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Research Article

Crossability Studies and Characterization by Molecular Markers in Rice (*Oryza sativa* L.) Accessions

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Abstract

Crossability of various accessions of *Oryza sativa* L. on the basis of pollen development and seed set was studied along with genetic diversity by RAPD and SSR markers. There was little difference in the average pollen fertility among the genotypes but large differences in seed set in crosses. There was a positive correlation between pollen fertility and seed set. Pollen germination in crosses ranged from 25.57 to 55.58 percent. Pollen germination had a positive correlation with seed set only after 10 minutes of pollination. The maximum seed-set was obtained by selfing than in crosses but the pollen tube growth in selfing and crosses did not show much difference. Eleven RAPD and 7 SSR primers were selected (out of 14 and 8, respectively) to assess the genetic diversity of 20 accessions and 2 rice varieties. A total of 42 RAPD amplicons were generated. The value of Jaccard's similarity coefficient ranged from 0.0 to 0.60 with an average value of 0.280. The polymorphic SSR primers generated a total of 20 alleles, with an average of 2.86 per locus. The value of Jaccard's similarity coefficient ranged from 0.0 to 0.857 with the average value of 0.308. The correlation coefficient and the significance of the correlation of the matrices based on RAPD and SSR data tested by the Mantel test showed that non-significant correlation (r = 0.18) existed between both matrices.

Keywords: Crossability; Indica Rice; Pollen Tube; Fruit Set; RAPD; SSR

The genetic improvement through hybridization and seed selection has been the main objective in the breeding programme in rice. Crosses between genetically diverse parents are important in hybridization programs to increase heterosis, maximize heterozygosity and maintain high levels of genetic variability in the progeny [1], ideally leading to an increase in grain yield. The intraspecific hybridization rate was 51% in O. sativa and 62% in O. glaberrima, whereas the interspecific hybridization rate was 39 - 42% [2]. The process of pollination and pollen germination leading to fertilization determines seed setting, and these processes are negatively affected by high-temperature stress coinciding with anthesis [3]. Shi-qiang., *et al*. [4] reported that the rice pollen grain starts to germinate at 2 minutes after pollination and the pollen tube penetrated stigma into style in 5 - 10 minutes, 30 minutes later the end of pollen tube reached the bottom of ovary, and only some pollen tubes arrived at embryo sac at 40 minutes after pollination. Variation in pollen tube growth rates in the pistil is frequently cited as a phenotypic manifestation of differences in gametophytic quality, leading to differences in the reproductive success among male gametes [5,6]. Jaitly and Khanna [7] reported that inadequate pollination, low % pollen germination and to some extent slow pollen tube growth and pollen tube abnormalities affect crossability in inter-varietal crosses and the backcrosses in *Oryza sativa* accessions.

Molecular markers are useful for evaluating genetic diversity and determining cultivar identity. The purpose of this study was also to evaluate the genetic diversity within a diverse collection of rice (*Oryza sativa* L.) accessions. The RAPD technique has advantages over isozyme and other DNA fingerprinting technologies in the speed of data-acquisition possible, the low cost of reactions, the small amounts of plant material required and the ability to per-

form analyses without the need for prior sequencing of the genome [8]. Microsatellites, or simple sequence repeats (SSR), are abundant and well distributed throughout the rice genome. SSR loci are co-dominant. The amplified SSR DNA bands with different alleles of an individual at each locus were scored as genotypes [9]. This paper reports pollen germination and pollen tube behavior in relation to crossability between various accessions of *indica* rice and seed set and genetic diversity using RAPD and SSR primers.

Materials and Methods

The materials used were NDR 9542, RNR 2354, NDR 9543, Kalanamak, CR 2613-1-5-2-7-2, LC, CR 2711-1, NDR 6330, Badshabhog, HUR 917, MGD 109, LC-1, CR 2713-11, R 1521-950-6-843-1, R 1536-136-1-77-1, Kalanamak-1, Bishnubhog, CN 1268-5-7, Tulasiphool and CR 2616-3-3-3-1. Among them 6 genotypes were selected to make crosses with CAU R1 and Shahsarang. The crosses were made by taking CAU R1 and Shahsarang as the female parents.

Five to six spikelets were taken at random from each of the parental varieties and kept in vials containing 70 percent ethanol. The anthers from spikelets were squashed in a drop of 2 percent acetocarmine and observed under the microscope. The pollen grains which absorbed the stain were classified as normal and fertile while those which did not absorb the stain were classified as sterile. Pollen grain size was measured with the help of a micrometer. Emasculation was done during the evening hours in a few florets from randomly selected plants. The emasculated plants were pollinated the next day between 10 - 11.30 AM, by the pollen of the male parent and these panicles were covered with butter paper bags to avoid pollination by the undesirable pollen. For each cross ten panicles were randomly chosen from plants. Spikelets from the top of the main axis of panicles, the primary rachis, as well as those belonging to the secondary and tertiary ones were detached, taking care that at least 35 - 40 florets from well-defined portions of the panicles were retained. Style length was measured with the help of a micrometer. The average value of three styles was taken. For pollen germination and pollen tube growth spikelets were detached after 10 minutes and 60 minutes of pollination and preserved in 70 percent alcohol till further use. For studies the spikelets were rinsed in distilled water and pistils were separated from the spikelets after which they were kept in a drop of 1N HCl for 10 minutes. They were again rinsed in distilled water and stained in cotton blue. After staining, the pistils were destained for 16 - 20 hours in a 1:1:1 mixture of 40 percent acetic acid: orthophosphoric acid: distilled water. The pistils were then rinsed in distilled water and mounted in pure lactic acid. The pollen grains and the pollen tubes stained deep blue.

