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Molecular Characterization of Doubled Haploid Rice Lines Derived from the F_1s of IR20 × Mahulata

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ABSTRACT: Characterization and variability evaluation is vital for the development of crop plant. Majority of the research on DH lines has focused on evaluating their overall agronomic performance in comparison to original genotype and/or lines developed through traditional breeding. However, this experiment focused to assess the molecular variation of rice (*Oryza sativa* L.) doubled haploid lines. DNA markers, specifically Simple Sequence Repeats (SSR) is a powerful tool for the evaluation of genetic variations and resolving cultivar identities. An overall of sixty-four doubled haploid rice lines derived from the F1s of IR20 × Mahulata through anther culture alongside its parents have been genotyped and clustered by using SSR markers markers. 95 SSR markers, out of 490 SSR markers have been found to be informative among the parents, have been utilized in genotyping the entire population. Based on dendrogram clustering, the DH-lines have been grouped into 3 clusters viz. cluster-I, cluster-II and cluster-III. Cluster I, cluster II and cluster III had 17, 24, 25 DHs respectively. The outcomes confirmed that there was a significant level of polymorphism among the DHs. This end result also showed that the lines that were grouped together, they may be much less diverse. Therefore, the DHs from more diverse cluster can be used for further breeding programme for crop improvement.

Keywords: Doubled haploids, Rice, SSR marker, Clustering, Variability, Genetic diversity.

INTRODUCTION

Anther culture (AC) techniques have proven to be a useful tool in plant breeding, allowing for the rapid synthesis of doubled haploids (DHs) (Suriyan *et al.* 2009). In comparison to traditional breeding, which requires 6–7 generations of self-pollination, the creation of DHs using AC allows for the quick fixation of homozygous lines. Double haploid techniques not only speed up the breeding cycle, but they also allow for more discriminating between progeny genotypes (Marassi *et al.*, 2006). The fixation of recessive characteristics in DH populations makes genetic research easier to undertake. Because of their homozygozity and uniformity, DH lines are ideal for genetic study, particularly on quantitative features. Because they may be duplicated and reproduced without genetic alteration over time, they are also considered a permanent mapping populace (Semagn *et al.*, 2006). These abilities enable the precise measurement of quantitative traits through multiple testing, as well as the minimization of the environmental component of phenotypic variance (Lu *et al.*, 1996).

Plant genetic diversity is currently being recognised as a distinct domain, owing to the fact that rising population with urbanisation and shrinking cultivable lands are the two most important causes contributing to food insecurity in developing countries. Breeding crops with improved yields and nutritious properties is the need of the hour, and the success of any breeding effort is dependent on germplasm diversity. The most significant piece of technology for assessing variability is molecular markers. The use of markers to do molecular characterization helps us in analysing genetic diversity.

The majority of research on DH lines has focused on evaluating their overall agronomic performance in comparison to original genotype and/or lines developed through traditional breeding. In comparison to the parental genotypes and/or lines employed as checks, these DH lines have demonstrated superior overall performance for a few agronomic traits (Winzeler *et al.*, 1987; Mitchell *et al.*, 1992; Courtois, 1993; Murigneux *et al.*, 1993). DH lines are thought to be stable and can be repeated endlessly in different environments. Several researchers used molecular and biochemical markers to examine anther-derived DHs for homozygozity as well as the presence of unanticipated phenotypes.

Characterization of AC-derived DHs in rice is limited and mostly relies on cytological and morphological studies. Assessment of genetic diversity on the DNA level is now possible thanks to advancements in polymerase chain reaction (PCR)-based markers. The experiment, on the other hand, used a limited number of markers to characterize the population.

Microsatellite loci, also known as simple sequence repeats (SSRs), are widely employed molecular markers nowadays (McCouch *et al.*, 1997, 2001, 2002; Cho *et al.*, 2000). They are simple to use and cost-effective (Gracia *et al.*, 2004). They're widespread, codominant, and interspersed throughout the genome, compared to other markers (McCouch *et al.*, 1997). SSRs can detect a far higher degree of polymorphism, making them ideal for studying genetic variation and doing in-depth genetic research (Ni *et al.*, 2002; Okoshi *et al.*, 2004; Semagn *et al.*, 2007 Kostova *et al.*, 2006; Tu *et al*., 2007). This study looked at the variability contained in the AC-generated sixty-four DH lines derived from a cross of two indica cultivars, as well as molecular evaluation using SSR markers.

