Application of RAPD, isozyme and protein markers for characterization of rice (*Oryza sativa* L.) varieties

Anuradha Bhhartiya*, Kamal K. Pandae*, Harpal S. Mewari* and Pankaj Sah**
* Department of Biotechnology, Government Post Graduate College, Haldwani, Nainital, Uttarakhand State, (India).
** Department of Applied Sciences, Higher College of Technology, Muscat, (Sultanate of Oman)
anuradhagbp@gmail.com , kamalpandey@yahoo.com , pankaj@hct.edu.om

Abstract: Morphological characters are usually used to identify the crop varieties because they can easily be observed at phenotypic level and provide the unique identification of crop varieties (Smith and Smith, 1992). However, these traits are under the control of many genes and show instability due to interaction with environments, which restrict the reliable identification (Patterson and Weather up, 1984). On the other hand increased the number of genetically released varieties through the efforts of plant breeders have created phenotypic uniformity which has further imposed restrictions on utilizing the morphological traits as markers especially for crops where genetic base is narrow. Thus reliable identification of genotypes is difficult to achieve on this basis. The advancement is genetics, biochemistry and molecular biology have made available new tools based on protein and DNA complements of individuals and are common in use (Smith and Smith, 1992). Molecular characterization was undertaken for nineteen rice varieties using twelve RAPD markers. four isozyme markers and protein marker.A total of amplicons were obtained with the average of 5.6 bands per primer. Of these, 48 were found to polymorphic and level of polymorphism was 70.58%. The number of bands generated was more primer dependent than the genotype dependent and ranged from 1 to 11. The percentage of polymorph bands ranged from 50% (EO 1598, EO 1594 to 100% (EO 1600, EO 1596). Primer EO1593, EO1600. EO1602 generated primer specific bands with Pant Majhera Dhan 7, UPRI 17B, Govind and Pant Dhan 6, respectively. The dendrogram revealed Pant Majhera Dhan 7 to highly diverse from rest of the eighteen varieties. Jaccard's pair wise similarity coefficient values 19 genotypes were calculated and the range of genetic similarity was found to 0.65 to 0.92 with average of 0.75± 0.05. A dendrogram was generated by UPGMA cluster analysis based on Jaccard's similarity coefficients, which showed poor grouping of genotypes due to genetic similarity among them. [Nature and Science. 2009;7(5):55-63]. (ISSN: 1545-0740).

Keywords: RAPD, Protein Markers, Molecular Taxonomy.

INTRODUCTION:

Rice (*Oryza sativa L.*) is world's most widely cultivated food crop. In India it is grown on 44.6 m ha area with the production of 93 million tones of milled rice. Globally India ranks first in area under rice cultivation (44.6 mha) and second in production (93.9 mt) after China and contributes to 23.5% of world rice production. Rice plays a vital role in the national food security, as it constitutes staple food for two-third of the population supplying about 33% of food calories.

In India, it is more than foodstuff; it's an entire culture and is a basis of both biological and cultural diversity**.** The quality preferences of rice consumers have resulted in a wide diversity of varieties specific to different localities. Although the exact diversity cannot be gauged, it is estimated to be around 140,000 different genotypes. India alone has 86, 330 accessions, of which 42,004 are in the national gene bank.

About 760 high yielding varieties have been released in India for various ecosystems. Thus varietal identification has attained a critical importance in India in view of increasing multiplicity of varieties and imminent implementation of Plant Variety Protection and Farmer's Rights Act, 2001. A breeder's variety must fulfill the criteria of Distinctness, Uniformity, and Stability (DUS) to be given protection under this Act. It is thus imperative to characterize all popular varieties and to prepare and maintain their comprehensive database.

However, to protect the rights of breeders/breeding institutions against misappropriations and plagiarization, it is desired that they are properly characterized on the basis of morphological and molecular characteristics. Gel electrophoresis of protein, RAPD markers and isozymes are a powerful tool to distinguish genotypes of plant species. Proteins being the last gene products reflect the genomic composition of varieties and hence are good candidates for varietal distinctness. These can provide useful supplementary information, which combined with morphological descriptors provide identification keys.

