Seed Science and Biotechnology ©2009 Global Science Books

Morphological, Biochemical and Molecular Characterization of 26 Rice Cultivar Seed and Seedlings for Cultivar Discrimination

Tamilmani Eevera[1*] • Karuppiah Vanangamudi[2]

[1] Department of Biotechnology, Periyar Maniammai University, Thanjavur, India
[2] Tamil Nadu Agriculture University, Coimbatore, India

Corresponding author: * **teevera2000@yahoo.com**

ABSTRACT

Seed morphological characters, SDS-PAGE of endosperm proteins and RAPD profile from different rice cultivars were studied to determine their genetic variation and phylogenetic relationship. Both seed and hulled seed characters of 26 cultivars of rice were examined and Cluster analysis was done by combining both the seed characters, which resulted in two major clusters. 'TKM 9' alone formed one cluster and the remaining 25 cultivars formed another cluster. Cluster analysis of the endosperm protein profile of the selected cultivars revealed two broad clusters. The cultivars viz., 'ADT 38', 'CR 1009' and 'TKM 9' formed one major cluster. The remaining cultivars formed another major cluster. The cultivars like 'PY 1' and 'ADT 40', 'MDU 5' and 'IR 50' showed a close relationship with short sub-cluster distance. 'ADT 38' and 'CR 1009' also formed one sub-cluster. The RAPD technique was used to differentiate 26 rice cultivars. Screening was done with 50 random primers. Consistent results were obtained with about 10 primers. The total number of scorable bands amplified using the 10 primers was approximately 58. Thus the average number of bands amplified per primer was six. The number of scorable bands generated per primer varied from nine to six. Of the 58 fragments amplified, 39 bands were polymorphic. The number of polymorphic bands generated per primer varied between two to eight. Of the 10 random primers, the percentage of polymorphism was more in OPH 19 (89%), followed by OPM 16 (75%). Least amount of polymorphism was observed in OPO 10 (33%).

Keywords: isozyme, morphological character, rice cultivar, RAPD, SDS-PAGE

INTRODUCTION

Cultivar identification is a pre-requisite for the effective provision of Plant Breeders Rights (PBR), which can be achieved by trade secrets, plant variety protection (PVP), or where available, through utility patents. All the three forms of protection require some measure of distinctness. Cultivar identification for the attainment of plant breeder's right is a taxonomic and genetic approach to determine cultivar distinctness. The chief goals are to promote the release of fresh genetic diversity into agriculture and to create an environment of continued funding for Plant Breeding Research and Genetic Resource Conservation. At the international level, cultivar identification and grain commodity usage become linked, because seeds are the encapsulated intellectual property, the protection of which forms an integral component of the General Agreement on Tariff and Trade (GATT) (Smith *et al.* 1995). Various methods are followed for cultivar identification depending upon the utility of the method, purpose and cost involved. Considering the importance of the role of cultivar identification, Cooke (1984) outlined the ideal features of the various methods, which include less environmental influences, high throughput, less amenable to personal bias and convenience to provide statistically significant results.

The traditional way to assess the genetic purity of seed of established crop cultivars is a grow-out test, where the crop is grown in isolation and vigorous rouging during different phases of crop growth is done with the aid of morphological descriptors available for that cultivar under consideration. The main problem for cultivar identification during field inspection of the seed crop is the lack of satisfactory standard characteristics for cultivar assessment. The authorities responsible for this task require stable characters for detecting the performance of registered variations and of new releases. Further characterizations such as laboratory tests like the phenol or KOH test, response of the variety to the added chemical, electrophoretic pattern and cytology allow the opportunity to improve the characterization of cultivars and could provide tools to improve efficiency of field inspection (Lorenzetti and Falcinelli 1987; Downey 1988; Rutz 1990). The International Seed Testing Association (ISTA 1996) has recommended the use of electrophoresis in seed purity testing. In this situation, with the advent of an array of molecular markers at DNA level for finger printing presently available and the new generation markers as a result of technology spillovers of genome projects. It may be possible to have a cultivar-specific fingerprint that reflects the stable genetic descriptor for inclusion in cultivar release proposal for unequivocal identification of cultivar and improved method of genetic purity testing other than those involving morphological descriptors. This has led us to use the technique of endosperm protein SDS-PAGE, seedling protein and isoenzymes, and RAPD for the characterization of rice cultivars in addition to seed and plant morphological characterization.

