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Performance of short duration rice mutants and assessment of genetic diversity using RAPD and ISSR markers

Swapan K. Tripathy[1*], Roshni Ekka[2], Devraj Lenka[1], Rajesh Ranjan[1], Shovina Pal[3], Bhumika Ray Mohapatra[3]

[1]*Sinha Molecular Breeding Laboratory, Department of Plant Breeding & Genetics, College of Agriculture, OUAT, Bhubaneswar-751003 (Odisha), India*

[2]*Department of Botany, Biotechnology Wing, Ravenshaw University, Cuttack, India*

[3]*MITS School of Biotechnology, Bhubaneswar, India*

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Abstract

Eleven short duration rice mutants along with their parent (Mandakini) and three standard checks (Jogesh, Nilagiri and Annapurna) were evaluated in the field. ORT 11, ORT 30 and ORT 35 out yielded significantly (>3250kg/ha) as compared to the parent (2830kg/ha) and also the best standard check variety Annapurna (2950q/ha). High yield performance of the aforesaid mutants were associated with high tiller number, number of grains/panicle, grain weight, and fertility percentage. 13 RAPD and 11 ISSR primers revealed 78.32% and 96.55% polymorphism respectively across the test genotypes and primers. RAPD had shown comparatively high genomic homology, whereas ISSR revealed divergent genotype pair with similarity coefficient value as low as 0.20 between Annapurna and Mandakini followed by Annapurna and ORT 30; and Annapurna and ORT 15.. The mutant ORT 38 and variety Jogesh clubbed together and could not be distinguished from each other at even 100% phenon level using RAPD markers. However, ISSR alone as well as the combined analysis could discriminate such genotypes. Considering the level of polymorphism, similarity coefficient value and clustering pattern; ISSR markers are preferred as these are comparatively more informative and potent enough for study of genetic diversity than RAPD markers. The genotype-specific RAPD and ISSR profiles would help to certify the genetic make up in rice genotypes.

*Corresponding Author: Swapan K. Tripathy ✉ swapankumartripathy@gmail.com

Introduction

In recent years, genetic improvement of rice in India and many other rice growing countries has already achieved yield platuae for medium land irrigated rice ecosystems, but areas pertaining to rain fed rice ecosystems are constrained with low productivity as these often experience water deficit owing to recurrent erratic rainfall and high temperature. About 45% of the world's rice is cultivated in rain fed ecosystems (Nazari and Pakniyat, 2008). The existing short duration rice varieties e.g., khandagiri, Parijat, Udaygiri, Naveen, Nilagiri have practically poor genetic potential for seed yield (<20q/ha) and lack abiotic stress tolerance; and are rarely adaptable to pre-monsoon drought situation. However, the local land races e.g., Sathiapia, Kalapank, Saria etc and some of the germplasm lines e.g., Jhu 11-26 and N 22 to name a few, harbour very high degree of tolerance to drought, but with poor productivity. Development of drought resistant cultivars will considerably improve rain fed rice production. However, little progress has been made in improving the genetic potential of rice for drought resistance due to lack of phenotyping facilities for precise drought resistance screening (Ribaut *et al*., 1997). Therefore, there is an urgent need to reorient breeding strategies to develop high yielding rice varieties to combat recurrent drought situation in coming years and characterize the genotypes based on molecular markers..

Recently, molecular tools facilitate the identification of genomic locations linked to traits of interest and help in indirect selection of such complex traits without the need for difficult phenotypic measurements. The molecular markers also provide precise information for varietal identification and genetic diversity. RFLP, AFLP, SSR, STS are too costly to employ in genotyping. On the other hand, RAPD and ISSR being multi-locus, simple, inexpensive and rapid techniques, can be aminable in various fields of crop improvement (Godwin *et al.,* 1997). But, dominant nature and low reproducibility of RAPD makes it less powerful than other markers. The ISSRs (inter simple sequence repeats) lie within the microsatellite repeats and offer great potential over RAPD, since they reveal variation in unique regions of the genome at several loci simultaneously (Zietkicwicz *et al* 1994). Besides, high variability among taxa, ubiquitous occurrence and high copy number of microsatellites in eukaryotic genomes (Weising *et al.*1998), make ISSRs extremely useful. ISSR even if being dominant in nature, overcome most of the limitations of other marker systems (Zietkiewicz *et al.,* 1994; Gupta *et al.,* 1994; Wu *et al.,* 1994; Meyer *et al.,* 1993) as they are cost effective, highly polymorphic, repeatable, need no prior sequence information and can exhibit specificity of sequence- tagged-site markers**.** In the present study, the potential of these markers in genotype profiling have been exploited.