Genomic DNA from cucumber leaves was isolated using CTAB method of Doyle and Doyle [10]. Young actively growing leaves of 15 - 20 days old plants were collected and used for DNA extraction. The quantification of DNA were done by staining DNA with ethidium bromide after electrophoresis in 0.8% agarose gel at 80V for 1 hour in TBE buffer (0.04M Tris borate, 0.001 M EDTA, pH 8.0) using known DNA concentration standards. One per cent agarose gel was prepared by dissolving appropriate amount of agarose in 1 X TBE buffer for RAPD markers while 2% agarose was prepared for SSR markers. EtBr was added to a final concentration of 2 µl/ml and mixed well. The melted agarose was poured into a gel mould and the gel was mounted in an electrophoresis. A pre-run of 15 minutes at 50 volts was given to the gel. The gel loading dye was mixed with DNA samples in 1:1 ratio and loaded in the gel with a micropipette. Electrophoresis was done at 90V for 1 - 2 hrs. The gel was visualized and photographed in a Gel Documentation system. Molecular weights of bands were estimated by using 1 Kb ladder for RAPD and 100 bp for SSR. The homology of bands was based on distance of migration in the gel. RAPD and SSR amplicons obtained from each entry were resolved as multiple and a single band on the agarose system, respectively and the data set were used to calculate pair-wise similarity coefficients following Jaccard [11]. The similarity matrices constructed were subjected to cluster analysis by unweighted pair group method of arithmetic average (UPGMA) analysis to generate dendrogram. These computations were performed using NTSYS-PC ver. 2.02 j, Exeter Software [12]. Mantel's correlation test [13] was performed by calculating correlations between Jaccard's similarity coefficients and cophenetic values for each pair of comparisons. The raw data matrix was used to calculate correlations between variables. The correlation matrix was subjected to “Eigen" vectors analyses, following which the principle components were extracted using the "Projection" module in NTSYS-PC. The first three most important PCA were used to construct a three dimensional plot of the accessions.

Results and Discussion

Pollen fertility

The pollen viability of all the species under this study revealed that the maximum viable pollen was recorded in CAU R1 (90.63 ± 0.57) whereas the lowest was in Shahsarang (77.12 ± 0.99) (Table 1). There was no correlation of pollen fertility with seed set.

Sl. No.	Parents	Pollen fertility (%)	Seed set (%)
1	CAU R1	90.63 ± 0.57	59
2	Shahsarang	77.12 ± 0.99	57
3	NDR 9542	84.81 ± 0.86	49
4	RNR 2354	81.60 ± 0.96	61
5	NDR 9543	84.49 ± 1.12	64
6	Kalanamak	83.93 ± 1.44	50
7	CR 2613-1-5-2-7-2	78.39 ± 1.06	43
8	LC	83.37 ± 1.11	45

Table 1: Percent of pollen viability in the parents in rice.

Pollen grain size

The size of the pollen grain did not show much variation among the parents. The diameter of the pollen grains ranged from 32 - 47µ. Among the parents the pollen grains of LC (47µ) were the largest and those of RNR 2354 (38.38µ) were the smallest in size (Table 2).

Sl. No.	Parents	Pollen grain size (µ)	Style length (µ)
1	CAU R1	41.19 ± 1.88	566.66 ± 72.64
2	Shahsarang	41.43 ± 0.75	646.66 ± 57.83
3	NDR 9542	42.41 ± 1.51	780.00 ± 15.27
4	RNR 2354	38.38 ± 2.52	840.00 ± 37.85
5	NDR 9543	39.71 ± 0.34	800.00 ± 76.37
6	Kalanamak	42.25 ± 1.60	756.66 ± 54.56
7	CR 2613-1-5-2-7-2	42.33 ± 1.35	813.33 ± 70.55
8	LC	47.22 ± 1.10	846.66 ± 51.74

Table 2: Size of pollen grains and length of styles (µ) in parents in rice.

Style length

The length of the style varied considerably with the maximum amongst the parents being that of LC (846.66 µ) and the least in CAU R1 (566.66µ) (Table 2). Competition among microgametophytes is an essential component of gametophytic selection [14]. Inter-male competition within a flower requires more pollen grains on the stigma than the number of ovules in the ovary. This may involve a single pollinator visit [15] or several visits. In the latter case competition may be possible between pollen from different visits if flowers have styles long enough to allow pollen tubes of the later-arriving pollen to overtake slow-growing pollen tubes from early deposits [15,16]. In flowers with relatively short styles, the intensity of gametophytic competition could be influenced significantly by the rate of pollen accumulation on the stigma and the timing of its germination.

Pollen germination

The maximum pollen germination after 10 minutes of pollination was recorded in Kalanamak (45.39%) and minimum in LC (28.56%) but after 60 minutes there was not much increase in germination (Figures 1-4). In crosses between parents, Shahsarang x NDR 9543 showed 55.58% pollen germination after 10 minutes of pollination, whereas in CAU R1 x NDR 9542 it was 25.57%. After 60 minutes Shahsarang x LC showed 75.33% pollen germination. The least germination was recorded in CAU R1 x NDR 9542 (25.57%) after 10 minutes and CAU R1 x CR 2613-1-5-2-7-2 after 60 minutes of pollination.

Rehydration of pollen upon landing on the stigma is the first crucial step in pollen germination, the process of production of long narrow pollen tubes. The pollen kit contains proteins including lectins which play an active role in pollen-stigma recognition and pollen germination. When there is competition between pollen tubes, some may fail to enter and will then continue growth on the surface of the branch or even in the air [17]. The activation of pollen grain depends on rehydration which depends on the inflow of water from the stigma after attachment of the grain. Hydration in the suitable medium leads to extrusion of the intine of the aperture [18]. In the present study there was regular increase in percent pollen germination after 10 minutes and 60 minutes in both selfing and in crosses (Figure 1, 2 and Table 3). Jaitly and Khanna [7] reported that there was no specific pattern of pollen germination for the parents, crosses or backcrosses. In many cases when the F_1's were backcrossed or selfed, the pollen germination was 100 percent. Selvanathan and Khanna [19] recorded maximum pollen germination on selfing the rice varieties as compared to the inter-varietal crosses. They also observed considerable differences in the reciprocal crosses. In a study aimed at increasing seed set in hybrid rice, Namari and Katoli [20] exposed a male sterile line to air borne pollen for different periods of time. Many spikelets set grain where only one pollen grain was deposited and 3 - 4 were almost always sufficient to do job. But Selvanathan and Khanna [19] made intervarietal crosses in rice and reported that inadequate pollination or low pollen germination seemed to be the cause of low seed set.