MATERIALS AND METHODS

The seeds of 19 rice cultivars ('CO 43', 'ADT 39', 'CORH 2', 'CR 1009', 'ADTRH 1', 'ASD 16', 'ADT 37', 'CO 45', 'IR 20', 'ASD 20', 'ADT 40', 'CO 46', 'ASD 19', 'MDU 5', 'TKM 9', 'ADT 38', 'White Ponny', 'IR 50', 'ADT 43') obtained from the Department of Rice, Tamil Nadu Agricultural University (TNAU), Coimbatore, Tamil Nadu, 5 rice cultivars ('Harsha', 'Aiswarya', 'Kanchana', 'Aathira' and 'Kairalee') obtained from the Regional Research Station, Pattampi, Kerala and 2 rice cultivars ('PY1' and 'PY 6') obtained from Krishi Vigyan Khendra, Pondicherry, were used for cultivar characterization.

Received: 15 November, 2008. Accepted: 16 July, 2009.

Original Research Paper

Seed morphological characters

Seeds were subjected to seed morphological characterization under laboratory conditions in the Department of Seed Science and Technology, Tamil Nadu Agricultural University, Coimbatore. Characters were observed based on Rosta (1975).

Endosperm protein extraction

Endosperm proteins were extracted from seeds of different cultivars of rice with 50 mM Tris-HCl buffer (pH 6.8) containing 100 mM NaCl, 10 mM EDTA, 100 mM glycine, 10% SDS and 1 mM phenyl methyl sulphonyl fluoride (PMSF). The crude homogenate was kept for 45 min at 40°C and centrifuged at 10,000 rpm for 15 min. The extracted proteins were recovered as clear supernatant. SDS-PAGE of the extracted proteins was carried out on 12% polyacrylamide slab gel under reducing conditions following the methods of Laemmli (1970). Samples containing 50 µg protein were loaded on 1.5 mm thick 12% acrylamide gels. Electrophoresis was carried out at a constant voltage of 60 V for 6 h. The gels were stained for 3 h in 0.25% (w/v) Coomassie brilliant blue R-250 followed by destaining in methanol: water: glacial acetic acid (4: 5.3: 0.7). Protein bands were visualized in a transilluminator under white light.

Seedling protein extraction

One gram of 14 days-old seedling samples was utilized for extracting seedling protein. The sample powder of each variety was taken in an Eppendorf tube and 1 ml of defatting solution (chloroform: methanol: acetone in the ratio of 2: 1: 1) was poured in each tube. After thorough shaking, the Eppendorf tubes were left for 3 h. The supernatant was decanted and this procedure was repeated three times. The samples were kept overnight at room temperature for drying. On the next day, 0.1 ml of extraction buffer (Tris HCl pH 7.5) was added and Eppendorf tubes were kept overnight at 10°C (inside the refrigerator). The next day, the samples were centrifuged in a refrigerated centrifuge (4°C) at 12,000 rpm for 30 min (Dadlani and Varier 1993). Supernatant solution from each sample was placed into a separate Eppendorf tube. Samples containing 200 µg protein were loaded on 1 mm-thick 12% polyacrylamide gels. Electrophoresis was carried out at a constant voltage of 60 V for 6 h. The gels were stained for 3 h in 0.25% (w/v) Coomassie brilliant blue R-250 followed by distaining in methanol: water: glacial acetic acid (4: 5.3: 0.7).

