NARDI FUNDULEA, ROMANIA
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ROMANIAN AGRICULTURAL RESEARCH, NO. 33, 2016
Print ISSN 1222-4227; Online ISSN 2067-5720

ANALYSIS OF GENETIC DIVERSITY IN NORTH INDIAN RICE (*ORYZA SATIVA* L.) GERMPLASM, USING SSR AND ISSR MARKERS

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ABSTRACT

Genetic diversity among thirty two accessions of rice representing landraces, released varieties, cultivars and breeding lines was analysed using simple sequence repeat (SSR), inter-simple sequence repeat (ISSR) and three dwarfing trait based markers. A total of 81 polymorphic bands were detected with 20 primer combinations. The genetic similarity among the accessions ranged from (0.60) to (0.97) with a mean of 0.737. All 32 accessions were clustered into 5 major groups. Of the 32 pair wise combinations generated by rice genotypes, the lowest coefficient of similarity 0.505 was observed between Sarbati-A and Sathi, Bagni and VB24 genotypes. The highest coefficient of similarity 0.964 was obtained between VD 62 and VLD86, which appear as the most similar and closely related accessions. Results illustrated the high-level polymorphism obtained by SSR and ISSR, especially trait-linked markers used in this study. These markers will applicable in discriminating different germplasm, and utility of these markers could be deployable in hybrid breeding programs, as well as in marker assisted selection (MAS).

Key words: rice landraces, cultivars. Lines, ISSR marker, SSR marker, dwarfing related markers, cluster analysis.

INTRODUCTION

Rice, *Oryza sativa* L. belongs to the family *Poaceae* and is one of the most important cereals, which feeds 50% of the population worldwide (Behera et al., 2012). It is cultivated on about one-fifth of the total arable land occupied by cereals and grown over wide geographical range under diverse cultural conditions. Large part of the Asian continent, which comprises more than half of the global population, cultivates and consumes 90% rice as primary food. Rice is annually planted over 11% crop land of the world, and ranks next to wheat (Chakravarti et al., 2012, 2013).

For the crop improvement, knowledge of genetic diversity is of utmost importance. The existing genetic diversity in the background of distinct plant types is the basic treasure which can be exploited by plant breeders to reconstruct the existing genotypes in the form of new varieties (Khera et al., 2012). Indian cultivars, landraces, wild and weedy relatives are the main source of rice diversity and are the rich source of worthy genes that plant breeders can exploit for crop improvement (Yadav et al., 2013).

For the estimation of diversity in germplasm, different methods are available, such as evaluation of phenotypic variation, biochemical and DNA polymorphisms. Both phenotypic and biochemical characterizations are not very reliable because they are influenced by environment, involve high labour cost, have numerical and phenological limitations. Opposite to this, DNA-based molecular markers are omnipresent, repetitive, stable and highly dependable (Kumar et al., 2011b). Several molecular markers have been used to characterize crop resources in rice and other crops, including restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), simple sequence repeat (SSR), and inter-simple sequence repeat (ISSR), (Saini et al., 2004; Kumar et al., 2011a, 2011b).

Received 21 January 2015; accepted 7 December 2015; First Online: March, 2016. DII 2067-5720 RAR 2016-16

Previously, comparative studies for diversity analysis in rice involving ISSR and SSR were successfully utilized by very few researchers, but these researches were mainly focused on basmati type (Saini et al., 2004; Singh et al., 2011; Kladmook et al., 2012; Rabey et al., 2012).

The objective of the present study was to evaluate the genetic diversity among the landraces, basmati lines and popular cultivars of rice by utilizing random SSR, ISSR and dwarfing trait based markers for their subsequent use in rice breeding program.

MATERIAL AND METHODS

Plant material

The experimental material comprised of 32 rice accessions representing landraces, released varieties, cultivars and breeding lines obtained from different breeding centres of north India. Details of these lines with their characteristic features are given in Table 6. The experimental trial was laid out in randomized complete block design with three replications during Kharif 2012 at the Crop Research Center, Chirodi farm of Sardar Vallabh bhai Patel University of Agriculture and Technology, following standard agronomic practices and management.

