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GENETIC DIVERSITY ANALYSIS OF RICE (*Oryza sativa* L) VARIETIES USING ISSR MARKERS

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ABSTRACT
A set of 66 rice varieties were analyzed using 8 ISSR primers to assess the genetic diversity. A total of 70 bands were detected out of which 56 were polymorphic, 14 were monomorphic and shows 78 percent polymorphism. The maximum number of polymorphic bands 10 were obtained in primers 843 and 879. The PIC value ranged between 0.15 (884) to 0.47 (807) with an average of 2.81 per locus. Out of eight ISSR primers unique specific allele was generated for the variety IR 50 with specific molecular weight of 672 bp, which can be used as molecular Id. Genetic similarity among genotypes varied from 0.5 to 1.0 with an average of 0.75. The first three principle components explained more than 77 percent of total genetic variation. This approach demonstrates the effective use and ability of ISSR markers for genetic diversity studies in rice.

KEY WORDS: Genetic diversity, polymorphism, genetic similarity, principle component.

INTRODUCTION

Rice (*Oryza sativa* L.) is a staple food for more than half of the world's population (Nagaraju *et al.,* 2001). It is cultivated in all the continents except Antarctica over an area of more than 150 million ha (Mha), with most rice production taking place in Asia (Jena and Mackill, 2008). For several good reasons, like finding a generic relationship among various varieties, their phylogeny etc., fingerprinting of various organisms including crops is being taken up. Further, a large number of new varieties are being released, but not all of these have been widely accepted, as many of them have been considered to be merely forms of acknowledged varieties. If these varieties are actually genetically distinct, conservation and purity of these varieties is needed for which a reliable and robust fingerprinting is absolutely required (Singh *et al.,* 1999). Conventionally, morphological descriptors are used for establishing the identity of varieties. Though morphological data provide the basis for DUS testing, these have some drawbacks. Morphological expression is influenced by environment leading to errors in scoring. Lack of knowledge on genetic control of phenotypic traits, insufficient variation, and long time required for appearance of the traits at appropriate growth stage are the key limitations that may necessitate the use of more reliable and faster to score marker systems (Bhat, 2001). Molecular markers had proven to be powerful tools in the assessment of genetic variation and in the elucidation of genetic relationships within and among species. ISSRs are non-functional and selectively neutral; they are known to be linked to coding regions, so that ISSRs are likely to mark gene rich regions (Kojima *et al.,* 1998). Several rice varieties have been released by several research institutions in India, to cater the needs of the farmers. The molecular characterization and fingerprinting of these released varieties using ISSR markers will provide sufficient knowledge on diversity among them at molecular level, which will help the breeders to develop strategies for the future, and the variety specific fingerprints will enable to identify and characterize each variety released.

MATERIALS & METHODS

Sixty six most popular rice varieties of different duration groups viz., short, medium and long (Table 1), including seven popular national check varieties were taken in the present investigation. Genetically pure seeds of these varieties were obtained from respective centre, where the varieties were bred is used for molecular characterization.

DNA Extraction

Genomic DNA from each variety was isolated from bulked leaf sample from five plants using CTAB method explained by Dellaporta *et al*. (1983). Bulking of leaf tissue for DNA isolation was carried out to ensure that it would make no difference to use a single individual plant or to pool different plants since the analysis of a single individual plant would representative enough of the entire variety.

ISSR marker analysis

Eight ISSR primers (UBC primers) shown high PIC value in the previous studies of Nagaraju *et al.,* 2002 & Saini *et al.,* 2004, were used for DNA profiling (Table 2). The primer sequences were synthesized by Agile life science technologies India Ltd. Mumbai. PCR reactions were carried out in Thermal cycler (Eppendorf. AG. Germany). The reaction volume of 15 µl is used for ISSR, containing 2 µl of genomic DNA, 1X assay buffer, 200 mM of deoxyribonucleotides, 2 µM of $Mgcl_2$, 0.2 µM of primer, 1 unit of Taq DNA polymerase (Bangalore Genei Pvt. Ltd., Bangalore) and 6.6 µl of sterile water. The PCR profile

