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Research Article

GENETIC DIVERSITY STUDIES ON 23 HYBRID RICE GENOTYPES USING MICROSATELLITE (SSR) MARKERS

GUPTA S.* AND SHARMA D.
Department of Genetics and Plant Breeding, College of Agriculture, Indira Gandhi Krishi Vishwavidyalaya, Raipur, 492012, Chhattisgarh, India
*Corresponding Author: Email - shwetagupta0912@gmail.com

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Abstract: A study conducted at Instructional cum Research Farm, Department of Genetics and Plant Breeding, College of Agriculture, Indira Gandhi Krishi Vishwavidyalaya, Raipur Chhattisgarh, to estimate the genetic diversity on 23 Hybrid rice genotypes using 18 microsatellite (SSR) markers, out of which fourteen SSR markers were found to be most informative for distinguishing the parental lines. Three rice CMS lines and twenty-three restorer lines used as parents in this study. Fourteen markers that were selected for the parental polymorphism study were: RM 517, RM 307, RM 228, RM 335, RM 251, RM 484, RM 09, RM 288, RM 302, RM 410, RM 444, RM 506, RM 411 and RM 535. The results shown in the study that considerable amount of genetic variability was present in the experimental material. Molecular markers like microsatellites (SSR's) markers should be used in the studies to have a better understanding of parental polymorphism in the genetic materials and thus helpful for the exploitation of heterosis.

Keywords: *SR Markers, Genetic Diversity, CMS lines, Restorer lines, UPGMA (NTSYS-pc Software)*

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Introduction

Molecular work was carried out for studying the parental polymorphism using 18 microsatellite (SSR) markers. Three rice CMS lines and twenty-three restorer lines used as parents in this study were analyzed for the parental polymorphism to determine the genetic diversity among genotypes using microsatellite markers.

The amplified product was scored as present (1) or absent (0) for each primer. Cluster analysis was performed using Jacquards coefficient of similarity matrices calculated from SSR markers to generate a dendrogram of twenty-three parental lines and three (CMS) female lines based on similarity coefficient using Unweighted Pair Group Method based on Arithmetic mean (UPGMA) using the NTSYS-pc Software.

Materials and Methods

For assessing the genetic diversity of parent's molecular studies was performed which includes DNA isolation, quantification, appropriate dilution of DNA, PCR amplification using SSR primers, electrophoresis, using polyacrylamide gel, scoring and analysis of data.

Genomic DNA extraction

Total rice genomic DNA was extracted from the leaves of 6 days old plants of the 23 parental lines and 3 CMS lines used in the present study. For the isolation of DNA, method described by Dellaporta *et al*., (1983) [1] was followed. The protocol was as follows:

1. Healthy leaf samples (2 cm long) have been collected into 1.5 ml eppendoff tubes and the tubes have been labelled with the plant number.
2. The leaf tissues have been cut into small pieces and put in a lyzer at 24,000 rpm, for 2min, 2 cycles and for 0.05 seconds.
3. 400 µl of DNA extraction buffer has been added into tubes and the tissues were grinded with a lyzer to a smooth paste with dark green color.
4. Again 400 µl of DNA extraction buffer has been added into green grinded paste. Mixed well and transferred 400 µl of this mixture into 1.5 ml eppendoff tube and the plant number has been written on tubes. The tubes were not placed back on ice as SDS will precipitate.
5. 400 µl chloroform has been added into grinded material kept in the eppendoff tube, mixed well and the tubes have spinned for 5 minutes at 12,000 rpm in centrifuge.
6. The top aqueous solution has been transferred into another 1.5 ml eppendoff tube and labelled.
7. 800 µl chilled absolute ethanol has been added into aqueous solution; mixed well and spinned for 5 minutes at 12000 rpm in a centrifuge. Supernatant have been decanted.
8. The pellet has been washed with 70% ethanol and then air-dried.
9. The DNA has been re-suspended into 100 µl of TE buffer.
10. 2 µl quantified and diluted DNA has been used for every PCR reaction.