DNA Isolation and polymerase chain reaction

A total of 10 ISSR, 10 SSR markers and three trait-linked SSR markers were used for diversity analysis (Tables 1, 2 and 3). SSR markers were selected from rice genes database (http://www.Gramene.org/microsat/ RM primers.html). Three trait linked markers, linked to dwarfing trait (Neeraja et al., 2009), *h* and *k* (Monna et al., 2002) were used for confirmation of presence of these genes. The primers were custom synthesized by IDT (Integrated DNA technologies) Pvt. Ltd.

DNA was extracted using the cetyl-trimethyl ammonium bromide (CTAB) method (Murray and Thompson, 1980). DNA concentration was estimated by the fluorescence intensities of ethidium bromide-stained samples on 0.8% agarose gels.

Table 1. ISSR markers used to amplify the *O. sativa* in the study

S.N.	Primer code	Primer sequence (5′→3′)
1	ISSR1	GGCGGCGGCGGCGGCAT
2	ISSR2	AAGAAGAAGAAGAAGGC
3	ISSR3	AAGAAGAAGAAGAAGTG
4	ISSR4	AAGAAGAAGAAGAAGCC
5	ISSR5	AGCAGCAGCAGCAGCCA
6	ISSR6	AGCAGCAGCAGCAGCCG
7	ISSR7	GGCGGCGGCGGCGGCTA
8	ISSR8	AGCAGCAGCAGCAGCGA
9	ISSR9	AAGAAGAAGAAGAAGCG
10	ISSR10	CCAGTGGTGGTGGTG

Table 2. SSR markers used to amplify the *O. sativa* in the study

Primer Code		Primer sequence (5′→3′)
RM 1	Forward	GCGTTGGTTGGACCTGAC
	Reverse	GCGAAAACACAATGCAAAAA
RM 10	Forward	CAGAATGGGAAATGGGTCC
	Reverse	TTGTCAAGAGGAGGCATCG
RM16	Forward	AACACAGCAGGTACGCGC
	Reverse	CGCTAGGGCAGCATCTAAA
RM20	Forward	GAAACAGAGGCACATTTCATTG
	Reverse	ATCTTGTCCCTGCAGGTCAT
RM21	Forward	GCTCCATGAGGGTGGTAGAG
	Reverse	ACAGTATTCCGTAGGCACGG
RM26	Forward	CTGCGAGCGACGGTAACA
	Reverse	GAGTCGACGAGCGGCAGA
RM41	Forward	AATTTCTACGTCGTCGGGC
	Reverse	AAGTCTAGTTTGCCTCCC
RM44	Forward	TCGGGAAAACCTACCCTACC
	Reverse	ACGGGCAATCCGAACAACC
RM152	Forward	GAAACCACCACACCTCACCG
	Reverse	CCGTAGACCTTCTTGAAGTAG
RM162	Forward	GCCAGCAAAACCAGGGATCCGG
	Reverse	CAAGGTCTTGTGCGGCTTGCGG

The PCR mixture contained 25-30 ng template DNA, 5 pmol of each primer, 0.05 mM dNTPs, 10× PCR buffer and 0.5 U of *Taq* DNA polymerase in a reaction volume of 15 μL. For SSRs, denatured at 94ºC for 5 min followed by 35 cycles (1 min denaturation at 94ºC, 1 min annealing at 55ºC and 1 min of primer extension at 72ºC) of PCR amplification was followed by final extension of 72ºC for 7 min. For ISSRs, annealing temperature was kept 50ºC for 1 min and rest of the cycles were

same. The amplification products were separated on 3.5 % metaphor agarose gels and run for 3 h in 1×TAE buffer. DNA fragments were visualized under UV trans- illumination using gel documentation system (Gel Doc™ XR+ Imager, Bio-Rad, USA).

Table 3. Three Dwarfing genes specific markers used for the identification of dwarfism in rice genotype

S.N.	Primer		Primer sequence (5′→3′)	Reference
1	sd-1	Forward Reverse	CACGCACGGGTTCTTCCAGGTG AGGAGAATAGGAGATGGTTTACC	Neeraja et al., 2009
2	k	Forward Reverse	AGCTGGACATGCCCGTGGTC TTGAGCTGCTGTCCGCGAAG	Monna et al., 2002
3	h	Forward Reverse	GACTCAACAGGCCCTCCAAA CCACGCGGTTATTGCAAGTT	Monna et al., 2002

Data analysis

Only consistent and reproducible bands were scored in binary format as 1 for presence and 0 for absence. Smeared and weak bands were excluded. Fragments of the same molecular weight were considered to represent the same allele. The PIC value for each locus was calculated as according to Kumar et al. (2011a), the pair-wise genetic similarities among all pairs of samples were estimated using Jaccard's coefficient (Jaccard, 1908). In order to group genotypes into discrete clusters, UPGMA based dendrogram was constructed using NTSYS-pc 2.1 software (Rohlf, 2000). The power of each primer to distinguish the studied genotypes was evaluated by the resolving Power (RP) (Provost and Wilkinson, 1999) as: $Rp=\Sigma Ib$ where *Ib* is the band informativeness, that takes the values of: 1-(2 x [0.5-p]), being p the proportion of the rice varieties containing the band.