In the present investigation, an attempt was undertaken to develop a few high yielding mutant lines of rice suitable under drought stress and characterize these along with four popular standard genotypes for varietal identification and study of *inter se* genetic diversity using RAPD and ISSR markers for their use in further breeding programme.

Materials and methods

15 short duration rice genotypes including 11 mutants (derived through recurrent mutagenesis with 0.5%EMS and 0.015%NG), their parent variety Mandakini, and three standard check varieties.(Jogesh, Nilagiri and Annapurna) were grown in the late Rabi season 2011-12 and their phenotypic performance for important agro-economic traits have been presented in Table 1**.** Genomic DNA of each of the test genotypes were isolated following Dellaporta *et al.*(1983) with little modification, purified by treatment with RNase A, quantified and diluted to a working concentration of 10ng/µl. 13 random decamer primers and 11 ISSR primers were screened out to amplify the genomic DNA. The amplifications were performed in a reaction volume 25µl containing 10mM Tris-HCl, pH 9.0, 1.5mM $MgCl_2$, 50mM KCl, 0.01% gelatin, 100µM each of dNTPs, 10ng of single random

primer, 10ng of genomic DNA and 1 unit of Taq polymerase (Genei, Bangalore). Amplifications were performed in a Gene Pro Thermocycler (Bioer Tech. Co., Ltd, Japan), programmed for 5min at 94°C, 40 cycles of 1min at 94°C for denaturation, 1min 30 sec. at annealing temperature as mentioned in Table 2 and 2min at 72°C for synthesis of daughter DNA strand, and final extension for 7 min at 72°C followed by storing at 4°C till loading to the agarose gel. The amplified products were loaded in agarose gel (1.5% in case of RAPD and 2.0% in case of ISSR) containing ethidium bromide @1.0µg/ml of agarose solution and electrophoresed at a constant voltage of 50V. The amplifications were checked for their reproducibility.

The gels were documented by gel doc system (Fire Reader-Uvtec, Cambridge, UK) for scoring the bands. The size of amplicons were determined by comparing with the lambda DNA ladder (500bp) with known fragment sizes. Gels were scored for the presence (1) or absence (0) of bands and the binary data score was analysed to estimate Jaccard's similarity coefficient (Jaccard 1908) values. The resultant distance/similarity matrix was used to construct an Unweighted Paired-Group Method with Arithmetic means (UPGMA)-phenograms (Sokal and Michener, 1958) employing Sequential Agglomerative Hierarchic and Non-overlapping Clustering (SAHN).

Results and discussion

The mutant genotypes had shown wide variation(Table 1) in important agro-economic traits e.g., maturity duration(90-125days), tiller number/m², panicle length, grain weight, fertility percentage and seed yield(kg/ha). The mutant cultures e.g., ORT 30, ORT 11 and ORT 35 were top yielders with significantly higher grain yield (>3250kg/ha) as compared to the best standard check variety Annapurna (2950kg/ha) as well as their parent variety Mandakini (2830kg/ha). High yield performance of these mutants could be attributed to high tiller number, more no. of seeds/panicle, high grain weight and fertility percentage. Elucidation of such a genetic variation at phenotypic level could be better characterized by genotyping. Therefore, the present investigation was further extended for molecular characterization to assess genomic homology, genotype-specific characteristics, heirarchical genotypic relationship in terms of clustering pattern based on genetic distance between paired genotypes.

