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Utilization of biochemical and molecular markers for assessment of distinctness in rice varieties

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ABSTRACT

Nineteen high yielding rice varieties were studied for morphological descriptors, total soluble protein, isozymes and RAPD molecular markers to determine distinctive features of each variety. In the present study out of 60 morphological descriptors 17 were monomorphic, 23 dimorphic and 20 found polymorphic. In six varieties namely Govind, Prasad, Pant Sankar Dhan 1, Pant Sankar Dhan 3, UPRI 95-17B, UPRI 92-133R and Saryu 52 were distinguished from rest of the varieties on the basis of different morphological discriptors. SDS-PAGE profile showed maximum number of bands (15) in Govind and Pant Dhan 6 and lowest number of bands (8) was obtained in Pant Dhan 10. On the basis of UPGMA cluster analysis of SDS-PAGE profile, variety Prasad was found distinct. A high degree of polymorphism was detected among the nineteen rice varieties through 12 random primers which generated a total of 68 bands with an average of 5.6 bands per primer. EO 1591, EO 1593, EO 1600 and EO 1602 generated unique bands in Pant Majhera Dhan 7, UPRI 95-17B, Govind and Pant Dhan 6, respectively. UPGMA cluster analysis revealed Pant Majhera Dhan 7 was highly diverse from other varieties.

Key words: *rice, distinctness, morphological descriptor, molecular marker, Isozyme, RAPD*

Rice is an important food crop with wide adaptation to a range of environments. In India, it is grown on 45.35 m ha area with the production of 99.15 million tonnes of milled rice in 2008 (Agricultural Statistics 2009). Globally, India ranks first in area under rice cultivation and second in production after China. In India, rice is more than food stuff; it's an entire culture and is a basis of both biological and cultural diversity. About 863 high yielding varieties have been released in India for various ecosystems through the efforts of plant breeders (Pandey *et al.,* 2010). Thus in context of increasing multiplicity of newly developed varieties and implementation of Plant Variety Protection and Farmer's Rights Act, 2001 varietal identification/ characterization has attained a critical importance. Plant Variety Protection and Farmer's Rights Act, 2001 require distinctness, uniformity and stability (DUS) criteria to be fulfilled for protection under this act. Implementation of this act encourage public/private investment in research and development of new plant varieties by giving protection to different categories of plant varieties including extant-notified and farmer's varieties against unauthorized multiplication of seeds or propagating materials for a specified period. Clear cut characterization is also necessary for the operational aspects in the seed trade. Accurate varietal profiling and distinctness based only on morphological and physiological parameters are not always possible by these parameters. Thus for precise description of varieties some laboratory based techniques may be very advantageous. Biochemical and molecular markers can be considered as additional descriptors for establishing the distinctiveness of the variety. The biochemical and molecular techniques group (BMT) of the International union for the protection of new varieties of plants (UPOV) is evaluating different DNA marker parameters in establishing distinctness, uniformity and stability (DUS) of plant varieties (Bredemeijer *et al*., 2002). Molecular techniques can provide new possibilities to characterize advanced genetic materials for registration purposes and for the protection of breeder's rights (Noli *et al.,* 2008). RAPD technique

has been used quite satisfactorily in discriminating genotypes and is having considerable advantage over morphological characteristics in varietal identification (Mackill, 1995). Gel electrophoresis of protein and isozymes are a powerful tool to distinguish genotypes of plant species (Cooke, 1995). Several authors have highlighted the new possibilities and perspectives in varietal characterization by the advent of biochemical and molecular markers and emphasised their advantages such as independence from the environment and rapidity of assessment (Smith and Smith, 1992; Preston *et al.,* 1999). Therefore, morpho-physiological, biochemical and molecular characterization of selected high yielding varieties were done to determine distinctive features of each variety.

MATERIALS AND METHODS

Nineteen high yielding varieties of Pantnagar were used in the study. Morpho-physiological characteristics were studied as per PPV&FR, DUS testing guidelines (Shobha Rani *et al.,* 2004).The experiments were conducted during the two wet seasons of 2005 and 2006 in randomized block design with 3 replications. Each variety under study was sown in 3 rows of 6 m length with 30×20 cm spacing. The observations were recorded at specified stages of crop growth period when characteristics under study had full expression. Out of 62 characteristics all characters were studied except culm attitude and expression of white core in polished grain as these two traits were not applicable to the material under study. Out of 60 agro-morphological traits 14 were quantitative and 46 were qualitative and visually measurable.