adopted was: (i) initial denaturation at 95ºC for 2 minutes, followed by (ii) 34 cycles of denaturation at 94ºC for 45 seconds, annealing at 55ºC and extension at 72ºC for 1 minute and 30 seconds and (iii) final extension at 72ºC for 10 minutes and at 4ºC for cooling. Annealing temperature was standardized for each primer and adopted for all the primers used in the study as identified by their specific Tm requirement. The amplified products were separated in 3 percent metaphor® agarose gel prepared in 1X TBE buffer stained with Ethidium Bromide (0.5µg/ml). The gel was run in 1 X TBE buffer (0.89M Tris borate, 0.02M EDTA, pH 8.0) at constant voltage of 80 V for a period of 2 hours to 2 hour 30minutes. The gel was visualized in UV transilluminator and photographs were taken using gel documentation system. (Model Alpha Imager 1200, Alpha Innotech Corp., USA).

TABLE 1. List of varieties used in the study

S. No	Varieties	Pedigree	Year of release	Duration	Breeding Station
1.	ADT 36	Triveni / IR 20	1981	Short	TRRI, Aduthurai
2.	ADT 37	BG 280-12 / PTB 33	1987	Short	TRRI, Aduthurai
3.	ADT 41	MUTANT FROM Bas 370	1993	Short	TRRI, Aduthurai
4.	ADT 42	AD 9246 / ADT 29	1996	Short	TRRI, Aduthurai
5.	ADT 43	IR 50 / WHITE PONNI	1998	Short	TRRI, Aduthurai
6.	ADT (R) 45	IR 50 / ADT 37	2001	Short	TRRI, Aduthurai
7.	ADT (R) 47	ADT 43 / JEERAGASAMBA	2005	Short	TRRI, Aduthurai
8.	ADT (R) 48	IET 11412 / IR 64	2005	Short	TRRI, Aduthurai
9.	ASD 16	ADT 31 / CO 39	1986	Short	RRS, Ambasamudram
10.	ASD 17	ADT 31 / RATNA / ASD 8 / IR 8	1988	Short	RRS, Ambasamudram
11.	ASD 18	ADT 31 / IR 30	1991	Short	RRS, Ambasamudram
12.	ASD 20	IR 18348 / IR 25863 / IR 53	1996	Short	RRS, Ambasamudram
13.	CO (R) 47	IR 50 / CO 43	2000	Short	PBS, Coimbatore
14.	PY 2	KANNAGI / CULTURE 2032	1980	Short	PKKVK, Puducherry
15.	PY 3	IR 3403-267 / PTB 33 / IR 36	1984	Short	PKKVK, Puducherry
16.	PY 5	SWARNADHAN / NLR 9674	1994	Short	PKKVK, Puducherry
17.	PY 7	IR 50 / CO 43	2007	Short	PKKVK, Puducherry
18.	IR 36	IR 1561-2281 / IR 244 / *O.nivara* / CR 94-13	1981	Short	IRRI, Philippines
19.	IR 50	IR 2153-14 / IR 28 x IR 36	1982	Short	IRRI, Philippines
20.	IR 64	IR 5657-33-2-1 / IR 2061-465-1-5-3.	1991	Short	IRRI, Philippines
21.	IR 72	TN 1 / Chianung 242	1989	Short	IRRI, Philippines
22.	IR 74	IR 19661 / IR 15795	1991	Short	IRRI, Philippines
23.	PMK 2	IR 13564-149-3 / ASD 4	1996	Short	ARS, Parmakudi
24.	PMK (R) 3	UPLRi 7 / CO 43	2003	Short	ARS, Parmakudi
25.	TKM 9	TKM 7 / IR 8	1978	Short	RRS, Tirur, Tamil Nadu
26.	TKM 11	C 22 / BJ 1	1998	Short	RRS, Tirur, Tamil Nadu
27.	TKM (R) 12	TKM 9 / TKM 11	2001	Short	RRS, Tirur, Tamil Nadu
28.	TRY (R) 2	IET 6238 / IR 36	2001	Short	ADAC and RI, Trichy
29.	TPS 1	IR 8 / KATTISAMBA	1983	Short	ARS, Thirupathisaram
30.	MTU 1010	KRISHNAVENI / IR 64	2000	Short	RRS, MARTERU
31.	JAYA	T(N) 1 / T 141	1968	Short	CVRC
32.	MDU 5	*O.glaberrima* / POKKALI	1996	Short	AC and RI, Madurai
33.	ADT 38	IR 1529-680-3-2 / IR 4432-52-64 / IR 7963-30-2.	1987	Medium	TRRI, Aduthurai
34.	ADT 39	IR 8 / IR 20	1988	Medium	TRRI, Aduthurai
35.	ADT (R) 46	ADT 38 / CO 45	2002	Medium	TRRI, Aduthurai
36.	ADT (R) 49	CR 1009 / JEERAGASAMBA	2011	Medium	TRRI, Aduthurai
37.	CO 42	RP 31-49-2 / LEB MUEY NAHNG		Medium	PBS, Coimbatore
38.	CO 43	DASAL / IR 20	1982	Medium	PBS, Coimbatore
39.	CO 45	R.HEENATI / IR 3403-267-1	1991	Medium	PBS, Coimbatore
40.	CO (R) 46	T7 / IR 20	1996	Medium	PBS, Coimbatore
41.	CO (R) 48	CO 43 / ASD 19	2007	Medium	PBS, Coimbatore
42.	CO (R) 49	C 20 / RNR 52147	2008	Medium	PBS, Coimbatore
43.	CO (R) 50	CO 43 / ADT 38	2009	Medium	PBS, Coimbatore
44.	ASD 19	LALNAKANDA / IR30	1996	Medium	RRS, Ambasamudram
45.	PY 1	PONNI / IR 8	1980	Medium	PKKVK, Puducherry
46.	PY 6	IR 19661 / CR 1009	2000	Medium	PKKVK, Puducherry
47.	P 2662	ADT 39 / ADT 43		Medium	PKKVK, Puducherry
48.	IR 20	IR 262 / TKM 6	1970	Medium	IRRI, Philippines
49.	TKM 10	CO 31 / C 22	1993	Medium	RRS, Tirur, Tamil Nadu
50.	TRY 1	IR 578-172-2-2 / BR-1-2-B-1	2000	Medium	ADAC and RI, Trichy
51.	TRY (R) 3	ADT 43 / JEERAGA SAMBA		Medium	ADAC and RI, Trichy
52.	TPS 3	RP 31-492 / LMN	1993	Medium	ARS, Thirupathisaram
53.	TPS (R) 4	TS 29 / ASD 16	2006	Medium	ARS, Thirupathisaram
54.	I.WHITE PONNI	TAICHUNG65 / 2 MAYANG EBOS-80		Medium	TRRI, Aduthurai
55.	PAIYUR 1	IR 1721-14 / IR 1330-3-3-2	1981	Medium	RRS, Paiyur
56.	BHAVANI	PETA / BPI 76	1976	Medium	PBS, Coimbatore