RAPD and ISSR have been standardized and employed successfully by different workers (Jeung *et al.*, 2005; Nasr *et al.,* 2009; Girma *et al., 2010;* Beverley *et al.,* 1997; Kaushik *et al.,* 2003; Bornet and Branchard, 2001 and Ye chunjiang *et al.,* 2005) to analyse samples of *Oryza* species. The success in generating wide range of polymorphic loci depends on proper choice of primers for DNA amplification. The optimum number of primers required to differentiate two or more cultivars may vary with the test materials used. When the variation in the cultivars is high the use of few primers can serve the purpose of generating useful information. Dhanaraj *et al.*(2002) used only seven primers, while only 2-3 primers were sufficient to distinguish between cultivars of broccoli (Hu and Quaros, 1991). However, it is *in vogue* to use more no. of primers to differentiate closely related cultivars in rice. Mackill (1995) could not differentiate a few number of japonica rice cultivars even using 21 RAPD primers due to low germplasm diversity. In the present investigation, initially twenty five random primers and fifteen ISSR primers (Chromos, India) were examined out of which three RAPD primers and two ISSR primers gave smeared background, one ISSR primer resulted all monomorphic bands, and a few of the primers of either RAPD and ISSR did not produce any amplified products. As a result, thirteen RAPD primers and eleven ISSR primers were finally screened for molecular characterization of 15 test genotypes. A list of such informative primers is presented in Table 2**.** In the present investigation, a wide array of PCR products in terms of amplicon size (bp) was revealed. Comparatively, RAPD produced amplicons of longer size than ISSR. It ranged from 380 to 2500bp in case of RAPD while, ISSR revealed

amplicon sizes within the range of 350 to 1650bp only. The total number of bands for all primers ranged from 4-8 with an average of 5.53 bands per RAPD primer. The ISSR primers produced 4-6 bands with an average of 5.54 bands per primer. RAPD produced as a whole 62 polymorphic amplicons out of a total 72 amplified products and thus, it resulted polymorphism to the tune of 86.1%. Whereas, ISSR was potent enough to reveal 60 polymorphic bands out of a total 61 bands scored resulting in a tremendous higher level of polymorphism (98.36%) in the present set of materials.

Table 1. Mean performance of 15 short duration rice varieties for agro-economic traits.

Sl. No.	Genotype	Days to maturity	Plant height (cm.)	Tiller No./m²	Panicle length (cm.)	No. of seeds /panicle	% fertility	100-seed weight	Seed yield (kg/ha)
1.	ORT-5	95	76	480	19.5	78	81.05	2.26	2410
2.	ORT 7	90	105	450	19.0	82	74.54	2.52	2930
3.	ORT 8	101	73	550	25.0	82	73.8	2.35	2950
4.	ORT 11	105	72	690	20.0	92	88.18	2.34	3430**
5.	ORT 15	110	89	550	20.0	73	70.24	2.32	3000
6.	ORT 22	108	67	580	19.0	86	84.84	2.23	2223
7.	ORT 26	103	84	586	19.0	88	81.60	2.21	2567
8.	ORT 30	125	77	580	23.0	105	91.23	2.65	3560**
9.	ORT 35	98	84	550	23.0	92	95.35	2.35	3260**
10.	ORT 36	97	83	624	25.0	115	88.46	2.53	3034
11.	ORT 38	106	74	560	18.5	103	85.21	1.86	2400
12.	Mandakini	105	70	410	19.5	78	83.87	2.15	2830
13.	Jogesh	103	83	400	16.5	76	83.33	2.52	2400
14.	Annapurna	112	90	420	20.0	78	84.5	2.00	2950
15.	Nilagiri	106	78	450	22.0	75	82.22	2.38	2540
$C.D._{0.01}$		5.8	15.8	45.6	2.68	12.8	5.60	0.40	230

Note: Annapurna -best standard check. **- significant at $P_{0.01}$

Besides, the total number of bands scored over RAPD and ISSR primers varied widely among the genotypes. It ranged from 36 bands in Mandakini to as high as 56 bands in ORT 26 (a mutant of Mandakini) using RAPD as against 17 bands in Annapurna to 41 bands in var. Jogesh in case of ISSR (data not shown). The binary data matrix of RAPD and ISSR resolved 542 and 420 polymorphic amplicons out of total 692 and 435 PCR products over the test genotypes and primers used which reveals 78.32% and 96.55% polymorphism in RAPD and ISSR respectively. The high level of polymorphism obtained in the present investigation may be ascribed to erstwhile mentioned higher genetic variation in the selected 15 test genotypes. Kaushik *et al.*2003 obtained a total 149 bands ranging from 200-3530bp in two rice varieties and the selected CSR 10 X HBC19 segregating F_3 plants using 26 ISSR primers.