Pollen tube growth

The emergence of pollen tube on the hairy stigma and style was seen immediately after pollination. After penetrating the stigma hairs, the tubes grew through these hairs into the style. Pollen tube growth increased with the passage of time. At 10 minutes after pollination some of the pollen tubes had just started their growth in the hairs and a few had already entered the transmitting tissue of the style. After 10 minutes maximum pollen tube growth was

observed in Shahsarang x NDR 9542 (173.33 ± 14.52) and minimum in Shahsarang x NDR 9543 (83.33 ± 8.81). At 60 minutes after pollination there was a still greater differentiation in the pollen tubes (Figure 5-8, Table 3). After 60 minutes maximum pollen tube growth was observed in Shahsarang x NDR 9542 (186.66 ± 12.01) and CAU R1 x Kalanamak (186.66 ± 8.81), and minimum in Shahsarang x Kalanamak (130 ± 15.27). According to Jaitly and Khanna [7], pollen tubes were observed in all the cases after 10 minutes of pollination. In some cases pollen tube length was not much after 10 minutes. Mostly there was a good increase in the pollen tube growth from 10 - 60 minutes of pollination. Selvanathan and Khanna [19] studied pollen development and hybridization between indica varieties of rice. Seed set was not correlated with a high rate of pollen tube growth.

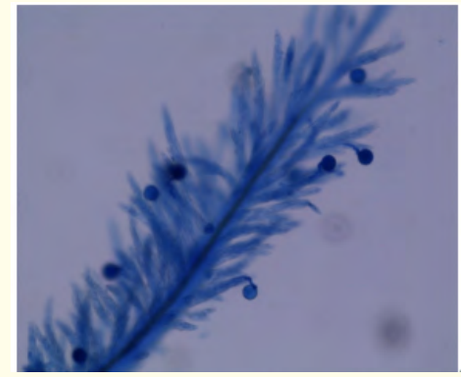


Figure 3: Pollen germination and pollen tube growth in RNR 2354 on selfing after 10 minutes of pollination (10 X).

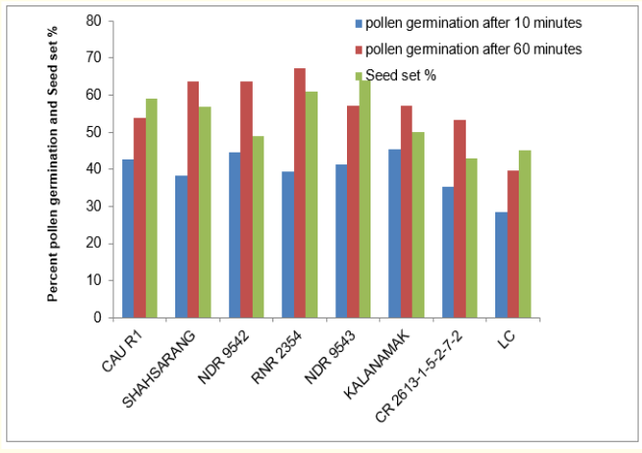


Figure 1: Pollen germination at different time intervals and percent of seed set in selfing.

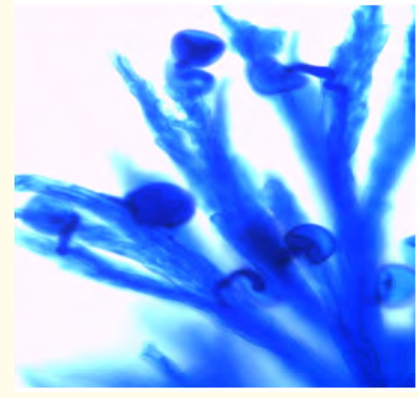


Figure 4: Pollen germination and pollen tube growth in Shahsarang x NDR 9543 after 60 minutes of pollination (40 X).

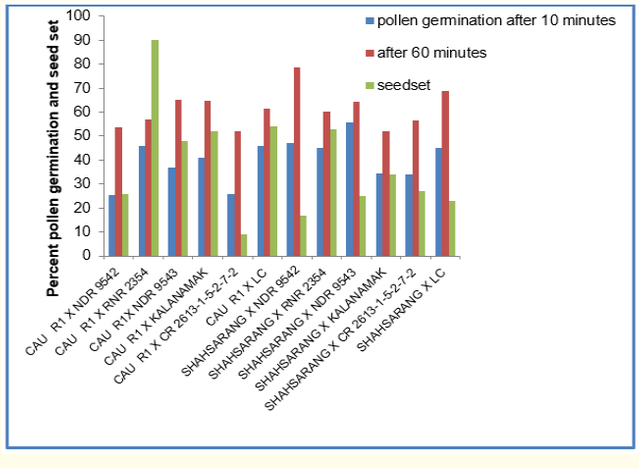


Figure 2: Pollen germination at different time intervals and percent of seed set in crosses.

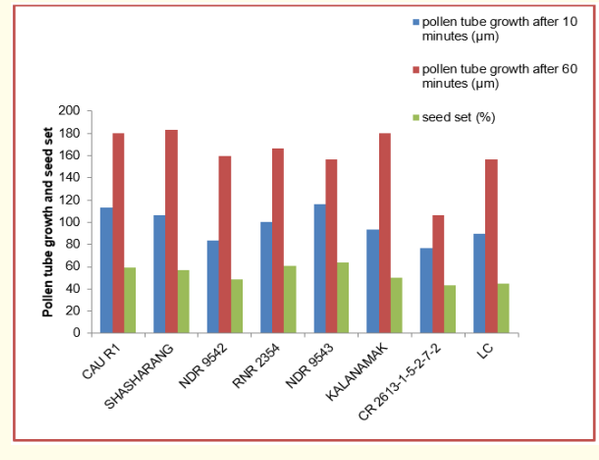


Figure 5: Pollen tube growth at different time intervals and percent of seed set in selfing.