RESULTS AND DISCUSSION

Fingerprinting patterns of SSR and ISSR

A total of 87 reproducible band positions were amplified by 10 ISSR (57 band positions) and 10 SSR primers (30 band positions), among which 81 were polymorphic (Table 4). The number of alleles generated by ISSR primers varied from two (ISSR 1) to ten (ISSR 7) with a mean of 5.7 alleles per locus, while that of SSR primers varied from two (RM-01, RM-10, RM-16, RM-20, RM-21, RM-152, and RM-41) to seven (RM-162) with a mean of 3 alleles per locus (Table 4). The size of the amplified products of ISSR and SSR markers ranged from 300-2000 bp and 200-300 bp respectively, which were in agreement with earlier studies (Saini et al., 2004; Rabbani et al., 2008; Kiani et al., 2011).

Table 4. Comparative analysis of SSR and ISSR marker

Components	ISSR	SSR
Total no. of Primers used	10	10
Polymorphic markers		
Total no. of bands amplified	57	30
Average no. of bands per primer	5.7	3.0
Maximum No. of bands amplified by a single primer	10	7
No. of polymorphic bands	53	28
Percentage of polymorphic bands	92	93
Maximum No. of polymorphic bands amplified by a primer	9	7
PIC		
Minimum	0.060	0.424
Maximum	0.943	0.920
Average	0.525	0.696
Resolving power		
Minimum	1.250	1.00
Maximum	13.312	7.737
Average	6.275	2.823
Size of PCR product	300-2000	100-3000

The maximum numbers of polymorphic bands (9 bands) were obtained using ISSR 4 primer. The average number of polymorphic bands was 4.05 per primer. About 93% polymorphism was obtained in the present

ISSR & SSR assay. Gene diversity expressed as polymorphism information content (PIC) values for the 10 ISSR loci varied from 0.060 for ISSR 1 to 0.943 for ISSR 3 with an average of 0.525 (Table 4).

The primer ISSR 3 was observed to be highly polymorphic (PIC value of 0.943). This was not surprising because ISSR markers are regarded as highly polymorphic, as they amplify unique regions in between. Our results were accordance with earlier reports in rice (Nagaraju et al., 2002; Saini et al., 2004) and *yellow sarson* (Kumar et al., 2011a). For SSR PIC values ranged from 0.424 (RM-44 primer) to 0.920 (RM-41 primer) with an average of 0.696 (Table 4). The higher the PIC value of a locus, the higher the number of alleles detected. This observed pattern was consistent with earlier findings in rice (Wang et al., 2009; Joshi et al., 2010). The maximum resolving power (RP) of ISSR marker was 13.312 by ISSR7 and minimum 1.250 with an average value of 6.275. In case of SSR marker maximum RP 7.737 was by RM 21 and minimum 1.00 with an average value of 2.823 (Table 4 and Figures 1 and 2). The greater the value of resolving power the more information is provided by that particular primer (Provost and Wilkinson's 1999). The extent of polymorphism in the present study was higher as compared to the earlier report in rice with arbitrary primers (Rabbani et al., 2008; Saini et al., 2004 and Skaria et al., 2011).