Seven out of thirteen RAPD primers resulted few monomorphic bands (Table 2). But, all amplicons produced by ISSR primers were polymorphic except the primer OUAT 11 which resulted one monomorphic (358bp) and three polymorphic loci. Three RAPD primers OU6, OU 9 and OU18; and seven ISSR primers OUAT 2, OUAT 6, OUAT 12, OUAT 13, OUAT 15, OUAT 16 and OUAT 18 revealed as high as six polymorphic amplicons each and hence, these could be considered as highly informative. Beverley *et al.*(1997) studied genetic variation of *Oryza sativa* from 19 localities using two PCR-based molecular marker systems (RAPD and ISSR-PCR). Employing RAPD, a set of 14 decamers of arbitrary sequence yielded amplification of 94 reproducible marker bands, 47(50%) of which were polymorphic Whereas, ISSR primers produced 71 PCR products with 56% polymorphism.

Table 2. Amplified products with different primers in 15 test genotypes.

Sl. No.	Primer Code	Sequences (5'-3')	GC content (%)	*Tm (°C)	*Tan (°C)	Polymorphic bands	No. of monomorphic bands	Total bands	% polymorphism	Range of fragment size in bp
					RAPD Primers					
1.	OU-1	CAGGCGGCGT	80.0	36.0	38.0	6	2	8	75.0	460-1800
2.	OU-2	ACTGAACGCC	60.0	32.0	34.0	4	-	4	100.0	500-2100
3.	OU-3	GGTGAACGCT	60.0	32.0	34.0	5	1	6	83.3	450-1750
4.	OU-4	TGGACCGGTG	70.0	34.0	36.0	5	-	5	100.0	580-2110
5.	OU-5	AGGACGTGCC	70.0	34.0	36.0	3	2	5	60.0	550-2000
6.	OU-6	GGGCTAGGGT	70.0	34.0	36.0	6	-	6	100.0	520-2000
7.	OU-9	GCGGGCAGGA	80.0	36.0	34.0	6	-	6	100.0	490-1700
8.	OU-10	AATCGGGCTG	60.0	32.0	34.0	4	1	5	80.0	455-1800
9.	OU-16	CTGAGACGGA	60.0	32.0	34.0	6	1	7	85.7	420-2000
10.	OU-18	GTGCGAGAAC	60.0	32.0	34.0	6	-	6	100.0	470-1900
11.	OU-20	AACGTCGAGG	60.0	32.0	34.0	3	1	4	75.0	**380**-2220
12.	OU-24	TCCGCTGAGA	60.0	32.0	34.0	3	2	5	60.0	445-**2500**
13.	OU-28	TGCTGCAGGT	60.0	32.0	34.0	5	-	5	100.0	385-1930
	Total					62	10	72		
					ISSR Primers					
1	OUAT-1	(AACC)6T	48.0	63.0	61.0	5	-	5	100.0	430-1250
2	OUAT-2	(GGTT)6C	52.0	64.6	61.0	6	-	6	100.0	**350**-780
3	OUAT-6	(AACT)6T	24.0	53.1	56.0	6	-	6	100.0	450-1300
4	OUAT-7	CCA(GTG)4	66.7	53.3	54.0	5	-	5	100.0	420-1260
5	OUAT-11	(TG)8C	52.9	52.8	56.6	3	1	4	75.0	358-1500
6	OUAT-12	(AG)8C	52.9	52.8	46.6	6	-	6	100.0	700-**1650**
7	OUAT-13	(GA)8C	52.9	52.8	46.6	6	-	6	100.0	520-980
8	OUAT-15	(GGC)5T	88.2	67.2	58.0	6	-	6	100.0	650-1230
9	OUAT-16	(AAG)5GC	41.2	47.9	51.2	6	-	6	100.0	550-1350
10	OUAT-17	(GGC)5T	88.2	67.2	60.0	5	-	5	100.0	490-1200
11	OUAT-18	(AGC)5CG	70.6	60.0	61.0	6	-	6	100.0	530-1050
	Total					60	1	61		

Note: Range of amplicon size as a whole for RAPD and ISSR marked bold. * -"Tm" and "Tan" denote melting and annealing temperature of primers respectively.