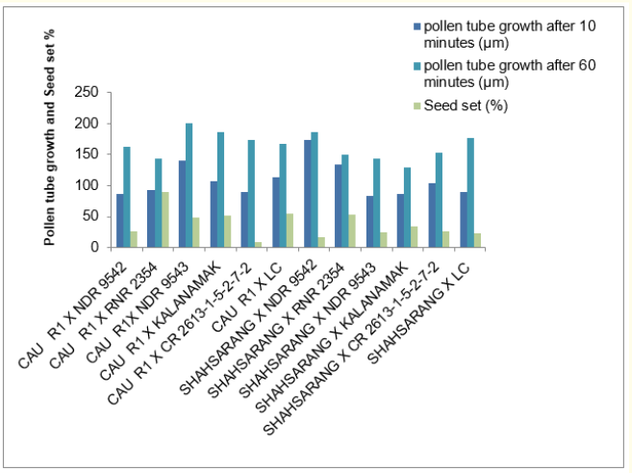


Figure 6: Pollen tube growth at different time intervals and percent of seed set in crosses.

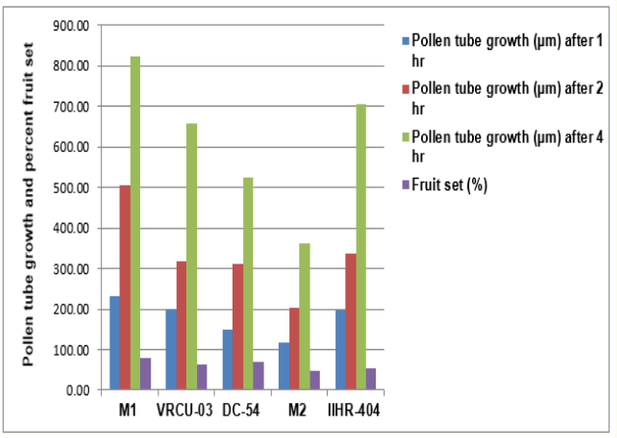


Figure 7: Pollen tube growth at different time intervals and percent fruit set in selfing.

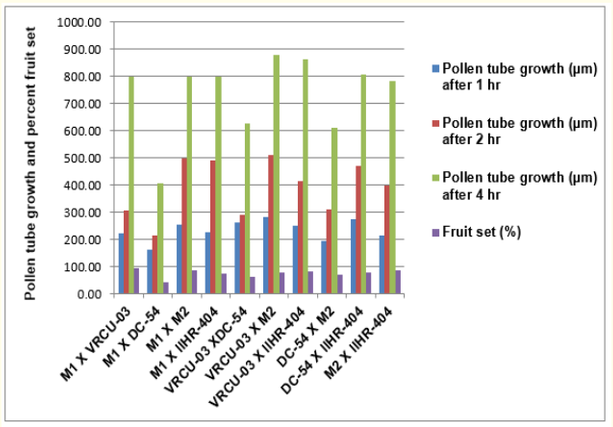


Figure 8: Pollen tube growth at different time intervals and percent fruit set in crosses.

Seed set

Within selfing, a good seed set was recorded in NDR 9543 (64%) and RNR 2354 (61%). High pollen germination was also recorded on selfing of RNR 2354. Among the crosses, CAU R1 x RNR 2354 showed maximum seed set (90%) (Figure 9, 10 and Table 3). According to Jagadish., *et al.* [21], high day temperatures beyond the critical threshold during sensitive developmental stages like gametogenesis and flowering leads to low seed-set. Water stress detrimentally affects flower induction, pollen production and subsequently leads to failure of fertilization and hence grain set [22]. According to Jaitly and Khanna [7] the maximum seed set was obtained on selfing the F1's between the different parents. In general, it was also good on selfing and crossing different parents as compared to the backcrosses. In nearly all the crosses, reciprocal differences were quite prominent. Though pollen sterility varied between 5 - 16.6 percent as reported by Jaitly and Khanna [7] no relationship between pollen sterility and grain yield was observed. Similar results were given by Selvanathan and Khanna [19] in intervarietal crosses in rice. In our study we got a positive and significant correlation of pollen fertility with seed set.

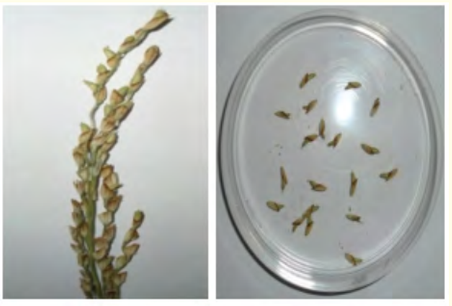


Figure 9: Seed set of NDR 9543 after selfing.

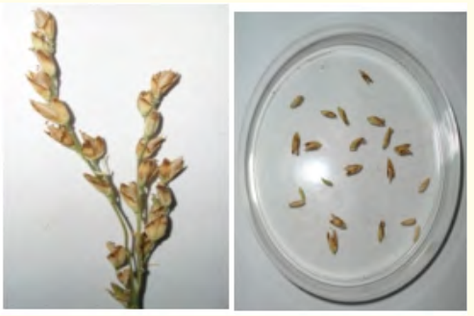


Figure 10: Seed set of CAU R1 x RNR 2354.

