis for tagging drought-tolerant QTLs/genes, fine mapping of those genes, and subsequently in marker-assisted breeding programs. The polymorphic markers linked with QTLs/genes associated with drought tolerance can be used in marker- assisted backcross breeding.

Keywords: Simple Sequence Repeats; Chuvannamodan; Jyothi; parental polymorphism; marker-assisted backcrossing

1. INTRODUCTION

Rice botanically known as *Oryza sativa* L., is a staple food crop for the majority of the world's population. It is a widely grown crop, with Asian nations alone accounting for more than 90% of total production

Comment [Ma1]: (Please add…primers)

Comment [Ma2]: Jyothi and Chuvannamodan rice varieties

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worldwide [13].It is a member of the Poaceae family, which also includes 12 other genera[43]. Jyothi is the ruling variety of Kerala released from the Regional Agricultural Research Station, Pattambi, India in 1974. This variety has a poor spikelet fertility percentage under drought, which shows that it is highly susceptible to drought. Traditional rice Chuvannamodan is a short-duration variety of 105 to 110 days with an average yield of 2200 kg per hectare. A study evaluated 15 cultivars for drought tolerance and found Chuvannamodan performed well for drought tolerant related traits like transpiration rate, stomatal conductance, and membrane stability [2]. Furthermore, proteome analysis indicated that Chuvannamodan was highly tolerant to drought. Among 80 traditional and high yielding cultivars, Chuvannamodan was found as one of the highly drought-tolerant varieties [4,19].

The advent of genetic markers has significantly boosted the efficiency of plant breeding by accurately following the inheritance of various important traits. They have proven to be useful tools for assessing genetic diversity and understanding genetic relationships within and across species [6]. There are several genetic markers for classifying genotypes and are not influenced by the environment. Marker data helps to analyze the genetic similarities and differences between the genotypes which will be of great interest in crop breeding programs[12]. The differences are known as molecular markers because they are often linked with specific genes and can act as a signpost to those genes. When such markers are very tightly linked to genes of interest, they can be used for the indirect selection of desirable alleles, which is known as marker-assisted selection [44,46]. These markers are being adapted by researchers as an effective tool for crop breeding programs like selecting suitable plants for hybridization, gene tagging, QTL mapping, DNA fingerprinting, etc.

Simple Sequence Repeat (SSR) markers are short, tandemly repeating DNA sequences of 2 to 6 base pairs. These microsatellites are the type of a variable number of tandem repeats (VNTR) that are abundant, multi-allelic, highly polymorphic, and co-dominant in the genome. Polymerase Chain Reaction (PCR) amplifications with primers designed from flanking regions of these VNTR enable targeted amplification of their locus. The size of the PCR products amplified would depend on the number of repetitive DNA units in the VNTR alleles. Due to the variation in the number of repeat motifs, the amplicons from diverse genotypes will show length polymorphism [7]. Molecular markers, when combined with linkage maps and genomics, aid in altering and improving the useful traits in plants [31].

In Marker assisted breeding (MAB), foreground selection and background selection are two important aspects performed in either concurrent or consecutive generations. The markers used in MAB must be polymorphic for efficient and accurate selection[16]. The use of markers in MAB considerably saves time as well as increases efficiency compared to conventional backcrossing. Hence, the present study was conducted to identify the polymorphic SSR markers between the rice varieties Jyothi and Chuvannamodan.

2. MATERIAL AND METHODS

2.1 Rice varieties used in the study

Jyothi is one of the high-yielding rice varieties, mostly suitable for direct sowing or transplanting and special planting in the Kole and Kuttanad regions of kerala, India. Traditional rice Chuvannamodan is a short-duration variety but tolerant to drought condition.

2.2Isolation of genomic DNA

The genomic DNA was isolated from the young leaves of Jyothi and Chuvannamodanby using the CTAB method with modification[25,38]. 100mg of leaf tissues were cryogenically grounded in pestle and mortar with 2% PVP and preheated 2X DNA extraction buffer (2% CTAB, 100mM TrisHcl, 20mM EDTA, and 1.4M NaCl) at 65^{o} C. The quality of isolated DNA was assessed by 0.8% of agarose gel electrophoresis. The purity and the quantity of the DNA were checked at O.D values of A260 and A280 nm by Nano spectrophotometer.