Quantification of genomic DNA

The visual as well as nano drop quantification was performed for this research work. Nucleic acid has maximum absorbance of ultra violet light i.e., about 260 nm. The crude DNA after quantification was diluted suitably for amplification. DNA was diluted in such a way that the diluted samples contained 25 to 50 ng/µl of crude DNA. Dilution was carried out according to the formula:

Required conc. of DNA (ng/µl) X Total volume required (µl)
Dilution = Available conc. of crude DNA (ng/µl)

PCR Amplification Using SSR Primers

PCR analysis was done using known microsatellite markers to identify the polymorphic loci between 23 parental lines listed in [Table-1] by referring the rice consensus map (Cornell SSR map, 2003).

PCR reaction

18 micro litres of cocktail were added to each PCR tube having template DNA. PCR mix for one reaction volume is 20 micro litres. PCR components for one reaction (Volume 20µl) with their quantity for microsatellite analysis.

Components	Concentration	Quantity
PCR Buffer (with 15 mM $MgCl_2$)	10X	2 µl
dNTPs	1mM	0.5 µl
$MgCl_2$	25mM	0.5 µl
Primer (Forward)	10µM	0.5 µl
Primer (Reverse)	10µM	0.5 µl
Taq DNA polymerase	1U/µl	0.3 µl
Sterile water	-	14.2 µl
Template DNA	40ng/ µl	2 µl
Total		20 µl

The mixture was overlaid with a drop of mineral oil before the amplification was carried out for 35 cycles of Veriti 96 well Thermal Cycler of Applied Biosystems Pvt. Ltd., USA.

Temperature profile used for PCR amplification using Microsatellite markers

The PCR tubes were then kept in a PCR machine PTC-100 of MJ research. The DNA was amplified using profile describe below:

SN	Temperature (°C)	Duration (Min.)	No. of cycles	Activity
1	94	5	1	Initial denaturation
2	94	1	35	Denaturation
3	55	1		Annealing
4	72	1		Extension
5	72	7	1	Final Extension
6	4	∞	1	Storage

Table-1 *Details of parents used in the study*

Genotypes Lines	Parentage	Source
IR 58025 A	IR 48483A/ Pusa 167-120-3-2	DRR, Hyderabad
CRMS 31 A	V 20A/ Manipur	CRRI, Cuttack
CRMS 32 A	V 20A/ Mirai	CRRI, Cuttack
Testers		
F_5-C1-PL-6-4-1	IR 58025A x Swarna	IGKV, Raipur
F_5-C1-PL-14-2-1	IR 58025A x Swarna	IGKV, Raipur
F_5-C1-PL-25-1-1	IR 58025A x Swarna	IGKV, Raipur
F_5-C1-PL-25-2-1	IR 58025A x Swarna	IGKV, Raipur
F_5-C1-PL-43-1	IR 58025A x Swarna	IGKV, Raipur
F_5-C1-PL-46-1-1	IR 58025A x Swarna	IGKV, Raipur
F_5-C1-PL-20-2-1	IR 58025A x Swarna	IGKV, Raipur
F_5-C1-PL-78-1-1	IR 58025A x Swarna	IGKV, Raipur
F_5-C1-PL-49-1-1	IR 58025A x Swarna	IGKV, Raipur
F_5-C2-PL-51-1-1	CRMS 31A x Swarna	IGKV, Raipur
F_5-C2-PL-53-1-1	CRMS 31A x Swarna	IGKV, Raipur
F_5-C2-PL-50-2-1	CRMS 31A x Swarna	IGKV, Raipur
F_5-C3-PL-57-1-1	DRR 3A x Swarna	IGKV, Raipur
F_5-C3-PL-57-2-1	DRR 3A x Swarna	IGKV, Raipur
F_5-C3-PL-62-1-1	DRR 3A x Swarna	IGKV, Raipur
F_5-C3-PL-63-2-1	DRR 3A x Swarna	IGKV, Raipur
F_5-C4-PL-69-2-1	IR 68902A x Swarna	IGKV, Raipur
F_5-C4-PL-69-1-1	IR 68902A x Swarna	IGKV, Raipur
D-200-GY-1-11-1	Dubraj 200 Gamma ray	IGKV, Raipur
D-200-GY-6-2	Dubraj 200 Gamma ray	IGKV, Raipur
D-200-GY-6-3	Dubraj 200 Gamma ray	IGKV, Raipur
D-200-GY-2-1	Dubraj 200 Gamma ray	IGKV, Raipur
D-300-GY-3-11	Dubraj 300 Gamma ray	IGKV, Raipur