Sample preparation for peroxidase and polyphenol oxidase isoenzymes

Seeds of each cultivar were soaked in distilled water for one day. On the next day the sprouted seeds were taken and kept on a blotter for removing excess moisture from the surface. Then, the one gram of seed was ground using a pestle and mortar on ice. Extraction buffer (Tris-HCl pH 7.5 0.1 M, 1.0 ml) was added and the sample was finely ground. It was placed into a clean Eppendorf tube and centrifuged at 12,000 rpm for 30 min in a refrigerated centrifuge at 4°C. The supernatant was used for running the alkaline gel. Peroxidase and polyphenol oxidase isoenzyme was analysed by alkaline PAGE procedure described by Dadlani and Varier (1993) and staining procedure of Reddy and Gasber (1971).

Random Amplified Polymorphic DNA

For isolation of total genomic DNA, healthy seeds of different cultivars were surface sterilized with mercuric chloride (0.01%) and then seeds were soaked overnight (16 h) in distilled water at room temperature. About a quarter of a seed, from 30-40 seeds per cultivar was pooled and used for DNA extraction. The DNA amplification mixture (25 µl) contained 25 ng template DNA (2 µl), buffer 2.5 µl, primer 1.0 µl, dNTPs 0.25 µl, Taq Polymerase 0.2 µl and sterile water 19.05 µl. PCR components were prepared as master mixes for each primer to minimize pipetting errors. Amplification were performed in two models of thermal cyclers viz., Model Gene Amp PCR system 2400, Perkin Elmer, USA and PTC-100 TM, MJ Research Inc. USA. Thermal cyclers were programmed for 35 cycles with an initial stand separation temperature at 94°C for 2 min and 94°C for 1 min, followed by annealing at 37°C for 1 min and extension at 72°C for 1 min. After 35 cycles there was a final extension at 72°C for 10 min. Amplification products were electrophoresed in a 1.6% agarose gel and stained with ethidium bromide. Each set of reactions was repeated in triplicate and only the reproducible one was included in the analysis.

Statistical analysis

Variation in morphological characters was determined by visual checking of the features on the seeds from each cultivar. Evaluation of variation in the endosperm proteins and RAPD profile was performed by calculating the individual band frequency for each cultivar. Polymorphism was scored for the presence (1) or absence (0) of bands. Cluster analysis was performed on the similarity matrix based on Jaccard's similarity index by the UPGMA method. All computations were performed with NTSYS-PC version 2.1 (Rohlf 1993).

RESULTS AND DISCUSSION

Based on the variety release proposal and IBPGR–IRRI cultivar descriptor, the plant morphological characters were observed under field condition in two different seasons. Based on our observation we identified field level key characters for identification of individual cultivars under field conditions. This key character will help in identifying the particular cultivar to facilitate seed growers, seed certification agencies, and other firms involved in quality seed production of rice.

The observed seed characters are given in **Table 1A**. Based on seed colour the cultivars were grouped into four different classes: golden ('ADT 40', 'MDU 5', 'Harsha', 'ADT 37', 'ASD 20', 'CO 43', 'ASD 19' and 'White Ponni'), yellow ('IR 50', 'ASD 16', 'ADT 38', 'IR 20', 'CORH 2' and 'CR 1009'), brown ('PY 1', 'ADT 39', 'CO 46', 'CO 45', 'PY 6', 'Kairalee' and 'Kanchana') and straw colour ('ADTRH 1', 'TRM 9', 'ADT 43', 'Aiswarya' and 'Aathira').

Cultivars like 'CR 1009', 'PY 1', 'White Ponni', 'PY 6' and 'ADT 37' grouped under short (less than 7.5 mm) seed length category. 'CO 45', 'CO 46', 'ASD 19', 'CORH 2' and 'ADTRH 1' grouped under long (9.0-10.0 mm) seed length category. Remaining cultivars fall under medium (7.5-9.0 mm) seed length category. Based on the seed width the cultivars were grouped into two categories i.e. ≤ 2 mm ('IR 50', 'ADTRH 1', 'PY 6', 'ADT 43', 'ASD 20', 'White Ponni') and 2.0-3.0 mm category (all the remaining cultivars).