MATERIALS AND METHODS

A. Plant materials

A total of sixty-four DHs from the cross of two *indica* rice cultivars, Mahulata and IR20 (total of 66 lines) were used for this study (Table 1). The DH lines were derived through the anther culture technique (Naik *et al.*, 2017).

B. DNA markers

Ninety-five rice microsatellite markers (SSR markers) (Table 2), ten markers representing for chromosome 1, eleven markers for each chromosome 2 and 3, eight markers for chromosome 4 and 9, seven markers for each chromosome 5,6,11 and 12, six and four markers for chromosome 7 and 10 respectively were used in the study to study the variation at genomic DNA level.

Table 1: List of 64 DH lines along with its 2 parents used in the study.

Line number	Parent/DH Line	Line number	DH Line	Line number	DH Line	Line number	DH Line
1	Mahulata	18	DH16	35	DH33	52	DH50
2	IR20	19	DH17	36	DH34	53	DH51
3	DH1	20	DH18	37	DH35	54	DH52
4	DH2	21	DH19	38	DH36	55	DH53
5	DH3	22	DH20	39	DH37	56	DH54
6	DH4	23	DH21	40	DH38	57	DH55
7	DH5	24	DH22	41	DH39	58	DH56
8	DH6	25	DH23	42	DH40	59	DH57
9	DH7	26	DH24	43	DH41	60	DH58
10	DH8	27	DH25	44	DH42	61	DH59
11	DH9	28	DH26	45	DH43	62	DH60
12	DH10	29	DH27	46	DH44	63	DH61
13	DH11	30	DH28	47	DH45	64	DH62
14	DH12	31	DH29	48	DH46	65	DH63
15	DH13	32	DH30	49	DH47	66	DH64
16	DH14	33	DH31	50	DH48		
17	DH15	34	DH32	51	DH49		

Table 2: List of polymorphic SSR markers used in molecular analysis of 64 DH lines.

Chromosome	Markers	Total markers
1	RM495, RM151, RM259, RM600, RM562, RM1106, RM1349, RM112, RM297, RM472	10
2	RM3148, RM110, RM5345, RM6374, RM561, RM327, RM475, RM497, RM250, RM482, RM207	11
3	RM14239, RM251, RM14379, RM232, RM15490, RM426, RM168, RM15981, RM16131, RM565, RM442	11
4	RM261, RM16626, RM16739, RM17034, RM17263, RM17337, RM255, RM3466	8
5	RM159, RM17819, RM18483, RM18550, RM163, RM18775, RM18877	7
6	RM204, RM539, RM136, RM527, RM19850, RM20228, RM201615	7
7	RM125, RM432, RM11, RM346, RM10, RM478	6
8	RM152, RM44, RM72, RM483, RM331, RM404, RM23076, RM223, RM195	9
9	RM23662, RM245, RM444, RM22613, RM296, RM434, RM410, RM278	8
10	RM24878, RM244, RM311, RM484	4
11	RM26213, RM26269, RM456, RM26529, RM26546, RM26656, RM26781	7
12	RM27683, RM27731, RM27818, RM101, RM28077, RM519, RM5282	7

C. DNA extraction

The modified CTAB method was used to extract DNA from the leaves of 18-20 days old seedlings from each DH-line and their parents (Murray and Thompson, 1980). The leaves were bulked and taken from at least 5–10 seedlings.

D. Purification and Confirmation of genomic DNA

Purification of DNA is essential for removing RNA, proteins, and polysaccharides, which are thought to be major contaminants in DNA precipitates. The addition of CTAB to the DNA extraction buffer allows polysaccharides to be removed. RNA was removed with the help of RNAase treatment, while proteins were removed with the use of phenol-chloroform extraction. The DNA obtained after extraction was validated using a horizontal gel electrophoresis apparatus and a 0.8 percent gel electrophoresis (containing ethidium bromide @ 0.5 mg/ml).