Gel electrophoresis

13 micro litres of PCR amplified SSR was mixed with 2 micro litres of loading dye (Bromophenol) and loaded on 5 percent polyacrylamide gel prepared in 1 X TBE buffer, ladder PBR-322 molecular marker was also loaded along with the DNA samples. Electrophoresis was done for 1 hour at 199 volts. The gel along with the DNA sample then stained with Ethidium Bromide (10ug/10ml) for 40-45 minutes. Gels were visualized on UV transilluminator and image can be seen in computer.

Results and Discussion

Detection of parental polymorphism using simple sequence repeat (SSR) primers.

The parental polymorphism was detected by using SSR primer. The primers used for this purpose are presented in [Fig-1]. Presence of band in particular base pair position was scored as "1" (One) and absence of band in that particular base position was scored as "0" (Zero). The SSR primers used for screening parental lines and their hybrids are given in [Table-2]. The amplified product was scored as present (1) or absent (0) for each primer. Cluster analysis was performed using Jacquards coefficient of similarity matrices calculated from SSR markers to generate a dendrogram of twenty-three parental lines and three (CMS) female lines based on similarity coefficient using Unweighted Pair Group Method based on Arithmetic mean (UPGMA) using the NTSYS-pc software.

Table-2 *List of microsatellite markers (SSR) primers used for detecting parental polymorphism*

SN	SSR Primers	Forward 5'-3'	Reverse 5'-3'
1	RM 517	CGTCTCCTTTGGTTAGTGCC	GGCTTACTGGCTTCGATTTG
2	RM 215	TGATCACCTCCTTCTCTGTAG	CAAAATGGAGCAGCAAGAGC
3	RM 221	TGCAAGAATCTGACCCGG	ACATGTCAGCATGCCACATC
4	RM 307	CTGCTATGCATGAACTGCTC	GTACTACCGACCTACCGTTCAC
5	RM 433	AGACAAACCTGGCCATTCAC	TGCGCTGAACTAAACACAGC
6	RM 247	CATATGGTTTTGACAAAGCG	TAGTGCCGATCGATGTAACG
7	RM 228	GCTTGCGGCTCTGCTTAC	CTGGCCATTAGTCCTTGG
8	RM 335	GCTCTATGCGAGTATCCATGG	GTACACACCCACATCGAGAAG
9	RM 251	ATGCGGTTCAAGATTCGATC	GAATGGCAATGGCGCTAG
10	RM 484	TGCTGCCCTCTCTCTCTCTC	TCTCCCTCCTCACCATTGTC
11	RM 09	GGTGCCATTGTCGTCCTC	ACGGCCCTCATCACCTTC
12	RM 288	CCGGTCAGTTCAAGCTCTG	ACGTACGGACGTGACGAC
13	RM 302	TCATGTCATCTACCATCACAC	ATGGAGAAGATGGAATACTTGC
14	RM 410	GCTCAACGTTTCGTTCCTG	GAAGATGCGTAAAGTGAACGG
15	RM 444	GCTCCACCTGCTTAAGCATC	TGAAGACCATGTTCTGCAGG
16	RM 506	CGAGCTAACTTCCGTTCTGG	GCTACTTGGGTAGCTGACCG
17	RM 411	ACACCAACTCTTGCCTGCAT	TGAAGCAAAAACATGGCTAGG
18	RM 245	ATGCCGCCAGTGAATAGC	CTGAGAATCCAATTATCTGGGG

**All microsatellite primers for analysis were obtained from Department of Genetics and Plant Breeding, College of Agriculture, Raipur.*