The present study was conducted to understand the pattern of genetic variability in nineteen rice varieties based on RAPD markers. For RAPD analysis, genomic DNA was isolated from fresh leaves (Murray *et al.,* 1980). One gm of fresh leaves of each of the nineteen varieties were crushed with a pre-chilled pestle and mortar to a fine powder for DNA extraction buffer (5 M NaCl, 0.5 M EDTA, pH=8, 1 M Tris base,PH=8, 10% CTAB). The homogenate was centrifuged to remove cell debris. The supernatant was treated with RNAse and DNA was precipitated with chilled ethanol. The quantity and quality was assayed by running DNA on a 0.8% agarose gel alongside a known quantity of lambda uncut DNA. Amplification reaction was carried out in 25µl reaction volume containing 75 ng of template DNA, 100ng/ µl of primer; 200 µM of dNTPs, 6U/ µl Taq (Bangalore Genei Pvt Ltd, Bangalore, India and 10X Taq buffer with $MgCl_2$). Twelve decamer primers were used for RAPD amplifications with minor modification. Amplification cycle consisted of denaturation for 1 min at 94^0C, 2 min of annealing at 36^0C followed by a 2 min at 72^0C, and primer elongation for 1 min at 72^0C. The PCR products were separated/resolved by electrophoresis on 1.5 % agarose gel from Genei, 1 X TBE buffer and ethidium bromide stained gel was photographed with a digital gel documentation system. Reproducibility of RAPD assay was tested by performing duplicate reaction at different times using identical genotypes and primer combinations under strict control of experimental condition and only the reproducible bands were scored. The RAPD bands were scored as present (1) or absent (0) for each genotype primer combination for all the nineteen rice genotypes, considering each amplified band as a unique locus. Band sharing data were used to calculate genetic similarities based Jaccard's coefficient (Jaccard, 1908) and UPGMA (Unweighted pair group method using arithmetic averages). Aloagrithim was employed to determine the genetic relationship of nineteen varieties (Sneath, 1973). All the analysis was performed using NTSYS-pc 2.02 software (Rholf, 2002).

For isozyme study, 7 days old etiolated seedlings were ground with 50mM tris Hcl buffer (PH=7.6) containing2mercaptoethanol and EDTA in ratio 1:2 (w/v). For extraction of Peroxidase, the buffer without 2 mercaptoethanol was used. Homogenate centrifuged at 15,000 x g and supernatants obtained were used for studying isozyme pattern respectively.

Isozymes were separated on 7% polyacrylamide gel using an anionic system [davis1964] and stained as described by Vallejos (10) for esterase[EST], peroxidases[POX], malate dehydrogenases[MDH] and alcohol dehydrogenases[ADH]. The bands were scored for construction of dendrogram using Jaccard's index. Data were entered as presence/ absence of bands ignoring the intensity for isozyme analysis.

Observations and Results:

Table A. The number of RAPD loci detected by using 12 RAPD primers on agarose gel (1.5%)

Code No.	Primer	Sequence	Total number of RAPD loci	Polymorphic loci	
				Number	%
1	EO1591	5'CACAGGCGG3'	10	8	80
2	EO1592	5'GTGTGCCCCA3'	7	5	71.4
3	EO1593	5'GGGAATTCGG3'	7	5	71.4
4	EO1594	5'CTGACCAGCC3'	4	2	50
5	EO1595	5'GGAAGTCGCC3'	7	5	71.4
6	EO1596	5'GGGAGACATC3'	4	4	100
7	EO1597	5'ACCGCGAAGG3'	1	0	0
8	EO1598	5'CCCGCCGTTG3'	6	3	50
9	EO1599	5'CCGCCCAAAC3'	3	0	0
10	EO1600	5'GTGCAACGTG3'	8	8	100
11	EO1601	5'GTTTCGCTCC3'	5	3	60
12	EO1602	5'TGCTGCAGGT3'	6	5	83.3