E. PCR amplification program

In a 96 well Thermal Cycler, PCR amplification subjected to the thermal profile (Table 3 and Fig. 1) was carried out to explore polymorphism among the parental lines and DH population (PEQLAB, Deutschland and Osterrtich, United Kingdom and EPPENDORF Master cycler nexus gradient, Hamburg, Germany). Table 4 shows the concentration and volume of all these chemicals used in a single PCR experiment.

Table 3: Temperature profile used for DNA amplification by STMS marker.

Step No.	Temp (°C)	Duration	Cycles	Function
1	94°C	5 min	1	Initial denaturation
2	94°C	1 min	35	Denaturation
3	55°C*	1 min		Annealing
4	72°C	1 min		Extension
5	72°C	10min	1	Final extension
6	4°C	Hold		Final hold

Note: * - Temperature varied as per primer

Table 4: Components, volume and concentration of PCR reaction mixture.

Sr. No.	Reagent	Stock concentration	Volume (µl)
1.	Sterile and nanopure H_2O	-	6.3µl
2.	PCR buffer with 15 mM $MgCl_2$	10X	1.0µl
3.	dNTPs (Mix)	10mM	0.1µl
4.	Primer (forward)	5µM	0.25µl
5.	Primer (reverse)	5µM	0.25µl
6.	*Taq* polymerase	5Unit/µl	0.1µl
7.	DNA template	20 ηg/µl	2.0µl
	Total		10µl

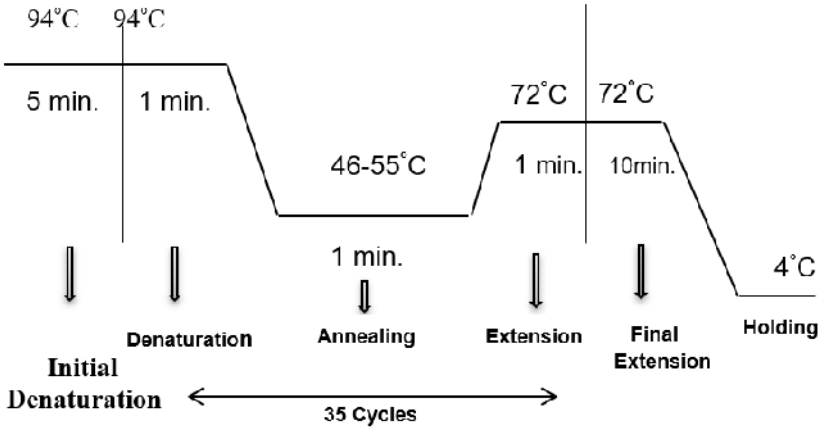

Fig. 1. Thermal cycles for PCR reaction.

F. SSR-PCR banding profile

PCR products (2-3µL) were resolved on a 3.5 percent agarose gel with a constant voltage of 150 volts for 2-3 hours after being combined with 6X loading dye (Thermo scientific # R0611). UV light was used to visualise PCR products in a gel documenting system (DNR Bioimaging system, Israel). The fragment size (bp) corresponding to the 100 bp molecular weight marker was used to score the clear cut and reproducible alleles amplified by each SSR marker among the DH plants (Sigma, Direct DNA PCR 100bp low D3687-1VL). Allelic bands for each locus were assigned a score based on the parental band, with 1 being Mahulata-type and 0 being IR20-type.

G. Statistical analysis

The molecular scoring data was subjected to statistical analysis for construction of dendrogram using

Dissimilarity Analysis and representation for windows (DARwin) version 6 (Perrier *et al.*, 2006). The dendrogram was constructed as using the dissimilarity coefficient. The dissimilarity matrix has been subjected to WPGMA (Weighted pair group method for arithmetic mean) for cluster analysis (Perrier *et al.*, 2003).