The profile value of the seed formed three different groups among the 26 cultivars (i.e.) semi-spherical ('ADT 37' and 'CR 1009'), semi-long ('ADT 40', 'MDU 5', 'Harsha', 'Kanchana', 'Kairalee', 'ASD 16', 'TKM 9', 'ADT 38', 'IR 20', 'PY 1', 'Aiswarya', 'Aathira') and elongated (all the remaining cultivars).

Based on the beak value the cultivars were divided into three groups: curved ('IR 50', 'ADTRH 1', 'PY 6', 'TKM 9', 'ASD 20', 'CO 45', 'PY 1', 'Aiswarya' and 'Aathira'), straight ('Kanchana', 'ADT 43', 'IR 20', 'White Ponni' and 'IR 1009') and slightly curved (all the remaining cultivars). Cultivars like, 'CO 45', 'CO 46', 'CORH 2' and 'ADTRH 1' were clearly distinct from the remaining cultivars due to the presence of an awn. Based on 1000-seed weight 26 cultivars were grouped into two categories: 28-30 g ('Aathira' and 'Kanchana') and < 28 g (all the remaining cultivars). The observed hulled seed characters are presented in **Table 1B**. Based on the colour of the silk integument the cultivars were grouped into white ('CO 43', 'ADT 38', 'ADT 39' and 'IR 20'), yellow ('ADT 40', 'Harsha', 'PY 6', 'ASD 20', 'CO 45' and 'CR 1009'), grey ('MDU 5', 'IR 50', 'ADTRH 1', 'ASD 16' and 'ADT 37'), brown ('CO 46', 'ADT 19', 'White Ponni', 'CORH 2', 'PY 1', 'Aiswarya' and 'Aathira') and red ('Kanchana', 'Kairalee' and 'ADT 43'). Hulled grains were grouped into four categories based

Table 1A Seed morphological characters (n = 400 seeds).

Variety	Seed characters						
	Seed colour	**Length (cm)**	**Width (cm)**	**Profile value**	**Beak**	**Awn**	**1000-seed wt. (g)**
CO 43	Golden	0.80	0.26	3.07	Slightly curved	Absent	20.00
CO 45	Brown	0.98	0.30	3.26	Curved	Present	24.90
CO 46	Brown	0.96	0.30	3.20	Slightly curved	Present	24.50
ADT 38	Yellow	0.88	0.30	2.93	Slightly curved	Absent	21.00
ADT 39	Brown	0.76	0.24	3.16	Slightly curved	Absent	18.00
IR 20	Yellow	0.84	0.30	2.80	Straight	Absent	19.00
ASD 19	Golden	0.98	0.23	4.26	Slightly curved	Absent	18.30
White Ponni	Golden	0.70	0.20	3.50	Straight	Absent	16.40
CORH 2	Yellow	1.00	0.24	4.16	Slightly curved	Present	23.70
PY 1	Brown	0.70	0.34	2.05	Curved	Absent	16.20
Aathira	Straw	0.84	0.30	2.80	Curved	Absent	26.20
Aiswarya	Straw	0.88	0.38	2.31	Curved	Absent	28.60
CR 1009	Yellow	0.70	0.30	2.33	Straight	Absent	23.50
ADT 40	Golden	0.80	0.30	2.66	Slightly curved	Absent	25.20
MDU 5	Golden	0.80	0.30	2.66	Slightly curved	Absent	21.10
IR 50	Yellow	0.90	0.20	4.50	Curved	Absent	20.40
ADTRH 1	Straw	1.00	0.20	5.00	Curved	Present	23.80
Harsha	Golden	0.80	0.30	2.66	Slightly curved	Absent	26.20
Kanchana	Brown	0.80	0.30	2.66	Straight	Absent	28.10
Kairalee	Brown	0.76	0.30	2.53	Slightly curved	Absent	21.30
ASD 16	Yellow	0.80	0.30	2.66	Slightly curved	Absent	24.20
PY 6	Brown	0.70	0.20	3.50	Curved	Absent	16.00
TKM 9	Straw	0.80	0.30	2.66	Curved	Absent	25.10
ADT 43	Straw	0.80	0.20	4.00	Straight	Absent	15.50
ADT 37	Golden	0.72	0.30	2.40	Slightly curved	Absent	23.40
ASD 20	Golden	0.90	0.20	4.50	Curved	Absent	22.10