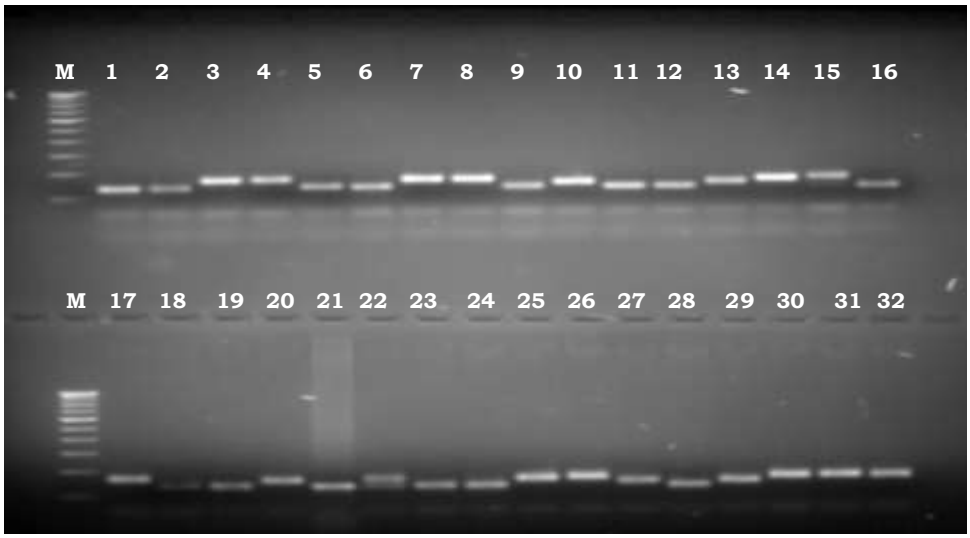

Figure 1. SSR profiling pattern of 32 rice varieties with RM 21 primer, M-50bp Ladder

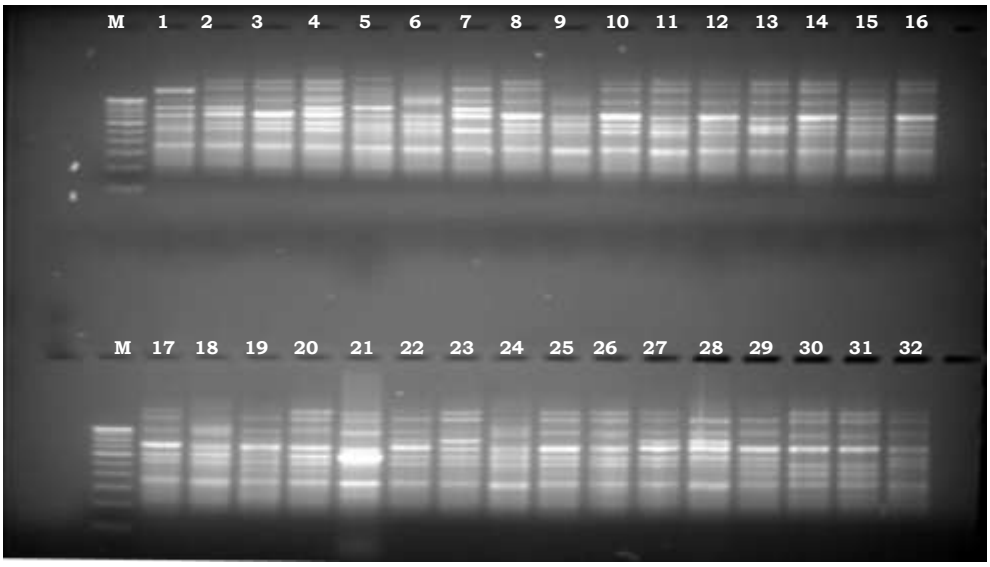

Figure 2. ISSR profiling pattern of 32 rice varieties with ISSR7 primer, M-50bp Ladder

Genetic similarity analysis

All the 87 bands, generated from 10 ISSR and 10 SSR primers, were used to calculate the genetic similarity index (GS) among the 32 accessions. Genetic similarities were calculated using the Nei-Li similarity co-efficient. Significant genetic variation was found among all rice accessions, with the GS value ranging from (0.60) to (0.97) with a mean of 0.737. Approximately similar results of similarity coefficients were observed among Indian and Pakistani rice varieties in previous studies (Rabbani et al., 2008; Kiani et al., 2011). Of the 32 pair wise combinations generated by rice genotypes, the highest coefficient of similarity (0.964) was obtained between VD 62 and VLD86, which appear as the most similar and closely related accessions. The lowest coefficient of similarity 0.505 was observed between Sarbati-A and Sathi, Bagni and VB24 genotypes.

Cluster analysis

The cluster analysis based on unweighted paired group method of arithmetic means (UPGMA) with 10 SSR and 10 ISSR primers allowed the discrimination of cultivars. The UPGMA based clustering grouped the 32 rice genotypes into five major groups (Table 5). Groups II had maximum number of genotypes (23) followed by group IV (4), group I (3), group III (1) and group V (1). The number of groups was similar to previous studies on genetic diversity and relationships among the different cultivars (Rabbani et al., 2008; Kiani et al., 2011).