2.3Parental polymorphism survey

A total of 208 SSR markers were used to generate polymorphism between Jyothi and Chuvannamodan. All the RM primer sequences and chromosome locations were retrieved from the https://www.gramene.org/ database covering 12 chromosomes of rice. Primers were synthesized with the help of Vision Scientific and used in the parental polymorphic study.Amplification of genomic DNA was

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performed in the Biorad T100 thermal cycler. PCR reactions were carried with 10 µl volume containing sterile water of 4.9 µl; 10X Taq buffer with 15mM $MgCl_2$ of 1.0 µl; 10mM dNTPs of 1.0 µl; 3U Taq DNA polymerase of 0.1 µl; 10 µM forward primer of 0.5 µl; 10 µM reverse primer of 0.5 µl and 50 ng DNA template of 2 µl. The template DNA was amplified with a PCR reaction program a) initial denaturation at 95^{o}C for 3 minutes, b) denaturation at 94°C for 50 seconds, c) primer annealing at 55°C for 30 seconds, d) primer elongation at 72°C for 1 minute, e) final extension at 72°C for 10 min., and a final hold at 4 °C until removal. 30 cycles of steps b) to d) were used to amplify template DNA. 3% agarose gel electrophoresis was used to examine the amplified PCR products. The amplified products were then visualized using the gel documentation unit, and the banding pattern was observed and recorded for further analysis.

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3. RESULTS AND DISCUSSION

The present study was conducted to identify the informative polymorphic microsatellite (SSR) markers. Parental polymorphism was generated between Jyothi and Chuvannamodan by using SSR primers for the marker-assisted backcross breeding (MABB) program. A total of 208 SSR primer pairs were synthesized and used to analyze the polymorphism. At OD260/OD280, the UV absorbance ratio of isolated DNA ranged from 1.82 to 2.0, which shows high purity of DNA. The concentration of extracted DNA ranged between 1278.3 to 1896.8 ng /µl. The final concentration of DNA was diluted to 50 ng /µl for PCR amplifications.

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Out of 208 SSR primers, 85 were found to be polymorphic between Jyothi (drought susceptible) and Chuvannamodan (drought tolerant), and 123 primers were monomorphic. The amplicon size ranged from 83bp (RM430) to 495bp (RM18919) among the different RM primers. RM212, RM490, RM3412, RM339, RM493, RM10871, RM10745, RM12091, RM264 are the some of polymorphic markers (Figure_1). Among the polymorphic markers, ten were on chromosome 1, six on chromosome 2, seven on chromosome 3, nine on chromosome 4, nine on chromosome 5, eleven on chromosome 6, five on chromosome 7, five on chromosome 8, eight on chromosome 9, five on chromosome 10, six on chromosome 11 and four on chromosome 12.

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The survey revealed 40.86 per cent of average polymorphism between the Jyothi and Chuvannamodan. The percentage of polymorphism depends on the number of SSR primers surveyed on the two varieties. The maximum polymorphic per cent was found on chromosomes 4 at 69.23 per cent, followed by chromosome 5 at 64.28 per cent. The minimum polymorphic per cent was found on chromosome 8 of 21.73 per cent(Table 1). Similar to the findings, a study recorded 21.28 per cent of average parental polymorphism between indica varieties Pratikshya and CR Dhan 801, where 108 SSR primers were polymorphic out of 510 [21]. A polymorphism study found 31.7 percent by using 230 SSRs between IR5541904 and Super Basmati for utilization in the identification of QTLs under drought[32]. Another study reported 40.5 per cent of polymorphism levels between *O. sativa* cv. IR64 and *O. glaberrima* parents using 464 SSRs for marker-assisted introgressing drought tolerant traits through backcross breeding[5].

Comment [Ma18]: The maximum polymorphic percentage was found on chromosomes 4 (69.23 %) followed by on chromosome 5 (64.28%) while minimum polymorphic percentage was found on chromosome 8 (21.73%)followed by on chromosome 7(29.41%).

SSR-based polymorphism is defined by the nature of repeat motifs like di, tri, and tetra in the genome. In the present study, dinucleotide repeats in the microsatellite region showed greater polymorphism than tri and tera-nucleotide repeats. The repeats can be differentiated based on highly repetitive sequences and moderately repetitive sequences for a possible polymorphism. Among 85 polymorphic markers, 66 markers contained dinucleotide repeats, 17 markers had trinucleotide repeats and 1 had tetranucleotide repeats. The frequency distribution of repeat motifs revealed that among the 66 dinucleotides, the GA motif occurred more frequently (20 times) than others, accounting for 33.33 percent , followed by CT repeats of 18 times, accounting for 30 percent (Figure2). Similar to the result, 36 percent of SSR primers correspond to poly (GA) motifs in developing 2740 microsatellite markers for rice[22]. The prevalence of dinucleotide repeats in parental polymorphism was reported by previous researchers [15, 21]. Out of 17 trinucleotide repeats, CTT and AAG repeated 8 and 3 times, respectively. The frequency of CTT accounted for 9.41 per cent of total polymorphism. Other trinucleotide repeats AAT, TAA, GCT, CGA, CTC, and ATC were repeated 1 time. The RM markers with GA, AT, ATT, and CTT repeat motifs will show the greatest variation in allele size. In this regard, our findings are also analogous to previously published SSR diversity data in rice[7, 8]which revealed a wide range of allelic variations in size for markers containing GA, AT, ATT, and CTT repeat motifs. Only one marker (RM10745) with a TATG