The parental lines were grouped into four major clusters [Fig-1] The similarity coefficient ranged from 0.41 to 0.98 with an average similarity index of 0.70. In pair wise comparison, the maximum similarity was obtained between CMS lines of IR 58025A and CRMS 31A with a similarity index of (0.98), whereas tester line D-200 GY-2-1 showed least similarity with similarity index of 0.41 with rest of the parents. The lower average similarity index among the genotypes indicated a greater genetic diversity among the parental lines. Similar results were reported by Akagi *et al*., (1996) [2]; McCouch *et al*., (1997) [3]; Wu and Tanksley, (1993) [4].

Cluster I consisting of 3 sub cluster of thirteen parental lines. Where parent line F5-C1-PL-6-4-1 is 82 percent similar to F5-C1-PL-14-2-1 , F5-C1-PL-25-2-1 is 84 percent similar to F5-C1-PL-46-1-1 , F5-C1-PL-78-1-1 is 76 percent similar to F5-C4-PL-69-2-1 , IR58025A is 98 percent similar to CRMS 31A and parental line F5-C1-PL-25-2-1 is 80 percent similar to F5-C1-PL-6-4-1 and F5-C1-PL-14-2-1. Similarly, parent line F5-C1-PL-20-2-1 is 81 percent similar to F5-C1-PL-25-1-1 and F5-C1-PL-46-1-1. Parent F5-C2-Pl-53-1-1 is 73 percent similar to IR58025A, CRMS 31A and CRMS 32A.

Cluster II consisted of 2 sub clusters of 5 parental lines viz., F5-C4-PL-69-1-1, F5-C1-PL-49-1-1, F5-C2-PL-50-2-1, F5-C3-PL-57-1-1, F5-C3-PL-57-2-1 where parental line F5-C4-PL-69-1-1 is 65 percent similar with F5-C1-PL-49-1-1, F5-C2-PL-50-2-1, F5-C3-PL-57-1-1, F5-C3-PL-57-2-1. Parent line F5-C1-PL-49-1-1 is 80 percent similar with F5-C2-PL-50-2-1, whereas parent line F5-C3-PL-57-1-1 is 76 percent similar with F5-C1-PL-49-1-1 and F5-C2-PL-50-2-1. Parent F5-C3-PL-57-2-1 is 58 percent similar with F5-C1-PL-49-1-1, F5-C2-PL-50-2-1 and F5-C3-PL-57-1-1.

In cluster III there were 2 sub clusters of 7 parental lines viz., F5-C3-PL-62-1-1, F5-C3-PL-63-2-1, F5-C2-PL-51-1-1, D-200-GY-1-11-1, D-200-GY-6-2, D-200-GY-6-3 and D-200-GY-3-11. Where parent line F5-C3-PL-62-1-1 is 71 percent similar with F5-C3-PL-63-2-1 and parent line F5-C2-PL-51-1-1 is 63 percent similar with F5-C3-PL-62-1-1 and F5-C3-PL-63-2-1. Similarly, parent line D-200-GY-1-11-1 is 90 percent similar with D-200-GY-6-2 and D-200-GY-6-3 is 89 percent similar with D-200-GY-1-11-1, D-200-GY-6-2, D-200-GY-6-3 and D-200-GY-3-11 and parent line D-200-GY-3-11 is 80 percent similar with D-200-GY-1-11-1, D-200-GY-6-2 and D-200-GY-6-3.

Cluster I and Cluster II were 55 percent similar and Cluster I and Cluster III were 50 percent similar. Cluster IV consisted of a single parent D-200-GY-2-1 which was 41 percent similar to rest of the parents. Since exploitation of heterosis is primary objective in hybrid development programme and diversity of parents play major role to get superior hybrids.