57.	JALDHIDHAN 6	-	-	Medium	-
58.	SWARNA	VASISTHA / MAHSURI	1979	Medium	RRS, MARTERU
59.	AKSHAYADHAN	BR 827-35 / SC 109-2-2	2008	Medium	CVRC
60.	NDR 359	BG 90-2-4 / OB 677	1993	Medium	CVRC
61.	SAMBA MASURI	GEB 24 / TN1 / MAHSURI	1986	Medium	ANGRAU, BAPATLA
62.	ADT 40	RPW 6-3 / SONA		Long	TRRI, Aduthurai
63.	ADT (R) 44	IET 14099-IR 56 / OR142-99	2000	Long	TRRI, Aduthurai
64.	PONMANI	PANKAJ / JAGANNATH	1983	Long	TRRI, Aduthurai
65.	PY 4	IR 8 x H 4	1988	Long	PKKVK, Puducherry
66.	KKL(R) 1	CR 1009 / ADT 39	2007	Long	PAJANCOA & RI, Karaikal

TABLE 2. Details of ISSR primers used for PCR amplification

Sl. No.	ISSR-UBC Primers	Sequence (5'-3')
1	807	AGA GAG AGA GAG AGA GT
2	808	AGA GAG AGA GAG AGA GC
3	810	GAG AGA GAG AGA GAG AT
4	842	GAG AGA GAG AGA GAG AYG
5	834	AGA GAG AGA GAG AGA GYC
6	879	CTT CAC TTC ACT TCA
7	884	HBH AGA GAG AGA GAG AG
8	885	BHB GAG AGA GAG AGA AYG

Scoring and Data analysis

Statistical analysis for ISSR marker data was done using NTSYS-pc (version 2.02). Amplified bands/ alleles were scored as present (1) or absent (0) for each genotype and primer combination. The data were entered into a binary matrix and subsequently analyzed using the computer package, NTSYS-pc (version 2.02) (Rohlf, 2001). PIC value was calculated for each of the ISSR loci using the formula developed by Rolden-Ruiz *et al.* (2000)

$PIC = 2f_i(1- f_i)$

Where, f_i is frequency of marker bands which were present and $1-f_i$ is frequency of marker bands which were absent.