Table 5. Distribution of rice genotypes into different clusters based on analysis

Groups	Sub group	Cluster	No. of genotypes	Genotypes
I	A	A1 A2	2 1	N22, MAUB 171 PD-12
II	B	B1	1	PUSA-2511
		B2	1	PUSA-677
		B3	4	PS-2, CSR-27, VLD-86 , VD62
		B4	3	MAUB-13, VB-24, SARBATI-B
		B5	2	SARBATI-A, PR106
		B6	2	CSR10, PB-1
		B7	2	VL-30177, TETEP
		B8	1	BAGNI
		B9	1	VLD-65
		B10	6	Govind, Pusa 1401,CSR30, CSR13, VLD61, VLD-81
III	C		1	VLD85
IV	D	D1	1	VB-22
		D2	3	BASMATI-370-A, PUNJAB BASMATI 2, BASMATI-370-B
V	O		1	SATHI

Genetic similarities obtained from ISSR and SSR data were used to create a cluster diagram. Cluster diagram revealed that Group I could be further divided into two clusters A1 and A2 at 0.76 similarity coefficients showing varied degree of similarities (Figure 3). The first cluster A1 includes two rice varieties N22 and MAUB 171 whereas second cluster A2 included Pant dhan 12. Group II was the largest and most diverse, consisting of 23 genotypes viz. PUSA-2511. Pusa 677, Pusa Sughand-2, CSR-27, VLD-86 , VD 62, MAUB-13, Vallabh Basmati-24, SARBATI-B, SARBATI-A, PR 106, CSR 10, Pusa Basmati-1, VL-30177, TETEP, Bagani, VLD-65, Govind, Pusa 1401,CSR 30, CSR 13,

VLD 61 and VLD-81. This group was further divided into ten clusters of varying degree of similarity. Cluster B1 includes one cross bred basmati variety PUSA-2511 derived from cross Pusa 3a/Haryana Basmati, whereas cluster B2 had variety Pusa 677. Cluster B3 contains four varieties namely, Pusa Sugandh-2, CSR-27, VLD-86, VD 62. Two cross bred basmati varieties MAUB-13 and Vallabh Basmati-24, along with Sharbati-B constituted cluster B4, whereas SARBATI-A and PR106 were in cluster B5. Cluster B6 includes two varieties CSR 10 and Pusa Basmati-1, whereas two varieties VL-30177 and TETEP were included in cluster B7. Bagni and VLD 65 represented Cluster B8 and B9 respectively. Cluster B10 had six accessions Govind, Pusa 1401, CSR 30, CSR 13, VLD 61 and VLD 81.

Group three had only one accession VLD-85, while Group four was separated into two clusters D1 and D 2 at GS= (0.708). D1 contains only one accession Vallabh Basmai-22 and two traditional basmati varieties Basmati-370-A and Basmati-370-B along with Punjab Basmati 2 were in cluster D2. The single genotype, Sathi was grouped in quite distinct cluster V and can be used for desirable characteristic in breeding programmes. Identical pattern of clusters differences were reported in earlier studies on rice including Basmati rice by using SSR and ISSRs (Nagaraju et al., 2002; Berilus et al., 2013). It is supposed that high heterosis could be obtained by crossing between the genotypes with low similarity coefficient (Abubakar et al., 2011; Biswas et al., 2012).