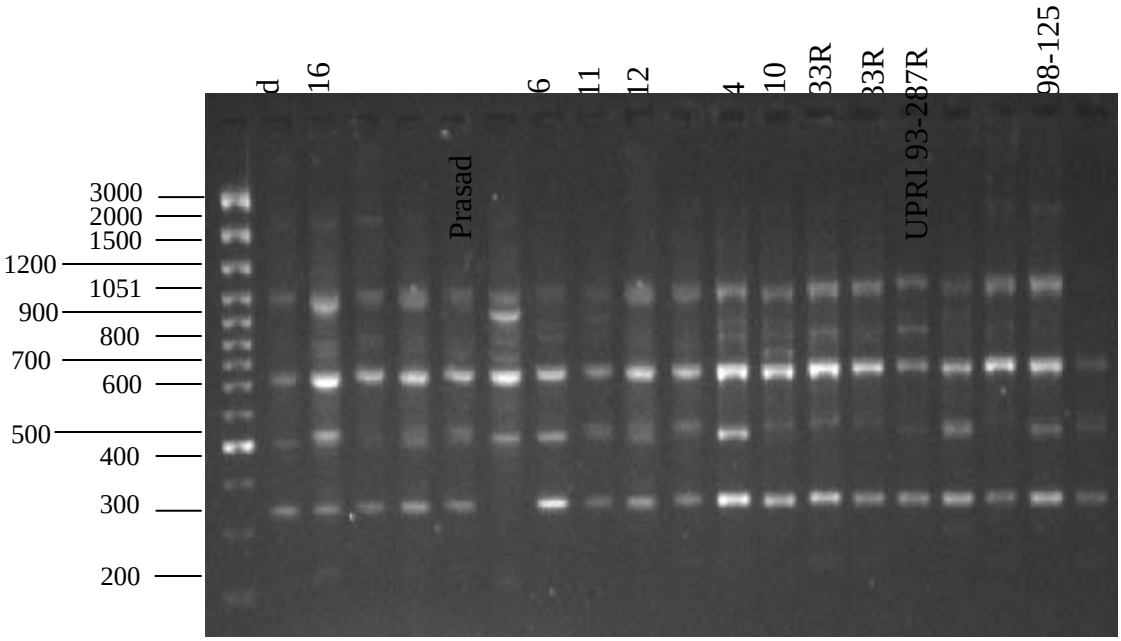

Plate.1. Banding profile of amplified DNA sequences from a RAPD reaction using Primer EO1591

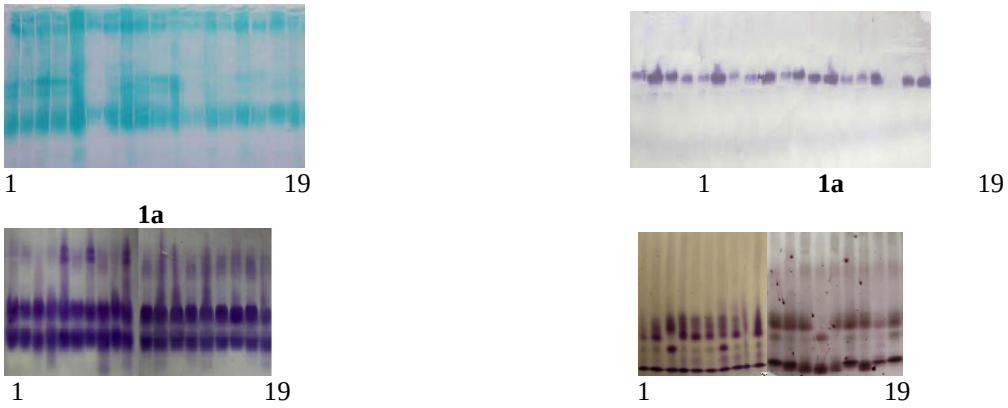

Plate.2. Isozyme pattern of seven days old seedlings of the 19 rice varieties.
1a. POX(Peroxidase),1b. ADH(alcohol dehydrogenase), 1c. MDH(Malate dehydrogenase), 1d.EST(Esterase)