Dice genetic similarity coefficients for ISSR were calculated and used to assess the genetic relationship among 66 rice varieties and then were used to construct dendrogram using unweighted pair group method using arithmetic averages (UPGMA) and sequential agglomerative hierarchial nested (SHAN) cluster analysis. Principle component analysis was performed to high light the resolving power of the ordination.

Analysis of DNA fingerprint patterns

A similarity index D expressing the probability that a fragment in one variety is also found in another for all pairwise comparisons was calculated (X_D represents the average similarity index for all pairwise comparisons). The probability that the DNA fingerprints of two varieties of rice will be identical by chance was then estimated as $(X_D)^n$ (Wetton *et al.*,1987).

RESULTS & DISCUSSION

ISSR analysis

A total of 70 bands were detected using 8 ISSR primers, out of which 56 were polymorphic and 14 were monomorphic and shows 78 per cent polymorphism (Table 3). The maximum number of polymorphic bands (11 bands) was obtained using primer 843, 879. The average of polymorphic bands was 7.0 per primer, percentage polymorphism ranged from 50% to as high as 100% with an average of 78% across all the varieties. The primers 808, 884 amplified less number of polymorphic bands (5 and 4) out of total bands amplified (10 and 7). The PIC value ranged between 0.15 (884) and 0.47 (807) with an average of 2.81. Figure 1 shows the amplification of multiple alleles in all the genotypes by 843 locus.

Genetic diversity analysis

UPGMA cluster analysis was performed using Dice's similarity co-efficient matrices calculated from ISSR markers to generate a dendrogram of 66 rice varieties investigated (Figure 2). The similarity coefficient ranged from 0.50 to 1.00 with an average of 0.75. The 66 varieties were clustered into two major clusters at 66 per cent similarity. The first major cluster IS I consist of seven sub clusters at a level of 75 per cent similarity, the first sub cluster IS I A consisting of 45 varieties which covers major part of total 66 rice varieties, in which IR 64 and IR 72, ASD 20 and PY 2 showed 93 per cent similarity among each other. The second sub-cluster IS I B consisting of five rice varieties (PY 5, CO 48, SAMBAMASURI, P 2662 and IR 20), third sub-cluster IS I C consisting of four varieties (ADT 41, ADT 43, ADT 45 and PMK 3) of which the variety PMK 3 constituted one separate cluster branched from third sub-cluster. TPS 1 separately constituted one separate cluster branched from the first major sub-cluster and forms fourth sub-cluster IS I D, a set of four varieties (ADT 47, PY 3, ADT 48 and PMK 2) were grouped in cluster five IS I E, ASD 19 and IR 36, CO 42 and CO 49 were grouped separately under cluster six IS I F and seven IS I G. The second major cluster IS II consisting of two sub-clusters at 75 per cent similarity, the first sub-cluster IS II A consisting of ADT 49 and PY 6, the second sub-cluster IS II B was formed by PY 1, separately under second major cluster. The distribution of genotypes under different clusters was given in table 4. The PCA is one of the multivariate

approaches of grouping based on similarity coefficients or variance-covariance values. It is expected to be more informative about differentiation of major groups.