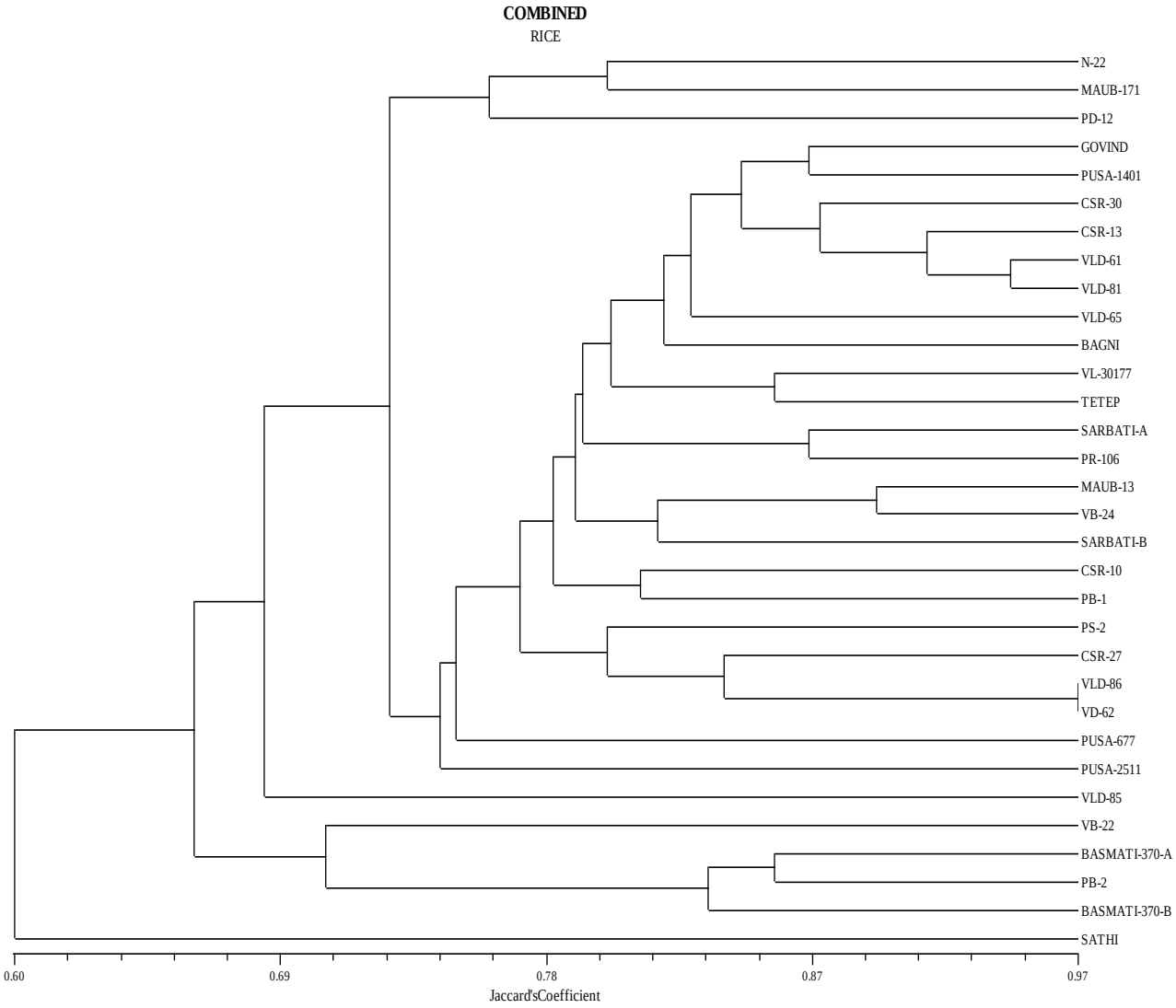

Figure 3. Dendrogram showing clustering of 32 rice varieties constructed using UPGMA based on Jaccard's similarity coefficient obtained from ISSR and SSR analysis

Microsatellite markers for dwarfism (sd-1) QTLs/gene

The molecular markers specific for the height trait in rice were used to detect bands among thirty two rice genotypes. Reproducibility of the amplification of banding pattern was confirmed by repeating each reaction at least thrice with deliberate alteration in the protocol. Although, a number of species-diagnostic marker bands were noticed, most of them were either rather faint or not repeatedly found in all thirty two rice genotypes. Thus, a large number of bands perhaps, might be potentially genotype-specific; therefore such informative bands were eliminated from consideration.

Table 6. Expression and characteristic features of dwarfing genes in rice varieties