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repeat motif was found polymorphic in the study. The chromosome wise distribution of all the polymorphic markers found in the study with physical position [22]is given in the form of physical map (Figure 3).
Out of 85 polymorphic markers, 28 markers were reported previously to be linked with drought-tolerant traits. A polymorphic marker RM208 on chromosome 2 was reported to be tightly linked with yield under drought [5]. On chromosome 3, a novel genomic region was identified to be flanked by RM168 and RM520 for the majority of the root-related traits [32], those markers are polymorphic in our study. The development of a root system is regarded as a key characteristic in rice for drought mitigation. In the same study, RM168 was also found to be associated with total water uptake which will help to overcome stress. A study revealed three polymorphic markers namely, RM212, RM302, and RM3825 on chromosome 1 linked to drought resistance QTLs like deep root mass, grain yield, deep root to shoot ratio, relative water content, and leaf drying[18,28]. The polymorphic markers associated with yield and other traits under drought were reported in the previous study listed in the table 2.

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Table_1 Chromosome wise polymorphic primers and polymorphic percent

Chromosome	No. of SSR markers used	No. of identified polymorphic markers	Polymorphism per cent
1	32	10	31.25
2	17	6	35.29
3	16	7	43.75
4	13	9	69.23
5	14	9	64.28
6	22	11	50.00
7	17	5	29.41
8	23	5	21.73
9	19	8	42.10
10	11	5	45.45
11	11	6	54.54
12	13	4	30.76
Total	**208**	**85**	**40.86**

Table_2 Association of identified polymorphic markers with different traits under drought

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S.No	Polymorphic markers	LG	Traits linked	Donor	Recipent
1	RM212	1	No. of grains per panicle[45] Biomass[24]	Zhenshan 97 Nootripathu	Minghui 63 IR20
2	RM3825	1	Leaf rolling and leaf drying[35] Panicle length[36]	Nootripathu Banglami	IR20 Ranjit
3	RM431	1	Grain yield[9] Grain yield[40]	Vandana ARC 10372	Way Rarem Ranjit
4	RM297	1	Harvest index[10]	Vandana	Way Rarem
5	RM490	1	Seedlings germination[26]	Ahlamitarum	Neda
6	RM493	1	Leaf rolling[47]	-	-
7	RM12091	1	Grain yield[14]	.Dhagaddeshi	Swarna and IR64

8	RM302	1	leaf rolling and leaf drying[35]	Nootripathu	IR20
9	RM263	2	Grain yield and days to flowering[34]	Kali Aus/2	IR64
10	RM208	2	Yield per plant Panicle fertility[5]	*O. glaberrima*	IR64
11	RM266	2	Grain weight[48]	TQ line	IRBB line
12	RM555	2	Grain yield[9]	Apo	Swarna
13	RM22	3	Grain yield[33]	WAB 450-I-B-P-157-2-1	Swarna
14	RM520	3	Grain yield[39]	Apo	IR64
15	RM168	3	Deep root length[32]	IR55419-04	Super Basmati
16	RM279	3	Grain weight[37]	-	-
17	RM518	4	Grain yield[27]	Kali Aus	IR64 and MTU1010
18	RM586	6	Grain yield[11]	IR55419-04/2	TDK1
19	RM587	6	Grain yield[11]	IR55419-04/2	TDK1
20	RM3	6	Grain yield[11,20]	IR55419-04/2 Milyang 23	TDK1 Taichung 189
21	RM339	8	Grain yield[42]	Swarna	Basmati334
22	RM566	9	Grain yield[10]	Aday sel	IR64
23	RM242	9	Root[17]	AERON1	MRQ74
24	RM257	9	Relative water content [3]	CR 143-2-2	Krishnahamsa
25	RM216	10	Grain yield[41]	N22	MTU1010
26	RM209	11	Grain yield[29]	Norungan	IR62266-42-6-2
27	RM17	11	Relative water content[30]	TKM9	Norungan
28	RM1261	12	Grain yield[1]	IR84984-83-15-481-B	FUNAABOR-2