RESULTS AND DISCUSSION

A. Polymorphism survey

Polymorphism was discovered in 19.4 percent of the two parents in a polymorphism survey. Out of 490 polymorphic SSR markers, 95 were used to characterise a total of 66 lines (64 DH lines along with two parents). Per chromosome, the number of polymorphic markers ranged from four on chromosome 10 to eleven on chromosomes 2 and 3.

B. Clustering

Basic understanding of the genetic diversity that exists in the germplasm available is fundamental to a successful breeding programme (Krishna *et al.*, 2018). The neighbour joining clustering divided the entire population under study into three major cluster. One of the parents Mahulata and other 16 DH lines were clustered in Cluster-I. Cluster-I grouped the lowest individuals *i.e.*, 17. However, Cluster-III bagged highest individuals among the 3 major cluster *i.e.*, 25 DH lines. Cluster-III grouped the other parent, IR20 along with 24 DH lines. Finally, the cluster-II included 24 DH lines. Again, each major cluster divided into 2 sub cluster (Table 5 and Fig. 2). Solitary clusters may be of distinct recombinant or rare segregants (Soundharya *et al.*, 2017; Mohanty *et al.*, 2020). More number of cluster formations is an indication of higher diversity.

Table 5: Clustering pattern of doubled haploid rice lines derived from IR × Mahulata.

Major cluster name	Sub cluster	Line Number
Cluster-I	Sub-cluster-I	1(Mahulata), 17, 18,19,20,57,59,65 and 66
	Sub-cluster-II	22,24,3,60,61,62,63 and 64
Cluster-II	Sub-cluster-I	49,50,51,52,53,54,55,56 and 58
	Sub-cluster-II	45,46,47,48,35,34,36,38,15,16,37,43,40,41 and 42
Cluster-III	Sub-cluster-I	2 (IR20),25,39,44,21 and 23
	Sub-cluster-II	28,26,27,32,33,29,30,31,4,5,6,7,8,9,10,11,12,13 and 14

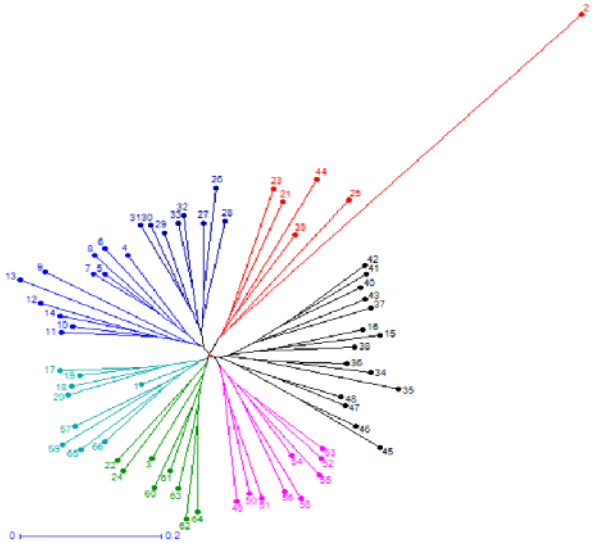

Fig. 2. Clustering of 64 doubled haploid lines along with its two parents using dissimilarity coefficient.

CONCLUSION

Through hybridization, genetic diversity has a direct relationship to the creation of variability. The DH-lines were divided into three major clusters in this study: cluster I, cluster II, and cluster III. To create variability and generate heterotic cross pairs for generating hybrid vigour, as well as for use in the hybridization programme, genotypes from different clusters should be employed rather than genotypes from clusters with low divergence (Narayanan and Murugan, 2013). Parents with a high yield potential and a large genetic variety are more likely to produce superior transgressive segregants in a small space of time in breeding programmes (Maurya and Singh, 1977). These genotypes can also be used in heterosis breeding to create recombinants with a high yield (Mohanty *et al.*, 2020). Furthermore, the effectiveness of identifying transgressive segregants or recombinants can be improved by using marker systems as a biotechnological tool. Higher molecular variability will be identified and understood in future breeding programmes aimed at creating more early maturing rice cultivars. To characterize the lines, a larger number of markers than those utilized in this work would be more efficient.

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Conflict of interest. None.

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