Total soluble proteins were extracted by hand grinding of 1g homogenization buffer decorticated grains in 2 ml chilled Tris-sucrose containing 0.1 M Tris, 0.4 M Sucrose, 10 mM KCl, 0.1 % v/v b-mercaptoethanol and 1 mM each of MgSO4, EDTA and PMSF. The homogenate obtained was centrifuged at 12,000 rpm for 30 min and the supernatant was further used for electrophoresis in a 12% SDS polyacrylamide gel.

For isozyme study, 7 days old etiolated seedlings were ground with 50mM tris Hcl buffer (PH=7.6) containing 2 mercaptoethanol and EDTA in ratio 1:2 (w/v). For extraction of Peroxidase, the buffer without 2 mercaptoethanol was used. Homogenate centrifuged at 15,000 rpm and supernatants obtained were used for studying isozyme pattern. Isozymes were separated on 7% polyacrylamide gel using an anionic system (Davis1964) and stained as described by Vallejos (1983) for esterase (EST), peroxidises (POX), malate dehydrogenases (MDH) and alcohol dehydrogenases (ADH).

Genomic DNA was isolated from fresh leaves (Murray and Thompson 1980). One gram of fresh leaves of each of nineteen varieties 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 75ng of template DNA, 100ng μl^{-1} of primer; 200 µM of dNTPs, 6U μl^{-1} 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 rise 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, 1X 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 biochemical and molecular profiles of 19 rice varieties were analysed using NTSYS-pc 2.02 software (Rholf 2002).The bands were scored as present (1) or absent (0) for each genotype primer combination of all the nineteen rice genotypes, considering each amplified bands as unique locus. The data entry was done into binary matrix as discrete variables. Band sharing data were used to calculate genetic similarities based Jaccard's coefficient (Jaccard 1908) and UPGMA (Unweighted pair group method using arithmetic averages). Alogrithim was employed to determine the genetic relationship of nineteen varieties (Sneath and Sokal 1973).

RESULTS AND DISCUSSION

Sixty morphological traits of the experimental material were recorded at specific stage of plant growth and development as per DUS test guidelines to make accurate description of varieties. Out of 60 morphological traits 17 traits had not shown any variation while 23 traits were dimorphic and 20 traits were found polymorphic in all the 19 varieties. In six varieties distinct morpho-physiological features were observed *viz.,* rice variety Govind showed absence of auricles and ligule, light green stigma colour and deflexed panicle curvature of main axis and these features were not found in rest of the varieties under study. Coleoptile and basal leaf sheath colour were found purple in Pant Sankar Dhan 1, Pant Sankar Dhan 3 and UPRI 95-17B which was distinguished as compared to green colour in rest of the varieties. In three varieties *viz.,* Pant Sankar Dhan 1, UPRI 92-133R and Saryu 52 purple stigma was the distinguishing feature. Colour of lemma and palea was gold and gold furrows on straw background, and light brown colour of decorticated grain was found as clear cut feature to identify Prasad variety. Eight grouping characters have been described in DUS test guidelines (Table1). One of these grouping trait namely decorticated grain aroma was found absent in all 19 varieties. Basel leaf sheath colour, time of heading, stem length, amylose content and decorticated grain colour were dimorphic while decorticated grain length and shape were observed to be polymorphic. Thus descriptors mentioned in DUS test guideline can distinguish some of the varieties with distinct features but not able to distinguish related varieties with similar phenotypic traits. In such a situation, biochemical and molecular markers can be useful to establish identity of a variety. Rice is one of the crop plants where detailed protein/isozyme analysis has been conducted and exhaustive information on the method of analysis and genetic control of isozymes are available. Various workers have employed electrophoretic profiles of total soluble proteins or isozymes for varietal identification (Malik and Khush 1996; Habib *et al.,* 2000).Total soluble protein profiling is considered as an undisputed

Table 1. Grouping Characteristics of 19 High Yielding Rice Varieties prescribed in DUS Test