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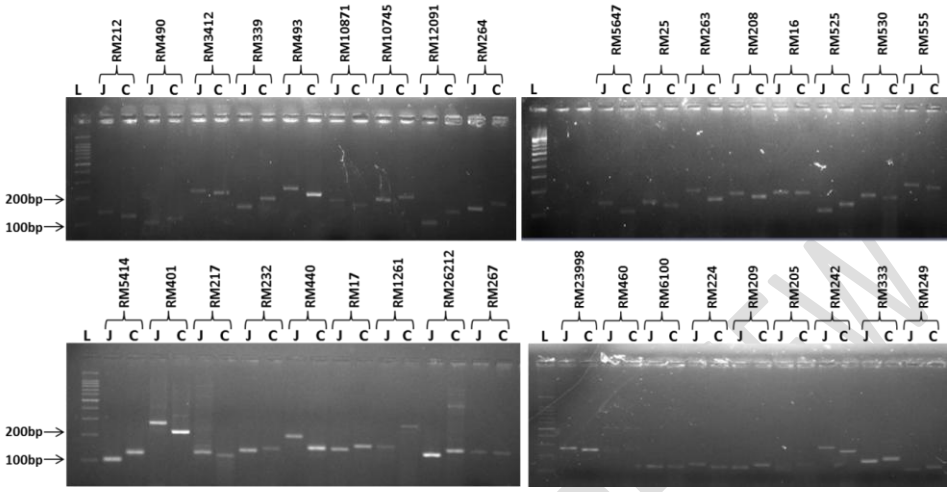

L - 100bp ladder; J - Jyothi; C - Chuvannamodan

Fig. 1 Amplification profile of polymorphic markers

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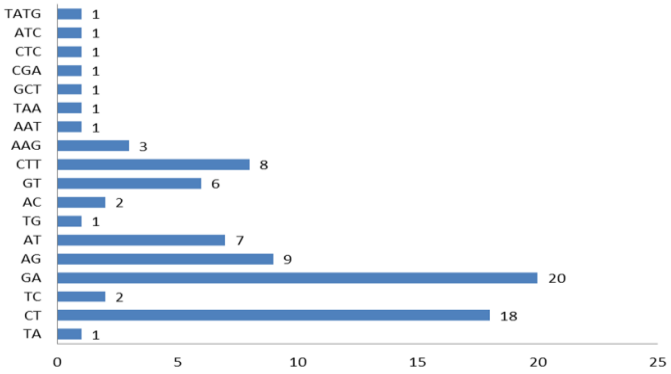

Fig. 2 Frequency distribution of polymorphic SSR repeat motifs

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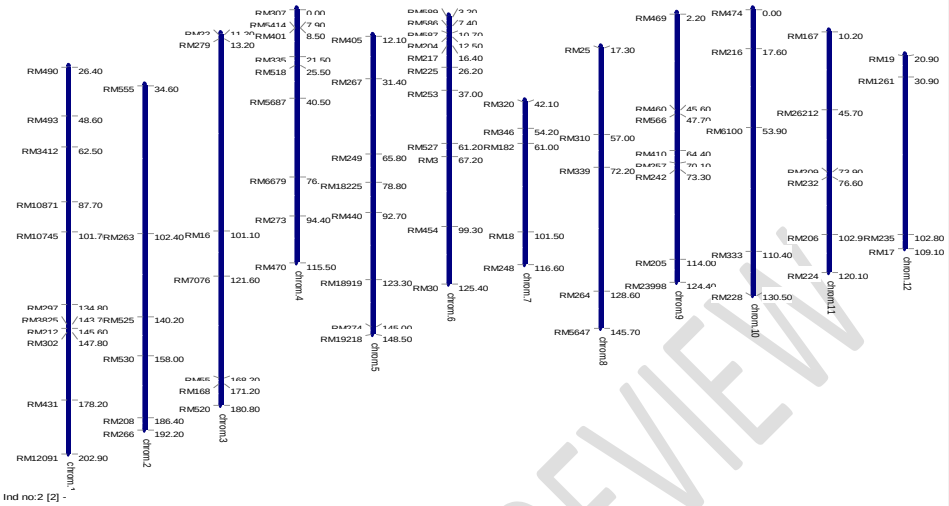

Fig.3 Chromosome wise distribution of 85 polymorphic SSR markers

4. CONCLUSION

All the identified polymorphic markers in the present study between Jyothi and Chuvannamodan may or may not associate with the drought-tolerant traits. Hence, QTL analysis for various traits should be performed in mapping populations developed from these parents to identify the putative markers for different traits under drought conditions. The identified 85 polymorphic SSR markers can also be used in diversity analysis, linkage studies, and bulk segregant analysis. Jyothi as the recurrent parent and Chuvannamodan as the donor parent can be used for developing the backcross population to introgressthe drought tolerant traits from the donor to the recurrent parent. These polymorphic markers can be used in foreground and background selection during marker-assisted backcross breeding programs.

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Comment [Ma30]: Is this terminology suitable here (which states the introduction of genes from one **species into the gene pool of another species,** occurring when matings between the two produce fertile hybrids)

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