S.No.	Varieties	Characteristic Features	Sd-1	H	RT01
1	N-22	Puddled, Semi dwarf, non-scented, Salinity resistant	+	-	-
2	MAUB-171	Super fine, mid-tall, medium duration	-	-	+
3	PANT DHAN-12	Semi dwarf, medium, duration, coarse	+	+	+
4	GOVIND	Dwarf, short duration, non-scented	+	-	+
5	SATHI SAFED	Dwarf, short duration, coarse	+	-	-
6	VALLABH BASMATI-22	Super fine, long grain, semi dwarf, short duration	+	+	+
7	PUSA-677	Puddled, Semi dwarf, non-scented	+	+	+
8	PUSA-1401	Semi dwarf, medium duration, disease resistant	+	+	+
9	BASMATI-370-(A)	Premium traditional Basmati rice variety			
10	SARBATI-A	Semi dwarf, short duration, lodging & disease resistant	-	+	-
11	MAUB-13	Semi dwarf, short duration, lodging & disease resistant	-	+	+
12	SARBTI-B	Semi dwarf, short duration, lodging & disease resistant	-	+	-
13	CSR-10	Semi dwarf, salt tolerant, coarse grain	-	-	+
14	CSR-30	Semi dwarf, salt tolerant, coarse grain	-	-	+
15	PUSA BASMATI -1	Super fine, semi dwarf, export quality	-	-	+
16	PUSA SUGHANDA-2	Semi dwarf, medium duration, aromatic	+	+	+
17	CSR-13	Semi dwarf, salt tolerant, coarse grain	-	-	+
18	PUNJAB BASMATI-2	Super fine, semi dwarf, export quality	+	-	-
19	PUSA-2511	Semi dwarf, scented & basmati type	+	-	+
20	CSR-27	Semi dwarf, salt tolerant, coarse grain	-	-	+
21	VLD-85	Semi dwarf, short duration, coarse			
22	VALLABH BAGNI	Dwarf, medium duration, violet color foliage	+	+	+
23	VB-24	Super fine, long grain, semi dwarf, short duration and owned.	+	-	-
24	Basmati-370 (B)	Premium traditional Basmati rice variety	-	+	-
25	VLD-61	Semi dwarf, short duration, coarse	-	-	+
26	VLD-81	Semi dwarf, short duration, coarse			
27	PR -106	Dwarf, short duration, coarse grain	+	-	+
28	VL-30177	Semi dwarf, short duration, coarse	+	-	+
29	TETEP	Blast resistance, tall, coarse grain	-	-	-
30	VLD-86	Semi dwarf, short duration, coarse	-	-	-
31	VD-62	Semi dwarf, short duration, coarse	-	-	-
32	VLD-65	Semi dwarf, short duration, coarse	-	-	-

Sd-1 gene specific bands were amplified in fourteen semi-dwarf varieties viz. N22, Pant Dhan-12, Govind, Sathi, Vallabh Basmati-22, Pusa-677, Pusa-1401, Pusa Sugandha-2, Punjab Basmati-2, Pusa-2511, Bagni, Vallabh Basmati-24, PR 106 and VL-30177 (Table 6). H gene specific bands were amplified in ten varieties viz. Pant Dhan-12, Vallabh Basmati-22, Pusa-677, Pusa-1401, Pusa Sugandha-2, Bagni, Sharbati-A, Sharbati-B, MAUB-13 and Basmati-370 (B). RT01 gene specific bands were amplified in

eighteen varieties viz. MAUB-171, Pant Dhan-12, Govind, Vallabh Basmati-22, Pusa-677, Pusa-1401, MAUB-13, CSR-10, CSR-30, Pusa Basmati -1, Pusa Sughanda-2, CSR-13, Pusa-2511, CSR-27, Bagni, VLD-61, PR-106 and VL 30177 (Table 6). Utilization of gene specific markers in previous studies was done for genotypes more closely related to *japonica* than *indica* group (Garris et al., 2005). The conspicuous differentiation of Basmati and non Basmati varieties with gene specific markers was studied by Nagaraju et al. (2002). This kind of classification with QTL linked/gene based SSR markers were well documented by Yadav et al. (2013) for diversity estimation of Asian rice germplasm and Indian rice germplasm.

CONCLUSION

North India holds an immense resource of rice cultivars that are great significance not only for breeders but also for farmers. An effort was made to assess genetic diversity in set of 32 accessions including landraces, released varieties, cultivars and breeding lines. The result of the present study proved the utility of microsatellite and ISSR markers in genetic diversity at random and functional regions of the genome of rice genotypes. All the genotypes analysed were distinct from each other at the molecular level. Sarbati-A and Sathi, Bagni and VB24 genotypes were assessed as useful for heterosis based rice breeding programmes, offered promise for their use in the genetic improvement of rice cultivars for grain quality.

Acknowledgements

The authors are thankful to Department of Biotechnology S.V.P.U.A & T, Modipuram, Meerut (U.P.) for providing all the facilities for the execution of this research work.

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