Banding pattern using RAPD and ISSR markers

DNA banding pattern of each of the genotypes is expected to differ if they are genetically different. Even subtle difference at genotypic level which other-wise sometimes could not be possible to differentiate by phenotyping, can be confirmed by use of markers. In the present pursuit, presence or absence of a few bands were found to be specific to certain test genotypes. RAPD band of amplicon size 1750 bp and 1000bp (amplified by Primer OU 4) were specifically absent in the mutant genotype ORT 7 and ORT 30 respectively, but were present in the parent variety Mandakini(Fig. 1). The ISSR primer OUAT 2 resulted six PCR products of amplicon size 750bp, 680bp, 600bp, 480bp, 450bp and 380bp. The 480bp amplicon was unique and was specifically amplified in mutant ORT 15(Fig. 2a). The ISSR primer OUAT 11 revealed four bands of fragment size 1500bp, 630bp, 500bp and 358bp among which amplicon 500bp was specifically observed only in mutant ORT 30 and its parent Mandakini (Fig. 2b). Whereas, the genotype Annapurna had shown absence of 1500bp and 630bp bands which were present in all other test genotypes. Such genotype-specific information could certainly help in genotype identification. Ye chunjiang *et al.*(2005) proposed that RAPD primers can be successfully used with ISSR primers for detection of new genomic loci and applied for genome mapping, finger printing and gene tagging. However, Jeung *et al.*(2005) compared the relative efficiency of three marker systems, RAPD, ISSR, and AFLP in rice. The AFLP assay

discriminated the genotypes effectively with a robust discriminating power (0.99), followed by ISSR (0.76) and RAPD (0.61).

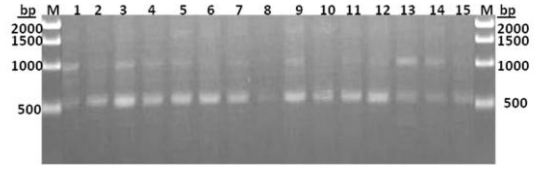

Fig. 1. RAPD profile of different short duration varieties of rice amplified with primer OU 4. M=DNA molecular marker, Lane 1-15: ORT 5, ORT 7, ORT 8, ORT 11, ORT 15, ORT 22, ORT 26, ORT 30, ORT 35, ORT 36, ORT 38, Mandakini, Jogesh, Annapurna, Nilagiri.

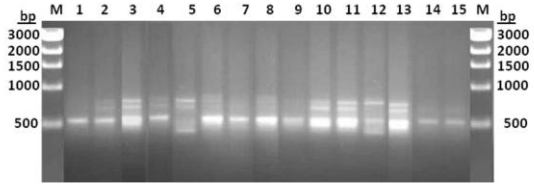

Fig. 2a. ISSR profile of different short duration varieties of rice amplified with primer OUAT 2. M=DNA molecular marker, Lane 1-15: ORT 5, ORT 7, ORT 8, ORT 11, ORT 15, ORT 22, ORT 26, ORT 30, ORT 35, ORT 36, ORT 38, Mandakini, Jogesh, Annapurna, Nilagiri

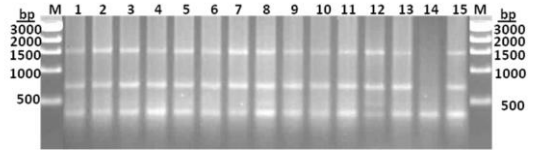

Fig. 2b. ISSR profile of different short duration varieties of rice amplified with primer OUAT 11. M= DNA molecular marker, Lane 1-15: ORT 5, ORT 7, ORT 8, ORT 11, ORT 15, ORT 22, ORT 26, ORT 30, ORT 35, ORT 36, ORT 38, Mandakini, Jogesh, Annapurna, Nilagiri.