Relationship of different parameters with seed set

Pollen fertility had a significant positive correlation with seed set (0.104). Number of seed formed had non-significant positive

Crosses	(%) Pollen germination after 60 minutes	Pollen tube growth after 60 minutes (µ)	Seed set (%)
CAU R1	53.84 ± 2.61	180 ± 15.27	59
Shahsarang	63.71 ± 2.38	183.33 ± 18.55	57
NDR 9542	63.74 ± 2.33	160 ± 23.09	49
RNR 2354	67.27 ± 2.51	166.66 ± 17.63	61
NDR 9543	57.06 ± 2.51	156.66 ± 23.33	64
Kalanamak	57.18 ± 2.50	180 ± 17.32	50
CR 2613-1-5-2-7-2	53.30 ± 1.65	106.66 ± 14.52	43
LC	39.6 ± 1.63	156.66 ± 8.81	45
CAU R1 x NDR 9542	49.95 ± 1.83	163.33 ± 17.63	26
CAU R1 x RNR 2354	54.92 ± 1.18	143.33 ± 14.52	90
CAU R1 x NDR 9543	64.57 ± 2.52	200 ± 5.77	48
CAU R1 x Kalanamak	64.77 ± 1.29	186.66 ± 8.81	52
CAU R1 x CR 2613-1-5-2-7-2	48.31 ± 1.87	173.33 ± 8.81	9
CAU R1 x LC	61.81 ± 2.17	166.66 ± 12.01	54
Shahsarang x NDR 9542	75.33 ± 1.77	186.66 ± 12.01	17
Shahsarang x RNR 2354	59.51 ± 1.20	150 ± 11.54	53
Shahsarang x NDR 9543	66.51 ± 1.12	143.33 ± 31.79	25
Shahsarang x Kalanamak	51.88 ± 1.84	130 ± 15.27	34
Shahsarang x CR 2613-1-5-2-7-2	53.97 ± 1.43	153.33 ± 14.52	27
Shahsarang x LC	68.42 ± 1.86	176.66 ± 14.52	23

Table 3: Pollen germination, pollen tube growth and percent seed set in selfing and crosses.

correlation with percent pollen germination at 10 minutes after pollination (0.277), and with pollen tube growth at 10 minutes after pollination (0.004), while number of seed formed had non-significant negative correlation with percent pollen germination at 60 minutes after pollination (-0.029), and with pollen tube growth at 60 minutes after pollination (-0.060) (Table 4). According to Jaitly and Khanna [7] per cent pollen germination was more directly correlated with seed set, and the rate of pollen tube growth and percent pollen tube abnormalities do not seem to be affecting seed-set to that extent.

RAPD analysis

All the 20 accessions and 2 cultivars were subjected to PCR amplifications using 14 RAPD primers. Three primers did not give any results so they were excluded from this study and thereby the results in the table list only 11 primers. A total of 42 amplification products were scored in the 20 accessions and 2 cultivars with different primers, which exhibited an overall 100% polymorphism (Table 5). The average numbers of amplification products formed were 3.84 with a maximum of 7 in OPD-08 and a minimum of 2 in OPD-07 and OPF 06. The size of the amplification products varied in case of each primer and the range was 0.35 kb to 2.5 kb. In general, the extent of polymorphism found was high (Figures 11-13).

The data obtained from RAPD analysis were subjected to UPGMA analysis to find out the relationship between the accessions and cultivars analyzed. The value of Jaccard's similarity coefficient ranged from 0.0 to 0.60 with the average value of 0.280 (Figure 11).

The clustering pattern obtained from the UPGMA analyses of the data is shown in figure 11. Overall, three distinct clusters were formed. Two accessions (LC-1 and CR 2713-11) grouped in cluster I and appear to be the most distinct from all others. Cluster II comprises of 5 accessions. Accessions within cluster II are further grouped in two sub-clusters. The first sub-cluster comprises of two accessions, Bishnubhog and CN 1268-5-7. Tulasiphool, R

	Pollen germination		Pollen tube growth			
	Pollen germination after 10 minutes	Pollen germination after 60 minutes	Pollen tube growth after 10 minutes	Pollen tube growth after 60 minutes	Percent viable pollen	Seed set %
Pollen germination after 10 minutes	1					
Pollen germination after 60 minutes	0.694	1				
Pollen tube growth after 10 minutes	0.247	0.494	1			
Pollen tube growth after 60 minutes	0.055	0.404	0.530	1		
Percent viable pollen	0.323	0.116	0.216	0.289	1	
Seed set %	0.277*	-0.029*	0.004	-0.060	0.104*	1

Table 4: Correlation studies for various characters in crosses of *Oryza sativa.*

Note: * denotes significant at 1% level of significance.

Sl. No.	Code	Sequence (5' - 3')	GC Content (%)	n	P	% P
1	OPB 01	GTTTCGCTCC	60	4	4	100
2	OPC 07	GTCCCGACGA	70	2	2	100
3	OPD 01	ACCGCGAAGG	70	3	3	100
4	OPD 06	GGGAATTCGG	60	6	6	100
5	OPD 08	GTGTGCCCCA	70	7	7	100
6	OPF 06	GGGAATTCGC	60	2	2	100
7	OPF 13	GGCTGCAGAA	60	4	4	100
8	OPF 14	TGCTGCAGGT	60	3	3	100
9	OPH 04	GGAAGTCGCC	70	5	5	100
10	OPK 19	CACAGGCGGA	70	3	3	100
11	OPY 02	TGCTGCAGGT	60	3	3	100

Table 5: Sequence, total no. of amplification products (n), total no. of polymorphic products (P) and percentage of polymorphism (% P) of RAPD markers.