Table 1B Seed morphological characters (n = 400 seeds).

Variety	Hulled seed characters						
	Colour of the silk integument	**Hulled grain colour**	**Vitreous character**	**Length (cm)**	**Width (cm)**	**Profile value**	**1000-seed weight**
CO 43	White	Brown	Side is small	0.60	0.20	3.00 Semi long	16.70
CO 45	Golden	Yellow	Absent	0.70	0.20	3.50 Slender	22.20
CO 46	Brown	Brown	Absent	0.70	0.20	3.50 Slender	19.50
ADT 38	White	White	Absent	0.70	0.20	3.50 Slender	17.60
ADT 39	White	Yellow	Absent	0.60	0.20	3.00 Semi long	14.60
IR 20	White	Brown	Side is extended	0.70	0.20	3.50 Slender	15.20
ASD 19	Brown	Brown	Absent	0.50	0.20	2.50 Semi long	14.50
White Ponni	Brown	White	Side is extended	0.50	0.20	2.50 Semi long	12.20
CORH 2	Brown	White	Side is small	0.70	0.20	3.50 Slender	17.20
PY 1	Brown	Yellow	Side is small	0.50	0.20	2.50 Semi long	12.80
Aathira	Brown	Red	Side is small	0.60	0.20	3.00 Semi long	22.30
Aiswarya	Brown	Red	Side is small	0.70	0.30	2.33 Semi spherical	23.60
CR 1009	Golden	Brown	Side is extended	0.50	0.30	1.66 Spherical	18.50
ADT 40	Golden	Brown	Absent	0.60	0.20	3.00 Semi long	19.10
MDU 5	Grey	Brown	Absent	0.60	0.20	3.00 Semi long	18.80
IR 50	Grey	Brown	Absent	0.60	0.20	3.00 Semi long	15.60
ADTRH 1	Grey	Brown	Side is small	0.70	0.20	3.50 Slender	17.70
Harsha	Golden	Brown	Side is extended	0.50	0.20	2.50 Semi long	20.00
Kanchana	Red	Red	Side centre is extended	0.60	0.20	3.00 Semi long	22.50
Kairalee	Red	Red	Side is small	0.60	0.20	3.00 Semi long	15.30
ASD 16	Grey	Brown	Side is extended	0.50	0.20	2.50 Semi long	18.20
PY 6	Golden	Brown	Side centre is extended	0.50	0.20	2.50 Semi long	12.80
TKM 9	White	Red	Side is small	0.60	030	2.00 Semi spherical	18.20
ADT 43	Red	White	Absent	0.50	0.20	2.50 Semi long	11.50
ADT 37	Gray	Brown	Side is extended	0.50	0.20	2.50 Semi long	16.90
ASD 20	Golden	Brown	Absent	0.70	0.20	3.50 Slender	17.60

on the colour of the hulled grain like white ('ADT 38', 'White Ponni', 'CORH 2' and 'ADT 43'), yellow ('CO 45', 'ADT 39' and 'PY 1'), red ('Aiswarya', 'Aathira', 'Kanchana', 'Kairalee', 'TKM 9') and brown (all the remaining cultivars).