Varieties: *Govind,. Manhar, Prasad, Pant Dhan* 4,*Pant Dhan* 6,*Pant Majhera Dhan* 7,*Pant Dhan* 10,*Pant Dhan* 11,*Pant Dhan* 12,*Pant Dhan* 16,*Pant Sankar Dhan* 1,*Pant Sankar Dhan* 3,*Saryu* 52,NDR 359,UPRI 92-133R,UPRI 95-17B,UPRI 93-287R,UPRI 99-1,UPR 2870-98-125

S.No.	Variety	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1.	Govind	1.00																		
2.	Pant Dhan 16	0.80	1.00																	
3.	Pant Sankar Dhan 1	0.84	0.77	1.00																
4.	Pant Sankar Dhan 3	0.75	0.81	0.82	1.00															
5.	Prasad	0.7	0.73	0.77	0.74	1.00														
6.	Pant Majhera Dhan 7	0.71	0.68	0.72	0.69	0.68	1.00													
7.	Pant Dhan 6	0.76	0.87	0.77	0.81	0.76	0.77	1.00												
8.	Pant Dhan 11	0.71	0.74	0.69	0.75	0.67	0.75	0.77	1.00											
9.	Pant Dhan 12	0.73	0.66	0.74	0.74	0.66	0.70	0.76	0.77	1.00										
10.	Maxhar	0.79	0.75	0.83	0.80	0.75	0.80	0.86	0.83	0.82	1.00									
11.	Pant Dhan 4	0.80	0.80	0.75	0.78	0.76	0.75	0.84	0.74	0.73	0.82	1.00								
12.	Pant Dhan 10	0.80	0.79	0.87	0.84	0.75	0.74	0.86	0.76	0.78	0.92	0.86	1.00							
13.	UPRI 92-133R	0.69	0.65	0.76	0.73	0.78	0.70	0.75	0.79	0.78	0.84	0.72	0.84	1.00						
14.	UPRI 95-17B	0.67	0.67	0.69	0.72	0.67	0.72	0.77	0.75	0.70	0.80	0.74	0.80	0.79	1.00					
15.	UPRI 93 287R	0.65	0.74	0.72	0.79	0.74	0.69	0.78	0.75	0.74	0.76	0.75	0.77	0.76	0.75	1.00				
16.	NDR 359	0.74	0.76	0.71	0.78	0.70	0.78	0.80	0.81	0.80	0.82	0.77	0.76	0.72	0.74	0.85	1.00			
17.	UPRI 99-1	0.75	0.75	0.76	0.76	0.82	0.80	0.82	0.76	0.71	0.81	0.75	0.78	0.80	0.76	0.76	0.82	1.00		
18.	UPR 2870-98-125	0.67	074	0.66	072	0.67	0.69	077	0.78	0.66	0.76	0.74	0.70	0.69	0.68	0.79	0.78	0.76	1.00	
19.	Saryu 52	0.69	0.68	0.70	0.73	0.65	0.67	0.72	0.76	0.71	0.81	0.69	0.75	0.70	0.69	0.77	0.76	0.71	0.80	1.00

Table 1. Pair wise similarity matrix based on Jaccard's coefficient or rice varieties by RAPD analysis