Varieties	Time of Heading	Stem Length	Decorticated Grain: Shape	Decorticated Grain: Length	Decorticated Grain: Colour	Endosperm: Amylose Content	Decorticated Grain: Aroma	Basal Leaf: Sheath Colour
Govind	Early	Long	Long Slender	Long	White	Medium	Absent	Green
Pant Sankar Dhan 1	Early	Long	Long Slender	Long	White	Medium	Absent	Purple
Prasad	Early	Long	Long Slender	Long	Light Brown	Medium	Absent	Green
UPRI 93-287R	Early	Long	Long Slender	Long	White	Low	Absent	Green
NDR 359	Early	Long	Medium Slender	Medium	White	Medium	Absent	Green
UPRI 92-133R	Early	Medium	Medium Slender	Medium	White	Medium	Absent	Green
Pant Dhan 6	Early	Medium	Medium Slender	Medium	White	Medium	Absent	Green
Saryu 52	Early	Medium	Medium Slender	Medium	White	Medium	Absent	Green
Pant Dhan 16	Early	Short	Short Slender	Short	White	Medium	Absent	Green
UPRI 95-17B	Early	Long	Long Slender	Long	White	Medium	Absent	Purple
Manhar	Late	Long	Long Slender	Long	White	Low	Absent	Green
Pant Majhera Dhan7	Late	Medium	Medium Slender	Medium	White	Medium	Absent	Green
Pant Sankar Dhan3	Late	Long	Long Slender	Long	White	Medium	Absent	Purple
Pant Dhan 10	Late	Long	Long Slender	Long	White	Medium	Absent	Green
Pant Dhan 12	Late	Long	Long Slender	Long	White	Medium	Absent	Green
Pant Dhan 4	Late	Short	Long Bold	Long	White	Medium	Absent	Green
Pant Dhan 11	Late	Long	Long Bold	Long	White	Low	Absent	Green
UPR 2870-98-125	Late	Long	Long Bold	Long	White	Low	Absent	Green
UPRI 99-1	Late	Long	Long Slender	Long	White	High	Absent	Green

Guidelines of PPVFR Act, 2001.

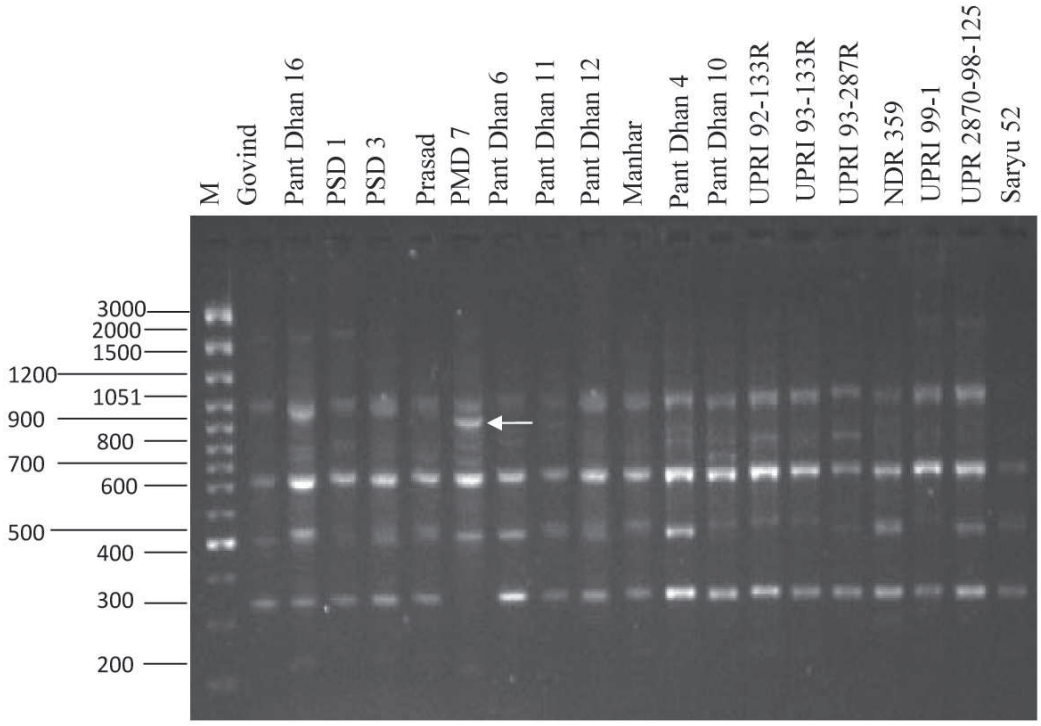

Fig. 1. Band profile of amplified DNA sequences from a RAPD reaction using Primer EO1591

technique for cultivar identification as proteins are the final gene products and reflect the genomic composition of varieties. UPOV (International Union for Protection of New Varieties of Plants) has also included electrophoresis of seed proteins in characterising wheat (UPOV 1994a) and barley (UPOV 1994b). Electrophoresis of total soluble seed protein by SDS-PAGE revealed a total of 15 bands in rice varieties under study. Maximum number of bands (15) were exhibited by Govind and Pant Dhan 6 and lowest number of bands (8) were obtained in Pant Dhan 10. Mahfooza *et al.,* (2004) also found differences among rice genotypes for total number of protein bands in a given zone. Protein bands were classified into three zones *viz.,* A, B and C on the basis of molecular weight. Polymorphic bands were obtained in zone B and C. On the basis of UPGMA cluster analysis variety Prasad was found distinct from rest of the varieties (Fig.2). While Govind and Pant Dhan 6; Pant Dhan 4, NDR 359 ands UPR 2870-98-1250; and Pant Dhan 10, UPRI 95-17B and UPRI 93-287 R formed groups and could not be discriminated. In this case due to relatedness of varieties there was a limited scope for total soluble protein to establish distinctiveness among varieties.