Cultivars like 'CO 45', 'ASD 20', 'ADTRH 1', 'CO 46', 'ADT 38', 'IR 20', 'CORH 2' and 'Aathira' form a group based on seed length whereas the remaining cultivars formed another group. Cultivars like 'TKM 9', 'Aathira' and 'CR 1009' were clearly distinct from the remaining cultivars with respect to their hulled seed width character. 'CR 1009' was clearly distinct from other varieties with respect to its spherical nature. The other cultivars form three groups: 'TKM 9' and 'Aathira' (semi-spherical), 'ADTRH 1', 'ASD 20', 'CO 45', 'CO 46', 'ADT 38', 'IR 20' and 'CORH 2' (slender) while the remaining cultivars fell under the semi-long category. Based on the 1000-hulled seed weight the cultivars were grouped into < 15 g ('ADT 39', 'ASD 19', 'White Ponni', 'PY 1', 'PY 6' and 'ADT 43'), 15-18 g ('CO 43', 'ADT 38', 'IR 20', 'CORH 2', 'IR 50', 'ADTRH 1', 'Kairalee', 'ADT 37' and 'ASD 20'), 18-21 g ('CR 1009', 'ADT 40', 'MDU 5', 'Harsha', 'ASD 16', 'TKM 9' and

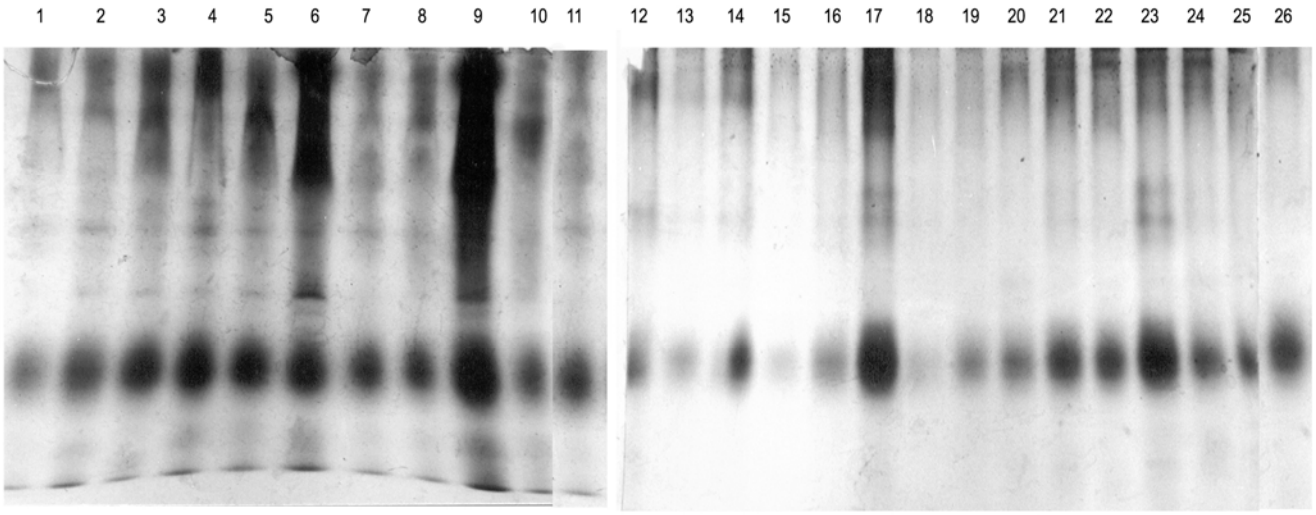

Fig. 1 PAGE profile of Peroxidase isoenzyme extracted from seedlings of different cultivars. Lane 1. 'IR 20' 2. 'ASD 16' 3. 'ADT 40' 4. 'ADT38' 5. 'IR50' 6. 'MDU5' 7. 'White Ponni' 8. 'Aiswarya' 9. 'PY6' 10. 'CO45' 11. 'ADTRH1' 12. 'PY1' 13. 'Kairalee' 14. 'CO46' 15. 'ADT39' 16. 'ADT43' 17. 'ASD20' 18. 'Aathira' 19. 'ASD19' 20. 'TKM9' 21. 'Harsha' 22. 'ADT37' 23. 'CORH2' 24. 'Kanchana' 25. 'CO43' 26. 'CR1009'.