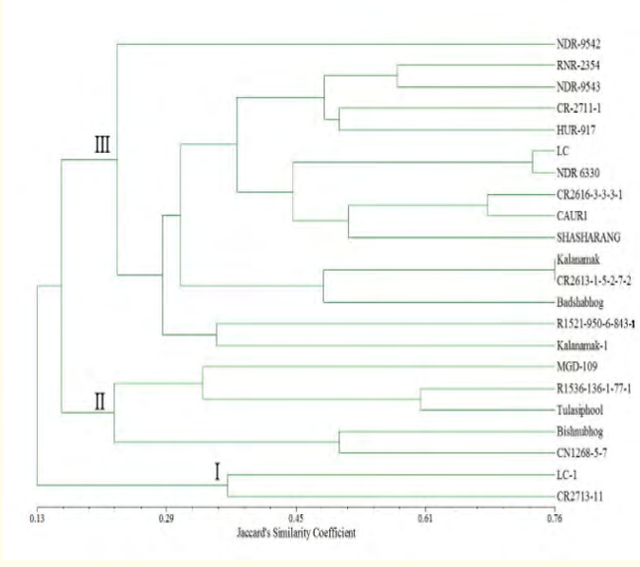


Figure 11: Phenogram generated using UPGMA analysis showing relationship between 20 accessions and 2 cultivars of rice using RAPD markers.

1536-136-1-77-1 and MGD 109 form a different sub-cluster in cluster II. Fifteen accessions/cultivars in cluster III further grouped in four sub-clusters. NDR 9542 appears to be the most distinct from all others in cluster III. The goodness-of-fit of the UPGMA dendrogram generated with RAPD data were tested by 2-way Mantel test [13]. High support for clustering patterns was observed for the cluster with Matrix correlation (r) of 0.84.

In the principal component analysis (PCA), the first three components explained 46.16% of the total variation, with 29.80% explained by the first component and 9.18% by the second component (Figure 12). In three-dimensional plot the grouping pattern of LC-1 was different as compared to the UPGMA cluster analysis.

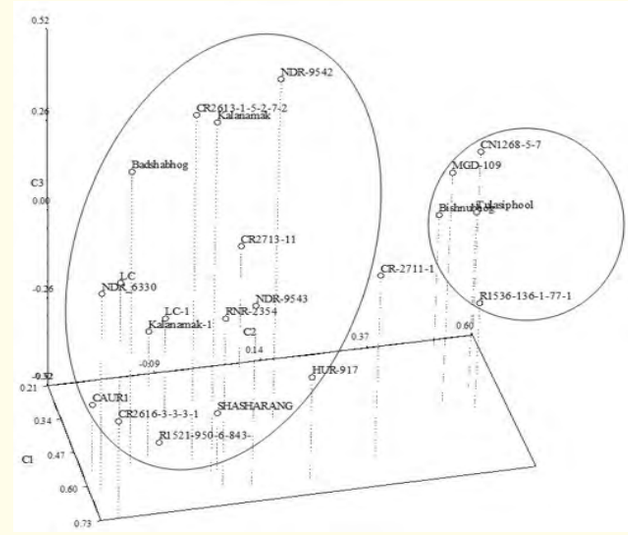


Figure 12: Three-dimensional plot of first three principal components extracted by Principal Component Analysis of 42 RAPD amplification products from the RAPD analysis of 20 accessions and 2 rice cultivars.

Molecular marker technology provides information that can help to define the distinctiveness of germplasm and their ranking according to the number of close relatives and their phylogenetic position (Kibria., *et al.* 2009). It is a complementary approach for genetic characterization.

The present investigation revealed the effectiveness of RAPD in detecting polymorphism among different accessions and cultivars of rice. The success of RAPD analysis in *O. sativa* accessions were also reported earlier (Rajani., *et al.* 2013). In the present study the percentage of polymorphism was found to be 100%. One of the reasons for this high level of polymorphism may be due to different parental combinations of accessions/cultivars as well as inclusion of traditional accessions/cultivars in the study. As reported by Sharma and Khanna (2009) 14 random oligonucleotide primers yielded a total of 217 amplified fragments (150 to 3000 bp in size) ranging from 7 to 21 polymorphic fragments per primer in 78 rice genotypes. Thus, genotype specific markers detected in these varieties have the potential to be used as genetic fingerprint for future varietal identification. Such markers were also detected in other rice studies (Raghunathachari., *et al.* 1999; Verma., *et al.* 1999; Bhuyan., *et al*. 2007).

Studies have demonstrated that amplification products obtained using short oligonucleotide primers reveals extensive polymorphism distinguishing genotypes in rice (Shivapriya and Hittalmani, 2006; Bhuyan., *et al.* 2007; Kibria., *et a*l. 2009). RAPD is a powerful technique to provide specific DNA fingerprints for protection of farmers' rights towards their contribution to conservation of landraces of traditional aromatic rice and prevent adulteration in the consignments of quality rice for export in order to maintain our credibility in the global market (Singh., *et al.* 2006). RAPD has proved to be useful as a genetic marker in the case of self-pollinating species with a relatively low level of intraspecific polymorphism (Heikal., *et al*. 2007).

Marsolais., *et al.* (1993) stated earlier that similarity level up to 50% in cluster analysis is indicative of plants derived from interspecific hybridization. In the present study the average similarity coefficient value for all 20 accessions and 2 rice cultivars was 0.280 which indicates the broad genetic base of studied cultivars. Two accessions (LC-1 and CR 2713-11) grouped in cluster I and appear to be the most distinct from all others. Accessions grouped in cluster I and II belong to short grain aromatic rice categories. However cluster III contains both short grain aromatic rice as well as slender grain aromatic rice. Rice accessions R 1536-136-1-77-1 and R 1521-950-6-843-1 were cross combination of R 302-111 and Ganga Baru but they grouped in different clusters. High level of genetic similarity (0.524) was observed between CAU R1 and Shahsarang (both from North-east region). The low level of average genetic similarity value may be attributed to the fact that majority of accessions/cultivars included in this study were either traditional aromatic landraces or selection from landraces. Landraces are heterogenous in their genetic makeup and generally adapted to a local environment and high level of population differentiation generally observed between them.