S.No.	Variety	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1.	Govind	1.00																		
2.	Pant Dhan 16	0.83	1.00																	
3.	PSD 1	0.83	0.83	1.00																
4.	PSD 3	0.84	0.84	0.84	1.00															
5.	Prasad	0.83	0.83	0.83	0.84	1.00														
6.	PMD 7	0.81	0.66	0.66	0.69	0.66	1.00													
7.	Pant Dhan 6	0.81	0.66	0.66	0.69	0.66	1.00	1.00												
8.	Pant Dhan 11	0.66	0.66	0.53	0.69	0.53	0.80	0.80	1.00											
9.	Pant Dhan 12	0.81	0.66	0.66	0.69	0.66	1.00	1.00	0.80	1.00										
10.	Manhar	0.84	0.86	0.84	1.00	0.84	0.69	0.69	0.69	0.69	1.00									
11.	Pant Dhan 4	0.61	0.61	0.75	0.76	0.61	0.72	0.72	0.72	0.72	0.76	1.00								
12.	Pant Dhan 10	0.61	0.61	0.75	0.64	0.61	0.72	0.72	0.58	0.72	0.64	0.81	1.00							
13.	UPRI 92-133R	0.78	0.78	0.78	0.92	0.78	0.64	0.64	0.64	0.64	0.92	0.71	0.71	1.00						
14.	UPRI 95-17B	0.61	0.61	0.50	0.64	0.50	0.72	0.72	0.90	0.72	0.64	0.66	0.66	0.71	1.00					
15.	UPR 93- 287R	0.84	0.84	0.84	0.85	0.84	0.69	0.69	0.57	0.69	0.85	0.64	0.76	0.92	0.64	1.00				
16.	NDR 359	0.91	0.91	0.91	0.92	0.91	0.75	0.75	0.61	0.75	0.92	0.69	0.69	0.85	0.57	0.92	1.00			
17.	UPRI 99-1	0.76	0.76	0.76	0.78	0.76	0.61	0.61	0.50	0.61	0.78	0.57	0.69	0.85	0.57	0.92	0.84	1.00		
18.	UPR 2870-98-125	0.91	0.91	0.91	0.92	0.91	0.75	0.75	0.61	0.75	0.92	0.69	0.69	0.85	0.57	0.92	1.00	0.84	1.00	
19.	Saryu 52	0.69	0.69	0.69	0.84	0.69	0.81	0.81	0.81	0.81	0.84	0.90	0.75	0.78	0.75	0.71	0.76	0.64	0.76	1.00

Table 2: Pair wise similarity matrix based on Jaccard's coefficient of rice varieties by isozyme analysis

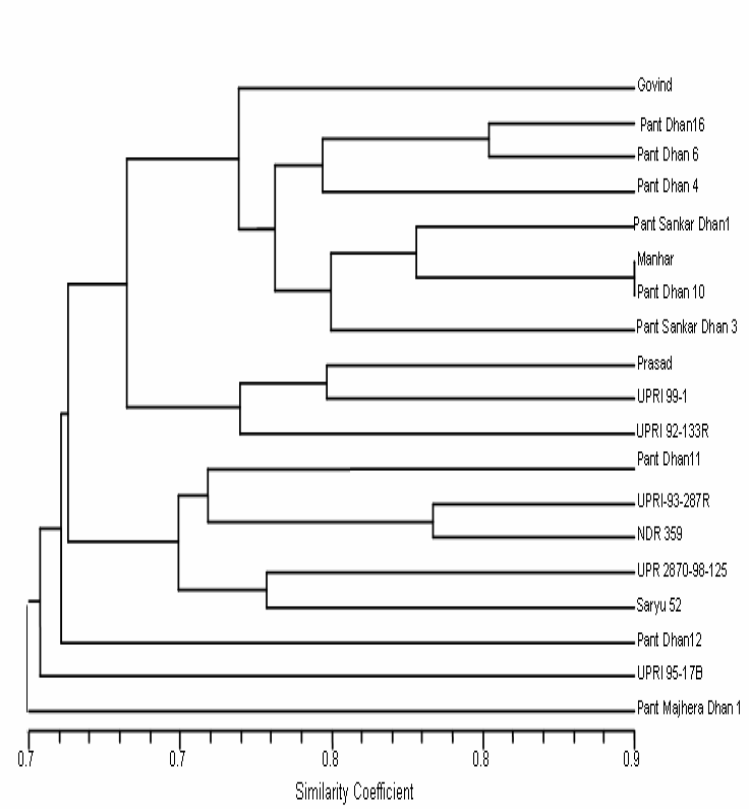

Fig. 1. Dendrogram of rice varieties based on RAPD analysis.