Native PAGE was conducted for four isozymes in present study. UPOV also included isozyme in maize (UPOV, 1994c), soybean (UPOV, 1994d) and sunflower (UPOV, 1994e) as additional characters. Isozyme POX, MDH, EST showed polymorphism, where as ADH generated monomorphic bands in all the varieties except NDR 359. Isozyme POX generated highest number of six bands. In Pant Majhera Dhan 7 POX_1, POX_2 and POX_5 were present which otherwise absent in all other varieties. In hybrid variety Pant Sankar Dhan 1 and its maintainer UPRI -17B showed a unique band EST_3, which was found absent in other varieties. MDH_2 was present in three varieties namely Prasad, Manhar and Pant Dhan 6. Analysis of combined isozyme pattern of all four isozymes by UPGMA cluster analysis was able to discriminate most of the varieties except NDR 359 and UPR 2870-98-1250; Pant Sankar Dhan and Manhar; and Pant Majhera Dhan 7 and Pant Dhan 6 (Fig. 3).These results can be supported by the fact that isozymes are the expression of part of coding region not of whole of the genome so only moderate level of polymorphism was generated by this. Isozyme marker can only be used as an additional tool for characterization of varieties.

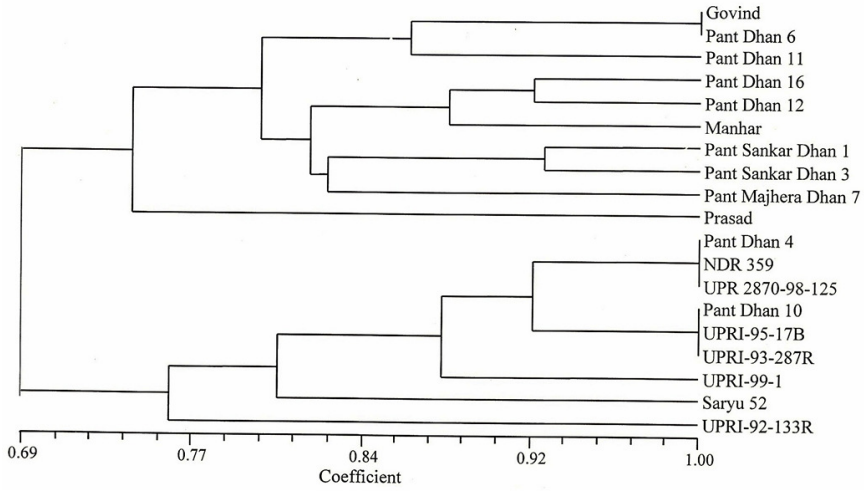

Fig.2. UPGMA cluster analysis of SDS-PAGE of total soluble protein profile in nineteen rice varieties

Molecular characterization by 12 RAPD markers generated a total of 68 bands with an average of 5.6 bands per primer. Of these, 48 were found polymorphic and level of polymorphism was 70.58%. The number of bands generated was more primer dependent and ranged from 1 to11. The range of polymorphic bands ranged from 50 % (EO 1598 & EO 1594) to 100 % (EO 1600 & EO 1596). EO 1591 (Fig. 1), EO 1593, EO 1600 and EO 1602 generated unique bands in Pant Majhera Dhan 7, UPRI 95-17B, Govind and Pant Dhan 6, respectively. UPGMA cluster analysis revealed Pant Majhera Dhan 7 was highly diverse from rest of 18 varieties (Fig.4). Pant Sankar Dhan 1 showed 76% similarity with UPRI 92-133R which indicated relationship among parent and its hybrid. UPRI 92-133R is one of the parents used in Pant Sankar Dhan 1. Pant Sankar Dhan 3 showed 72% similarity with its restorer parent UPRI 93-287R. Pant Dhan 12 and Govind were found 73% similar as Govind is one of the parents of Pant Dhan 12. Govind and Pant Dhan 10 were 80% similar because both have IR 20 as common parent in their ancestry. Advantage of molecular markers is their