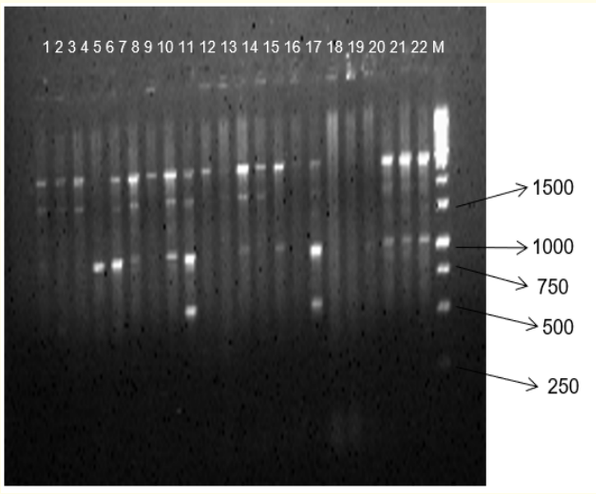


Figure 13: RAPD profile generated by OPD 06 primer.

Sl. No.	Name of accessions and cultivars
M	1 kb DNA ladder
1.	NDR 9542
2.	RNR 2354
3.	NDR 9543
4.	Kalanamak
5.	CR 2613-1-5-2-7-2
6.	LC
7.	CR 2711-1
8.	NDR 6330
9.	Badshabhog
10.	HUR 917
11.	MGD 109
12	LC-1
13.	CR 2713-11
14.	R 1521-950-6-843-1
15.	R 1536-136-1-77-1
16.	Kalanamak-1
17.	Bishnubhog
18.	CN 1268-5-7
19.	Tulasiphool
20.	CR 2616-3-3-3-1
21.	CAU R1
22.	Shahsarang

SSR analysis

Out of 8 primer pairs used for the genetic diversity analysis, 7 were found to be polymorphic. The polymorphic primers generated a total of 20 alleles. The number of alleles ranged from two (Primer 3 and primer 5) to four (primer 6), with an average of 2.86 per locus (Table 6).

The data obtained from SSR analysis were subjected to UPGMA analysis to find out the relationship between the accessions and cultivars analyzed. The value of Jaccard's similarity coefficient ranged from 0.0 to 0.857 with an average value of 0.308. The clustering pattern obtained from the UPGMA analyses of the data is shown in figure 13. Overall, four distinct clusters were formed. Two accessions (Kalanamak and Badshabhog) grouped in cluster I and appear to be the most distinct from all others. Cluster II comprises of 7 accessions. Accessions within cluster II are further grouped in three sub-clusters. The first sub-cluster comprises of two accessions, R1536-136-1-77-1 and R1521-950-6-843-1, both are semi-bold type and cross combination of R 302-111 and Ganga Baru. CR2616-3-3-3-1, Shahsarang, CAU R1 and MGD-109 form a different sub-cluster in cluster II. HUR-917 appears to be the most distinct from all others in cluster II. Cluster III comprised of 3 sub-clusters. Ten accessions within cluster IV are further grouped in

two sub-clusters. The first sub-cluster comprises of seven accessions CR-2711-1, LC, CR 2613-1-5-2-7-2, NDR-9543, Bishnubhog, NDR 6330 and RNR 2354. NDR 9542, Kalanamak-1 and Tulasiphool were present in another sub-cluster.

The goodness-of-fit of the UPGMA dendrogram generated with RAPD and SSR data were tested by 2-way Mantel test [13]. High support for clustering patterns was observed for the cluster with Matrix correlation (r) of 0.72.

Sl. No.	Code	Sequence (5' - 3')	GC Content (%)	No. of alleles
1	MRG 2431	F: ATCCAAATCCAATGGTGCAG	45	3
		R: GTGGCGAAAGGGAACATTCT	50	
2	MRG 4963	F: CGAAAAGTGGGAAGCAAATG	45	3
		R: GCGTACCCCTAGTGGCTGTA	60	
3	MRG 5836	F: TATAAGCCGCAGCCAAATTC	45	2
		R: AAAAACCTAGAAAATGGGAAAATG	29.2	
4	SSR 236	F: TTCATGAAAAGCCCAAGCAT	40	3
		R: TTCCCCGATTTTAGCTAGGC	50	
5	SSR 287	F: TTCGCCATGAAGTCTCTCG	53	2
		R: CCTCCCATCATTTCGTTGTT	45	
6	SSR 288	F: TTCGCCATGAAGTCTCTCG	53	4
		R: CCTCCCATCATTTCGTTGTT	45	
7	SSR 293	F: CCGAGGAGAGGAGTTCGAC	63	3
		R: GTGCCAATTTCCTCGAAAAA	40	

Table 6: Forward and backward primer sequence for polymorphic SSR primers.

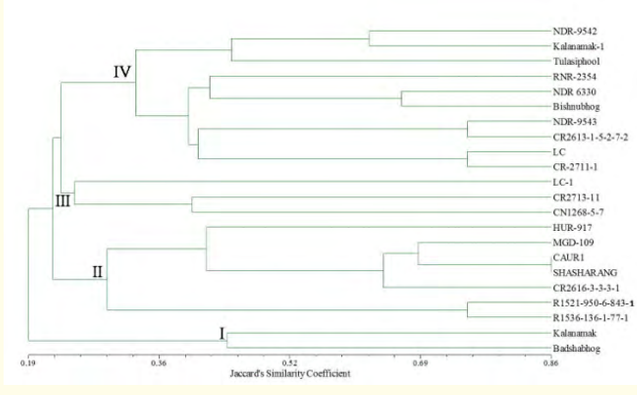


Figure 14: Phenogram generated using UPGMA analysis showing relationship between 20 accessions and 2 cultivars of rice using SSR markers.