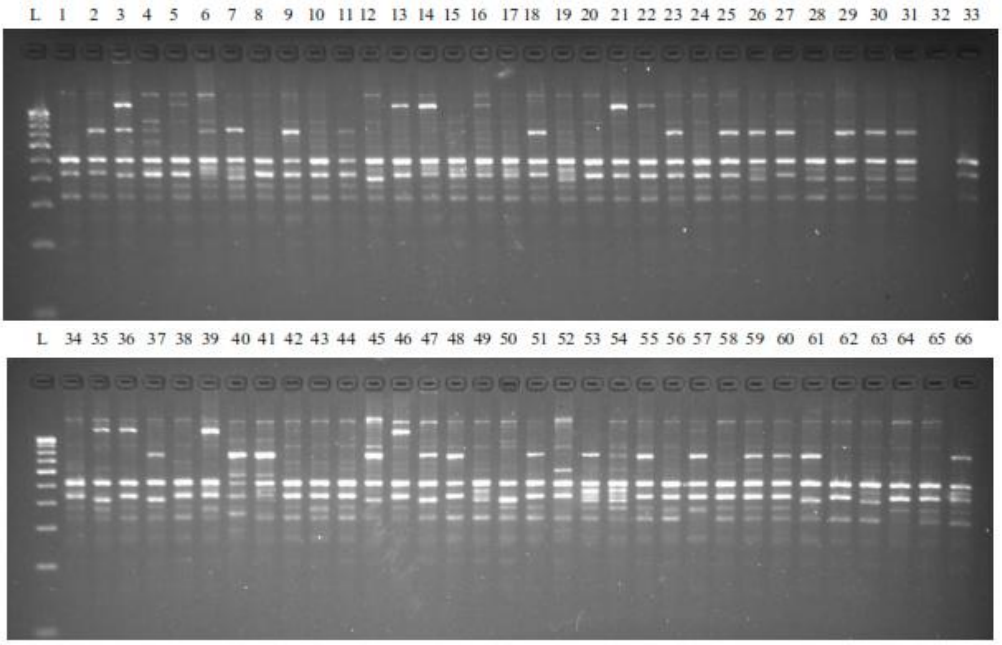

FIGURE 1. Gel profile showing the amplification of ISSR primer 843 in 66 rice varieties. The lane number corresponds to a rice genotype as given in the table 1, 1 kb DNA ladder.

TABLE 3. Band variation and PIC values for ISSR markers

S.No	Primers	Total number of bands	Number of monomorphic bands	Number of polymorphic bands	PIC value	Polymorphism percentage	Product size (bp)	Unique allele (bp)
1.	807	8	0	8	0.47	100	264-1002	-
2.	808	10	5	5	0.38	50.0	258-1123	-
3.	810	10	1	9	0.33	90.0	206-1191	-
4.	842	5	2	3	0.32	60.0	644-1006	-
5.	843	11	1	10	0.48	90.0	376-1145	672 (IR 50)
6.	879	11	1	10	0.43	90.0	164-990	-
7.	884	7	3	4	0.15	57.1	444-1039	-
8.	885	8	1	7	0.25	87.5	648-1118	-
Total		70	14	56	Mean: 2.81	Mean: 78.07 %		

() – values Figures in parenthesis are variety for unique allele

TABLE 4. Distributions of varieties to different clusters based on UPGMA method in ISSR dendrogram

Cluster no.	Total no. of varieties	varieties
IS I A	45	ADT 36, ADT 37, ADT 42, PY 7, KKLR 1, MDU 5, CO 47, JAYA ASD 20, PY 2, IR 64, IR 72, IR 50, ASD 18, ASD16, ADT 38, CO 45, ADT 46, TRY 3, JD 6, CO 50, IR 74, TRY 2, ADT 44, CR 1009, PY 4, CO 46, ASD 17, TPS 3, TKM 9, TRY 1, MTU 1010, ADT 40, TKM 11, TKM 12, BHAVANI, NDR 359, SWARNA, AKSHAYADHAN, ADT 39, CO 43, TKM 10, PAIYUR 1, TPS 4, IWP
IS I B	5	PY 5, CO 48, SAMBAMASURI, P 2662, IR 20
IS I C	4	ADT 41, ADT 43, ADT 45, PMK 3
IS I D	1	TPS 1
IS I E	4	ADT 47, PY 3, ADT 48, PMK 2
IS I F	2	ASD 19, IR 36
IS I G	2	CO 49, CO 49
IS II A	2	ADT 49, PY 6
IS II B	1	PY 1