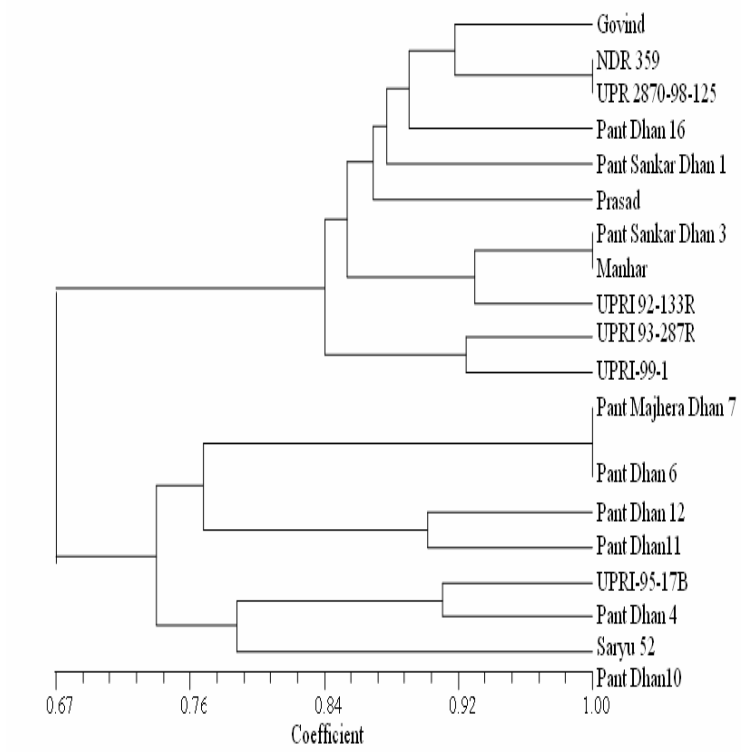

Fig. 2: Dendrogram of rice varieties based on isozymes (peroxidase, esterase, malate dehydrogenase and alcohol dehydrogenase)

Discussion:

PCR amplification of DNA extracted from nineteen varieties was done following the same protocol for all 12 RAPD primers. Before amplification with primers, optimization was carried out and it was observed that 75ng/µL DNA sample and 100 ng/µL concentration of primer was best. A typical example with primer EO1591 is shown in **Plate1**. A total of 68 amplicons were obtained with this primer with an average of 5.6 bands per primer. The number of bands generated was more primer dependent than genotype and ranged from 1 (EO1597) to 10 (EO1591). Out of these 68 bands, 48 bands were polymorphic and the level of polymorphism was 70.59%. **(TableA).** Two primers (EO1596, EO1600) out of twelve showed 100% polymorphism, while two primers namely EO1597, EO1599 did not show any polymorphism. Primer EO 1597 was found to be monomorphic as it gives single band. Various authors have also reported the variation in total bands produced and level of polymorphism generated**.** All the 19 genotypes have been discriminated in the present RAPD analysis involving twelve decamer primers. Though it is difficult to prepare a key for identification of all the varieties on the basis of polymorphism involving a small set of primers, a few primers have generated a very specific bands for only a limited number of genotypes. Primer EO1591, EO1593, EO1600, EO1602 generated primer specific bands with *Pant Majhera Dhan 7*, UPRI-95-17B, *Govind* and *Pant Dhan 6* respectively. Jaccard's pair wise similarity coefficient values for nineteen varieties were also calculated. The range of genetic similarity was found to be 0.65 to 0.92 **(Table1)**. The average similarity index based on Jaccard's coefficient was 0.75 $\pm$ 0.05. Overall view suggested that the grouping of the varieties is very poor as the similarity among the varieties. The dendrogram **(Fig.1)** revealed *Pant Majhera Dhan* 7 to be highly diverse from rest of the nineteen varieties since it showed only 73% similarity with rest of the group as it is selection from local germplasm of Pithoragarh district. The dendrogram further delineated the above varieties into two groups, by clearly demarcating UPRI 95-17B as highly distant from rest of the varieties by showing only 73.4% similarity with them.