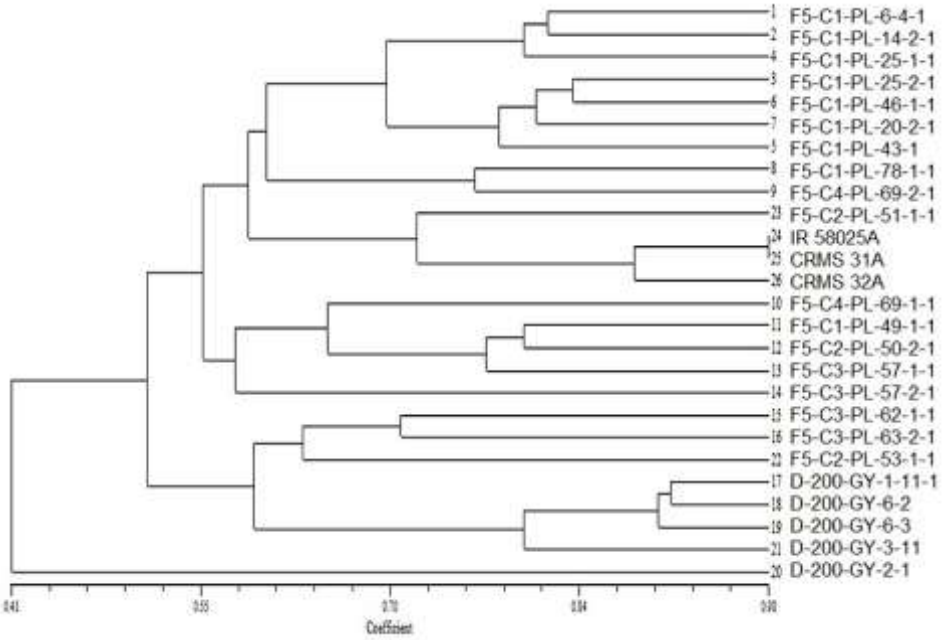

Fig-1 Dendrogram representing the parental polymorphism between 26 genotypes using 14 SSR markers

Estimation of heterosis based on genetic diversity of parents at molecular level play significant role in selecting diverse parents at molecular level.

Correlation coefficient measures the degree and direction of relationship between two or more variables. Correlation between genetic diversity and mid parent heterosis for yield shows positive correlation which showed genetic diversity in present study is favorable for higher yield. Parents having maximum genetic diversity at molecular level D-200-GY-2-1 (0.41) showed higher yield followed by F5-C3-PL-57-2-1 (0.58), F5-C2-PL-51-1-1 (0.63) and F5-C4-PL-69-1-1 (0.65) whereas with least genetic diversity for lines IR58025A and CRMS 31A (0.98) with lowest yield.

Similar results were also shown by Xiao *et al*., (1996) [5] ; Xu Weijun *et al*., (2002) [6], Wang ShengJun *et al*., (2006) [7], Zheng *et al*., (2007) [8], Sudararam *et al*. (2008) [9], Tamilkumar *et al*., (2009) [10], Sharma *et al*., (2009) [11], Jaikishan *et al*., (2010) [12] that SSR analysis might be a better method to study the diversity of parental lines [13].

Conclusion

In the present investigation, it was found that considerable amount of genetic variability was present in the experimental material under study. Parental polymorphism studied using 14 most informative and known microsatellite (SSR) markers showed sufficient genetic diversity in the parent material, thus useful for exploitation of heterosis. Molecular markers like microsatellites (SSR's) markers should be used in the studies to have a better understanding of parental polymorphism in the genetic materials and thus helpful for the exploitation of heterosis.

Application of research: The study showed that considerable amount of genetic variability was present in the experimental material under study. Molecular markers like microsatellites (SSR's) markers should be used in the studies to have a better understanding of parental polymorphism in the genetic materials and thus helpful for the exploitation of heterosis.

Research Category: Genetics and Plant Breeding

Abbreviations: PCR polymerase chain reaction, SSR simple sequence repeats

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Study area / Sample Collection: Instructional cum Research Farm, College of Agriculture, Raipur, 492012, Chhattisgarh, India

Cultivar / Variety / Breed name: Swarna and Dubraj

Conflict of Interest: None declared

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.
Ethical Committee Approval Number: Nil

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