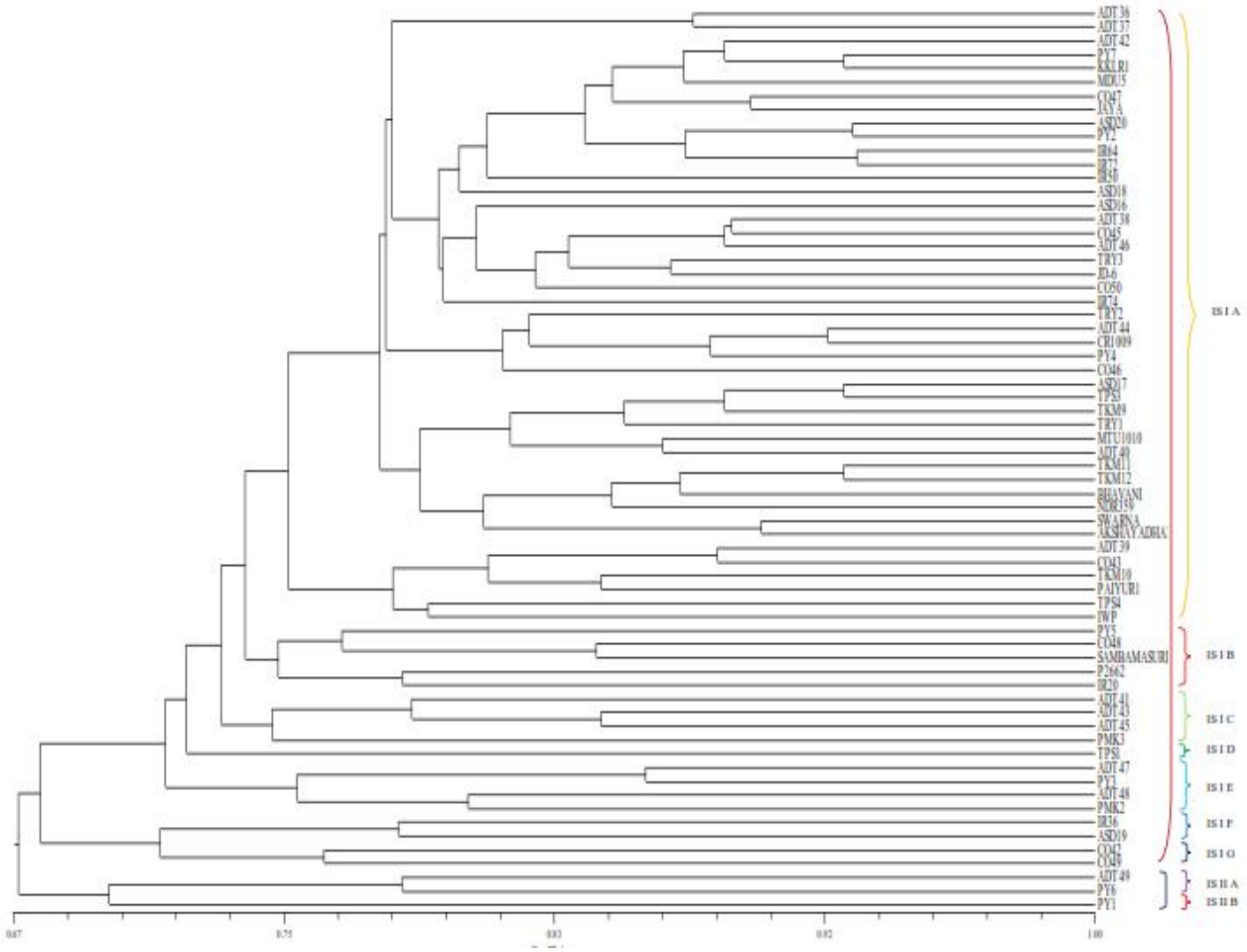

FIGURE 2. Dendrogram showing genetic relationship using ISSR markers

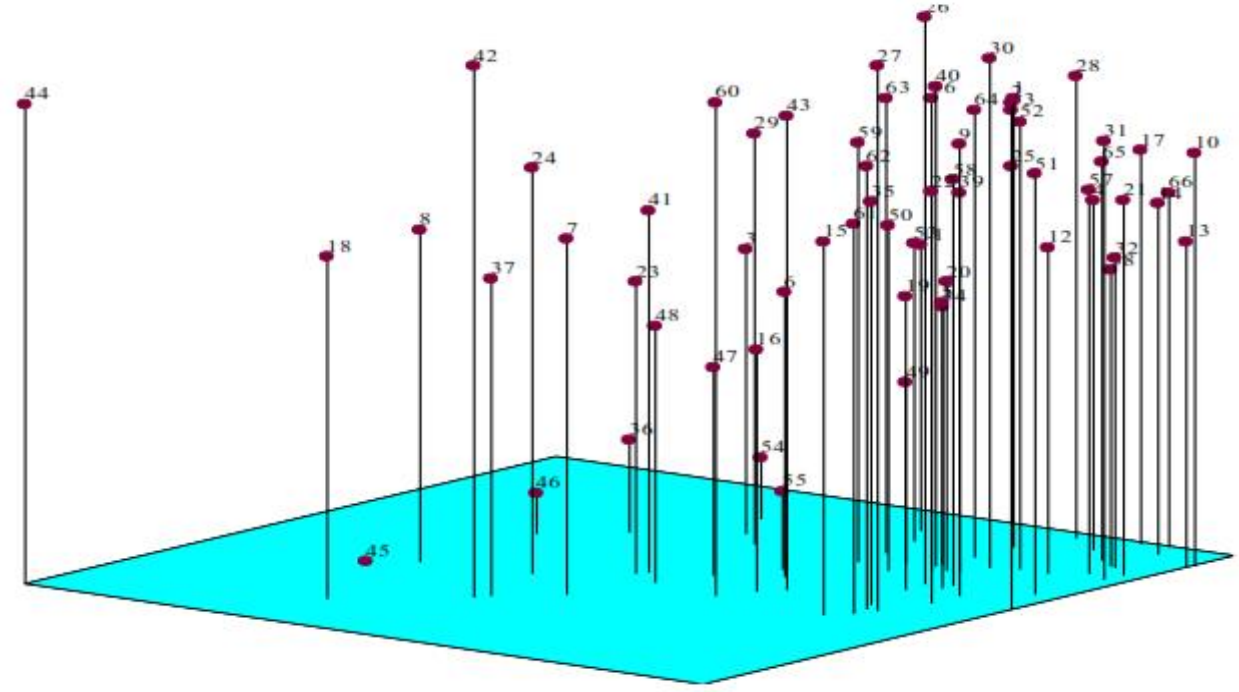

FIGURE 3. Three dimensional plot of principal component analysis using ISSR markers – Biplot. 1 – 66 varieties listed as per Table 1

The groupings identified by PCA were comparable to those identified by UPGMA cluster analysis. More than 77 per cent of the variation in the estimates of genetic similarity was explained by the first three components (Figure 3). The first principal component explained significant variation (approx. 75 per cent) and clearly separated all the rice varieties into nine relative distinct clusters, further these clusters were divided into 15 relative distinct clusters by second and third principal components that explained approximately four per cent variation of the data. PY 1, TPS 4, PAIYUR 1, CO 49, IR 36, and ASD 19, all these rice varieties were formed as separate clusters individually.

Varietal identification using ISSR markers

In the present study, amplification reliable DNA fingerprinting has been identified in one rice variety using ISSR primers. Specific marker alleles has been identified as molecular identities for the variety, based on the banding pattern generated by eight ISSR primers with molecular weight ranging from 164 bp (UBC-879) to 1191 bp (UBC-810) (Table 3). Out of eight ISSR primers unique specific allele was generated for the variety IR 50 with specific molecular weight of 672 bp, which can be used as molecular Id. The low probability of obtaining identical match by chance estimated for the ISSR markers (2.16×10^{-9}) revealed their high discrimination power. In the ISSR-PCR analysis, the eight primers amplified a total of 70 bands (loci) out of which 56 were polymorphic and shows 78 per cent polymorphism which was in contrast to the findings of Joshi *et al.* (2000) and Singh *et al.* (1999). The primers 807, 843 and 879 showed maximum polymorphic information content (PIC) of 0.47, 0.48 and 0.43 respectively. The primers 808, 884 and 885 amplified less number of polymorphic bands (5, 4 and 7) out of total bands amplified (10, 7 and 8). This may be because of the similar parentage of many of the cultivars used in the present study. In summary the ISSR markers provide a powerful tool for the generation of potential fingerprinting diagnostic markers for different rice varieties. The polymorphic ISSR markers also provide better resolution with respect to relatedness among rice cultivars.

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