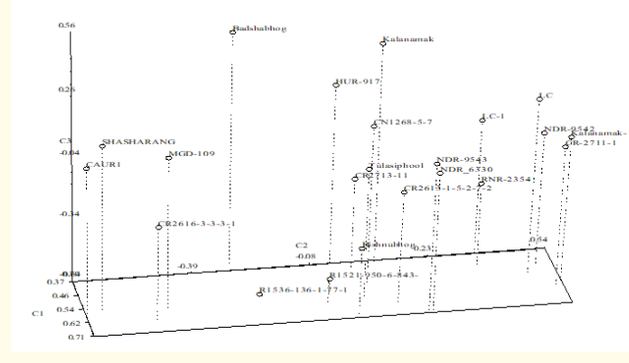
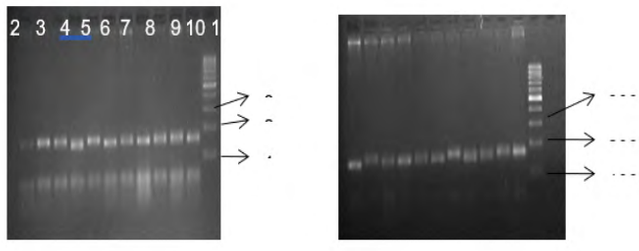


Figure 15: Three-dimensional plot of first three principal components extracted by Principal Component Analysis of 20 SSR amplification products from the SSR analysis of 20 accessions and 2 rice cultivars.

In the principal component analysis (PCA), the first three components explained 52.82% of the total variation, with 31.56% explained by the first component and 13.30% by the second component (Figure 15). In general, the three-dimensional plot grouping pattern was not comparable to the UPGMA cluster analysis.

The correlation coefficient and the significance of the correlation of the matrices based on RAPD and SSR data tested by the Mantel test showed that non-significant correlation (r = 0.18) existed between both matrices. In the present study genetic diversity

among 20 accessions and 2 rice cultivars was also assessed by using SSR markers. SSR is an important tool for genetic variation identification of germplasm (Powell., *et al.* 1996; Ma., *et al*. 2011). SSR markers are highly polymorphic, more reproducible, co-dominant and distributed throughout the genome. More than 2200 microsatellite markers have been mapped to specific locations in rice genome (Mc Couch., *et al*. 2002). A random set of these mapped markers providing genome wide coverage should facilitate an unbiased assay of genetic diversity and thus giving a robust, unambiguous molecular description of rice landraces.



16 A **16 B**

Figure 16: A and B: SSR profile generated by MRG 4963 primer.

SSR markers are tandem repeats interspersed throughout the genome and can be amplified using primers that flank these regions (Giovannoni., *et al.* 1999). SSR are highly polymorphic PCR- based markers and may be expected to be very powerful in cultivar discrimination (Sobotka., *et al.* 2004). SSRs have been used for cultivar identification in rice (Saini., *et al*. 2004; Lu., *et al*. 2005; Chakravarthi and Naravaneni, 2006; Ram., *et a*l. 2007; Chattopadhyay., *et al*. 2008; Kibria., *et al*. 2009).

A total of 20 alleles were detected using 7 polymorphic SSR loci with the mean value of 2.86 alleles per locus. The mean value of alleles per locus is low as compared to earlier study in the rice crop (Zhang., *et al*. 2009; Kumar., *et al*. 2010; Pandey., *et al.* 2012). This may be due to less number of genotypes included in this study. All SSR markers included in this study are polymorphic and able to differentiate between the 22 genotypes. The overall genetic similarity computed through SSR analysis (0.308 is relatively higher as compared to RAPD analysis (0.280). The low genetic similarity between genotypes confirms the diverse nature of rice cultivars included in this study. The clustering of genotypes is different in both types of analysis and the correlation (0.18) between both types of cluster is low. Two cultivars CAU R-1 and Shahsarang (common cultivars in North East hilly areas) show 0% with NDR 9542 (a cross combination of NDR 30030 and Swarna), Kalanamak-1 and LC-1 (both used as checks in initial variety trial on aromatic short grain). CR 2616-3-3-3-1 (Medium seed type; a cross combination of Pusa 44 and Dubraj) also shows 0% similarity with Kalanamak-1. However, they were not found to be much dissimilar in RAPD analysis. Less number of SSR primers included in this present study may be the reason for the absence of any similarity.

According to Sharma and Khanna (2009) 8 SSR primers yielded a total of 11 amplified fragments (100 to 300 bp in size) ranging from 1 to 2 polymorphic fragments per primer. None of the primers yielded any unique band in 76 germplasm lines of Kalanamak rice. Hence, SSR primers were not found to be suitable in the identification of closely related germplasm. Similar results were also reported by Ram., *et al* (2007). Since rice is a self-pollinating species, variation within the same germplasm is expected to be very low. However, microsatellite markers have been found to be ideal markers for characterizing genetic diversity at the intra-varietal level by Olufowote., *et al* (1997). The suitability of mapped sequence tagged microsatellite site markers has also been studied by Singh., *et al*. (2004) for establishing distinctiveness, uniformity and stability in aromatic rice.

Kalanamak is a traditional aromatic rice landrace and used as a check in initial variety trial on aromatic short grain rice and advanced variety trial on aromatic slender grain rice. Two samples of Kalanamak included in this study show very less genetic similarity in both RAPD (similarity coefficient is 0.318) and SSR (similarity coefficient is 0.273). Variation between different accessions of landraces was also reported in earlier works (Pusadee., *et al.* 2009; Kumar., *et al*. 2010).

Conclusion

Pollen fertility had a significant positive correlation with seed set (0.104), so it is useful to know that before selecting parents for crossing. Maximum seed set was in CAU R1 x RNR 2354 so these can be crossed with each other easily. The low genetic similarity between genotypes confirms the diverse nature of rice cultivars included in this study. Two cultivars, CAU R-1 and Shahsarang show 0% similarity with NDR 9542, Kalanamak-1 and LC-1. CR 2616-3-3-3-1 also shows 0% similarity with Kalanamak-1. Two samples of

Kalanamak included in this study show very less genetic similarity in both RAPD (similarity coefficient is 0.318) and SSR (similarity coefficient is 0.273). All these diverse types may be used for future breeding programmes for crop improvement.

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