All the varieties except *Pant Dhan* 12, *Pant Majhera Dhan* 7, and UPRI 95-17B divided into two major clusters 1 and 2 related at only 74.2% similarity level. Cluster 1 containing six genotypes and it were further divided into two sub clusters A and B being differentiated at 78% similarity between them. Cluster

A comprised of two genotypes *Saryu* 52 and UPRI 2870 95-125, sub cluster B was comprised of *Pant Dhan 11*, UPRI 95-287R, and NDR-359. UPRI 93-287R and NDR-359 with 86% similarity with each other. Cluster 2 had eleven varieties *Govind, Pant Dhan* 16, *Pant Dhan* 6, *Pant Dhan* 4 *Pant Sankar Dhan* 1, *Manhar*, *Pant Dhan* 10, *Pant Sankar Dhan* 3, *Prasad*, UPRI 99-1 and UPRI 99-133R and it was further divided into two sub clusters C and D differentiated with 76.3% similarity between them. Cluster C had three varieties UPRI 95-133R, UPRI 99-1 and *Prasad*. UPRI 99-1 showed relatedness with *Prasad* at 83% similarity level. Sub cluster D was again subdivided into two mini clusters with 81% similarity. One mini cluster had *Pant Sankar Dhan* 3, *Pant Dhan* 10 and *Manhar* with 83% similarity. *Manhar* showed close relation with *Pant Dhan* 10 (92% similarity) and seems to be identical as depicted in dendrogram , however it is impossible to prove that two varieties are genetically identical without sequencing their genomes. Varieties that are found identical or similar based on molecular analysis data may differ in just one important character. The other mini cluster had three genotypes *Pant Dhan* 4, *Pant Dhan* 6 and *Pant Dhan* 16. *Pant Dhan* 6 showed close relationship with *Pant Dhan* 16 with 87.7% similarity. The present study revealed existence of sufficient genetic variation at DNA level in nineteen varieties. This information will help breeder to characterize the varieties on the basis of polymorphism in addition to morphological marker, to select the diverse varieties based on variation at the DNA level to be used in crossing programme for realization heterosis identifying donor parents for useful agronomic attributes and markers linked to different polygenic traits.

Data scored from nineteen rice varieties with four isozymes (esterase, peroxidase, malate dehydrogenase and alcohol dehydrogenase) were used to generate Jaccard's pairwise similarity matrix. According to this, similarity coefficient ranged from 0.5 to 1.00. **(Table.2)** represents the similarity index among the varieties and **Fig.2** represents the dendrogram drawn on the basis of Jaccard's coefficient.

According to cluster analysis, there were two major cluster groups. The closely related varieties were grouped in one major group. Between two major cluster groups there is not too much distance observed. This showed that these varieties are closely related at biochemical level. This was entirely unexpected. The dendrogram was divided into two major clusters namely, cluster 1 and cluster 2, related at 67% similarity level. Cluster 1 was divided into two sub clusters, A and B differentiated at 73.5% similarity level. Sub cluster A contained three varieties namely, *Pant Dhan* 4, *Saryu* 52 and *Pant Dhan* 12. the variety *Pant Dhan* 10 was related to *Saryu* 52 and *Pant Dhan* 4 at 78.5% similarity level. There were five varieties in sub cluster B *viz.*, UPRI 95-17B, *Pant Dhan* 11, *Pant Dhan* 12, *Pant Dhan* 6 and *Pant Majhera Dhan* 7. Out of these five varieties, *Pant Majhera Dhan* 7, *Pant Dhan* 6 and *Pant Dhan* 12 showed 100% similarity with each other. *Pant Dhan* 11 and UPRI 95-17B showed 89.5% similarity with each other.