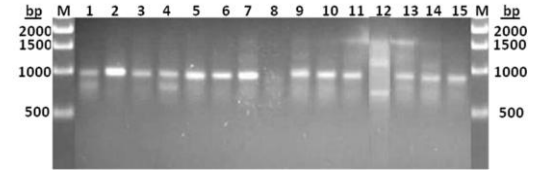

Fig. 2c. ISSR profile of different short duration varieties of rice amplified with primer OUAT 12. M=DNA molecular marker, Lane 1-15: ORT 5, ORT 7, ORT 8, ORT 11, ORT 15, ORT 22, ORT 26, ORT 30, ORT 35, ORT 36, ORT 38, Mandakini, Jogesh, Annapurna, Nilagiri.

The ISSR primer OUAT 12 generated six bands of amplicon size 1650bp, 1300bp, 1200bp, 950bp, 730bp and 700bp(Fig. 2c). Out of these, 700bp band was found to be unique which was specifically amplified only in Mandakini. The ORT series of mutants were developed from Mandakini following recurrent chemical mutagenesis with EMS (ethyl methane sulphonate) and NG (N-nitro-N-nitroso-guanidine). The absence of 700bp band in the mutants as compared to their parent Mandakini, and also the mutant-specific finger printings as mentioned above are indicative of clear genetic variation which might be due to deletion or point mutation by mutagenic action at DNA level in the parent variety Mandakini. Besides, the mutants also exhibited wide variation in agro-economic traits and few of them had also shown genetic improvement in overall productivity as mentioned earlier.

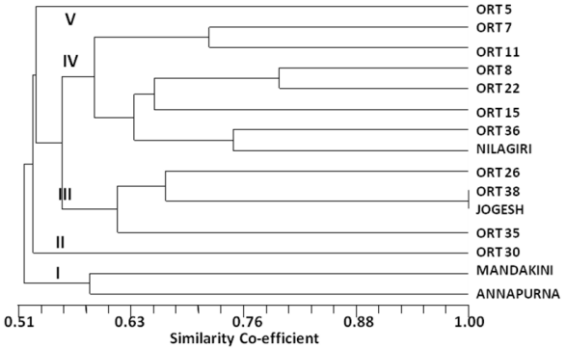

Fig. 3. Dendrogram showing genetic diversity in a set of 15 short duration rice genotypes based on RAPD markers.

Genetic relationship

The binary data scored in terms of presence or absence of band for each of the test genotypes were used to estimate similarity index (data not shown). The overall average similarity values estimated using RAPD, ISSR and pooled binary data score were 0.556, 0.534 and 0.544 respectively. Similarity coefficient value between each pair of genotypes is likely to give a clear picture of the extent of genomic homology in terms of gene content and nucleotide sequence. Similarity index value in each pair of genotypes using RAPD markers ranged from 0.43 to as high as 1.00. Whereas, it varied from 0.20 to 0.90 and 0.37 to 0.96 in case of ISSR and combined analysis respectively. Using RAPD, it was not

possible to find a divergent genotype pair below 0.43 whereas, but ISSR was found to be potent enough to sort out divergent genotype pair with similarity index value as low as 0.20. Superiority of ISSR over RAPD markers has been reported in rice (Qian *et al.,* 2001) and barley (Nagaoka and Ogihara, 1997). Based on ISSR technique, the most divergent genotype pair exhibiting highest genetic distance between them was identified to be Annapurna and Mandakini followed by Annapurna and ORT 30; and Annapurna and ORT 15. The same divergent genotype combinations were also identified in combined analysis but with higher level of similarity index value.

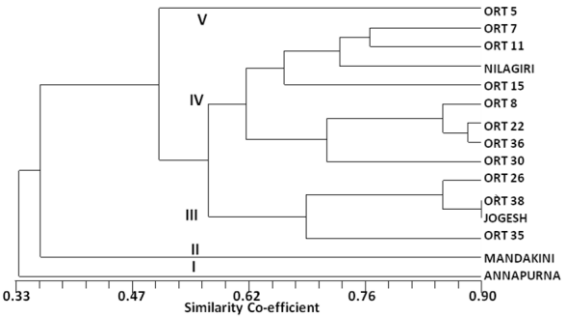

Fig. 4. Dendrogram showing genetic diversity in a set of 15 short duration rice genotypes based on ISSR markers.

Clustering pattern

Genetic diversity can be studied based on morphological, biochemical and molecular level. However, molecular markers have special advantages over others as they show detail genetic differences in a faster way and without any environmental influence (Saker *et al*., 2005; Souza *et al.*, 2008). Therefore, in recent years, considerable emphasis has been placed on the development and use of molecular markers in all major crops.