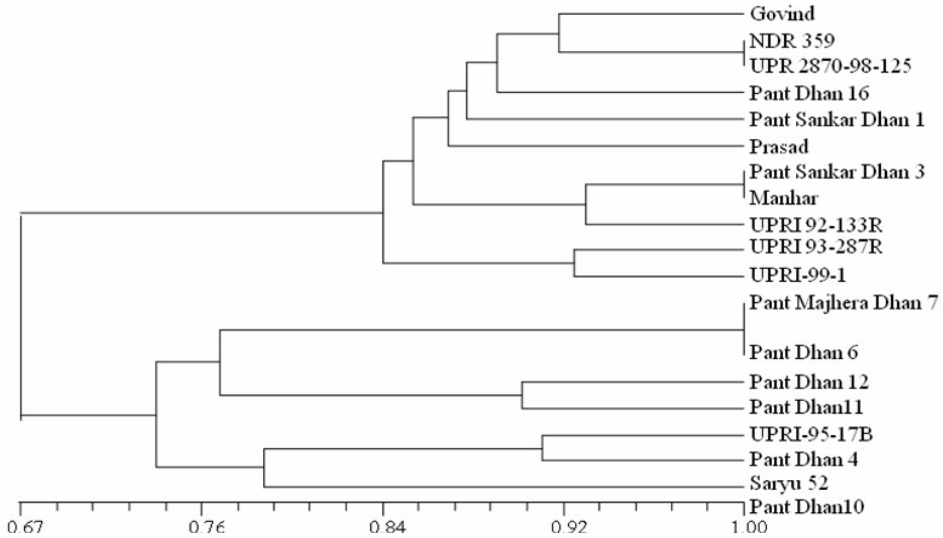

Fig. 3. Combined UPGMA cluster analysis of four isozymes viz. Peroxidase (POX), Esterase (EST), malate Dehydrogenase (MDH) and Alcohol Dehydrogenase (ADH) in nineteen rice varieties

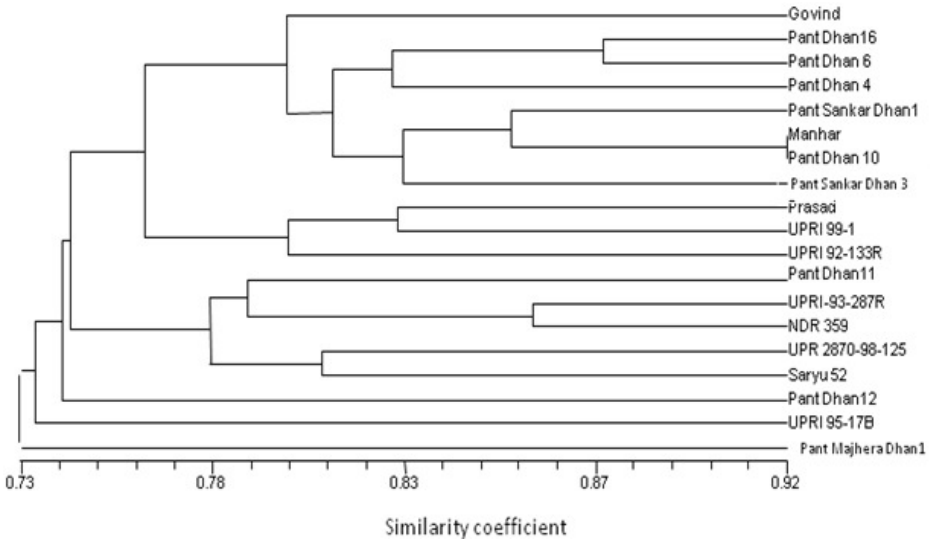

Fig. 4. UPGMA cluster analysis of RAPD banding profile in nineteen rice varieties

relatively higher discrimination power than biochemical markers. Molecular markers have been shown to be a powerful tool for genotype characterization (Law *et al.* 1998; Lefebvre *et al.* 2001). Based on the observed results, it can be concluded that agro morphological descriptors mentioned in DUS test guidelines, can facilitate the maintenance of purity of varieties, and help in resolving disputes related to the genetic lineage of the varieties and situations where the morpho-physiological DUS descriptors fails to establish distinctiveness of a variety then biochemical and molecular markers may be used as additional descriptors. Among the biochemical markers, the efficacy of total seed protein and isozyme marker markers was found limited and generated a moderate level of polymorphism in distinguishing closely related rice varieties. Hence biochemical markers can be used as additional descriptor with morphological traits for varietal profiling. Joshi and Chawla (2010) suggested that introduction of molecular markers for varietal profiling can be very advantageous as molecular markers showed better resemblance with the pedigree as compared to morphological markers.

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