Cluster 2 was divided into two sub clusters C and D. Sub cluster C comprised of five varieties *viz*., UPRI 92-133R, UPRI 99-1, UPRI 93-287R, *Pant Sankar Dhan* 3 and *Manhar*.

Sub cluster C was comprised of UPRI 99-1 and UPRI 93-287R which were closely related (92%).

Sub cluster D is divided into two min clusters d` and d``. Mini cluster d` had six varieties *viz.*, *Govind*, NDR 359, UPR 2870-98-125, *Pant Dhan* 16, *Pant Sankar Dhan* 1 and *Prasad*. *Prasad* showed 85.5% similarity with *Govind*, NDR 359, UPR 2870-98-125, *Pant Dhan* 16 and *Pant Sankar Dhan* 1. Out of these six varieties of this sub cluster NDR 359 and UPR 2870-98-125 showed 100%
relatedness with each other and these varieties were related with *Govind* at 91.5% similarity level. *Pant Sankar Dhan* 1 was related with *Govind*, NDR 359, UPR 2870-98-125 and *Pant Dhan* 16 at 87% similarity level. Mini cluster d" consisted of *Pant Sankar Dhan* 3 and *Manhar* which showed 100% similarity with each other. The homology of genotypes (% similarity) for protein bands varied from 50 to 100% among different varieties. Results on homology between the varieties are presented in **Table 4.4.3.** Varieties NDR 359 and *Pant Dhan 4*, UPRI95-287R with *Pant Dhan 10* and UPRI 95-17B with *Pant Dhan10* and *Pant Dhan 6* with *Govind* showed complete homology of 100%.

Fig.4.4.2. depicts the relationship among nineteen rice varieties under study in the form of dendrogram. On the basis of cluster analysis, the varieties were divided into two major groups (Cluster 1 and 2). Within one major group there were closely related varieties. However, there was not too much distance between two major groups. This showed that these varieties were closely related at biochemical level. This was not entirely unexpected because *O. sativa* is an autogamous crop. Cluster 1 and 2 were related at 69% similarity level.

In cluster 1, UPRI 92-133R is related to other genotypes (*Pant Dhan 4*, NDR 359, UPRI 95-17B, UPR 2870-98-125, *Pant Dhan* 10, UPRI 93-287R, UPRI 99-1 and *Saryu* 52) at 75.5% similarity level. Saryu 52 has 80% similarity to rest genotypes in cluster 1. The dendrogram depicted 100% similarity

among *Pant Dhan* 10, UPRI 95-17B and UPRI 93-287R at biochemical level. The similarity observed among *Pant Dhan* 4, NDR 359 and UPR 2870-98-125 was 100%.

In cluster 2, Prasad was 74% similar to *Pant Majhera Dhan* 7, *Manhar*, *Pant Dhan* 12, and *Pant Sankar Dhan* 1. *Pant Sankar Dhan* 3, *Pant Dhan* 11, *Pant Dhan* 16, *Pant Dhan* 6 and Govind.

Cluster 2 was further divided into two sub clusters, A and B, differentiated at 79.5% similarity level. Sub cluster A consisted of six varieties, *Pant Majhera Dhan* 7 is related to *Manhar*, *Pant Dhan* 12, *Pant Sankar Dhan* 1, *Pant Sankar Dhan* 3 *and Pant Dhan* 16 at 81.5% similarity level. *Pant Sankar Dhan* 1 and *Pant Sankar Dhan* 3 were 92% similar with each other. In sub cluster B, *Govind* and *Pant Dhan* 6 exhibited 100% similarity with each other.

ACKNOWLEDGEMENT:

The authors are grateful to Head, Genetics and Plant Breeding Department, the Director of Experiment Station and Dean, College of Agriculture, G.B. Pant Agricultural University for providing the necessary facilities for conducting this investigation.

Authors for correspondence:

anuradhagbp@gmail.com , kamalpandey@yahoo.com , pankaj@hct.edu.om

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3/27/2009