The whole range of thirteen RAPD primer based DNA profiles comprising 72 distinct scorable bands in 15 test genotypes revealed five genetic clusters at 55% phenon level (Fig. 3). Among the test genotypes, Mandakini and Annapurna were separated from rest of the genotypes and constitute Cluster-I**.** Next onwards two single genotypes e.g. ORT 30 and ORT 5 were distinguished which constituted Cluster-II and cluster V respectively. Cluster III included four genotypes e.g., ORT 35, Jogesh, ORT 38 and ORT 26. Among these, Jogesh and ORT 38 revealed similar RAPD profiles and these could not be discriminated even at 100% phenon level. The Cluster IV is a highly multivariety cluster and it constituted seven genotypes e.g., Nilagiri, ORT 36, ORT 15, ORT 22, ORT 8, ORT 11 and ORT 7. The dendrogram shows that the best standard check variety and the parent variety (Mandakini) of the ORT series mutants have high degree of genomic homology, while most of the ORT mutants maintained high genetic distance from their parent. This signifies mutational change in the resulting mutants compared to the parent used. The mutational changes are hereditary and have direct bearing in some way for phenotypic variation in terms of agro-economic traits studied. Among the ORT mutants ORT 11, ORT 30 and ORT 35 gave significantly higher yield during late Rabi season, 2011-12. ORT -11 has shown high genetic distance from its parent variety Mandakini and the best standard check Annapurna. Exceptionally high yield potential (3430kg/ha) of this mutant is attributed to dwarf plant type (72cm.), high tillering (690/m^2), moderate maturity duration (105days), high fertility percentage (88.18%), moderate number of grains per panicle(92) and medium grain type(100 grain weight 2.34gm)(Table 1).

Based on ISSR markers, the dendrogram revealed five distinct clusters at 55% phenon level (Fig. 4). In contrast to RAPD, initialy the genotype Annapurna and the parent variety were separated out into single variety clusters e.g., Cluster-I and Cluster-II. Similar to RAPD, Cluster III included the same four genotypes i.e., ORT 35, Jogesh, ORT 38 and ORT 26; and also Cluster V contained same genotype ORT 5. It is worth to note that the genotype ORT 30 was initially separated out from rest of the test genotypes in case of RAPD, but clustering pattern based on ISSR markers clubbed it in a large multivariety cluster (Cluster IV). Cluster -IV included eight genotypes e.g., ORT 30, ORT 36, ORT 22, ORT 8, ORT 15, Nilagiri, ORT 11 and ORT 7. Thus, RAPD

and ISSR analysis revealed a clear cut picture that genotypes, e.g., Annapurna, Mandakini, and a mutant genotype ORT 5 are most divergent from rest of the genotypes. Saini *et al.*(2004) and Girma *et al.*(2010) also studied clustering pattern of cultivated and wild rice using different molecular markers.

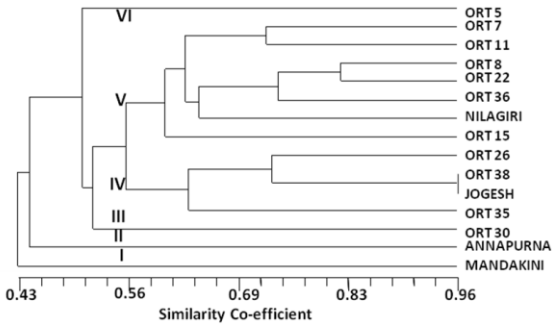

Fig. 4. Dendrogram showing genetic diversity in a set of 15 short duration rice genotypes based on combined RAPD and ISSR markers.

The clustering pattern based on combined data of both DNA markers (RAPD and ISSR) exhibited six clusters (Fig. 5) and separated each of the aforesaid divergent genotypes as well as erstwhile mentioned ORT 30 into single variety clusters e.g., Cluster I, Cluster II, Cluster III and Cluster VI at the same 55% phenon level. Thus, for fine tuning, DNA markers generated from different marker systems may be combined together for conclusive information relating to genetic relationship in a set of test genotypes.

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