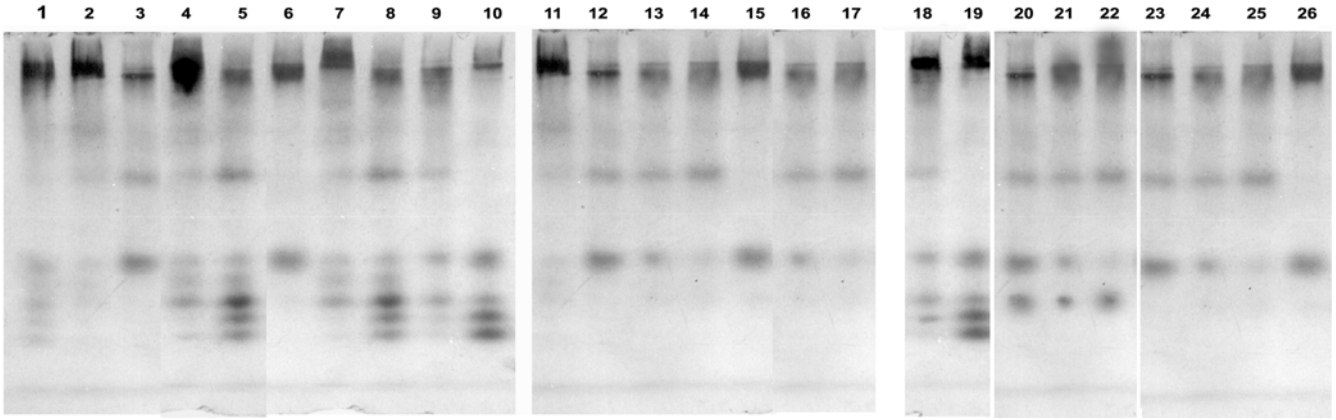

Fig. 2 Variation in polyphenol oxidase isoenzyme profile in rice cultivars. Lane 1. 'CO45' 2. 'Aiswarya' 3. 'CO46' 4. 'Kanchana' 5. 'White ponni' 6. 'CORH2' 7. 'PY1' 8. 'ADT40' 9. 'MDU5' 10. 'IR50' 11. 'PY6' 12. 'ADT38' 13. 'Aathira' 14. 'CR1009' 15. 'Harsha' 16. 'IR20' 17. 'ASD19' 18. 'Kairalee' 19. 'TKM9' 20. 'ADT43' 21. 'ADT37' 22. 'ASD16' 23. 'CO43' 24. 'ASD20' 25. 'ADT39' 26. 'ADTRH1'.

'CO 46'') and 21-24 g ('CO 45', 'Aiswarya', 'Aathira' and 'Kanchana'). This was in line with the findings of Rosta (1975), Sivasubramaniam and Ramakrishnan (1978) and Vanngamudi *et al*. (1988) in rice.

Seed protein and seedling protein and isoenzymes

In rice, several researchers have analysed isozymes. Noteworthy is the work of Glaszman *et al*. (1983) who analyzed 1688 traditional rice from Asia with 15 polymorphic loci coding for 8 different enzymes and grouped them into six groups. However, the isozyme analysis cannot be employed in assessing the genetic structure of cultivated cultivars of a specific region since the polymorphism level observed will usually be low. In the present study two isozymes, peroxidase (**Fig. 1**) and polyphenol oxidase (**Fig. 2**) were utilised for cultivar discrimination.

Based on cluster analysis two major clusters were formed in peroxidase isoenzyme profile analysis (**Fig. 3**). The cultivars like 'ADT 38', 'ASD 16', 'White Ponni', 'Aiswarya', 'IR 50', 'PY 6', 'MDU 5' and 'ADT 40' formed one major cluster. The remaining cultivars formed another major cluster. In the final subcluster level includes the following rice cultivars viz., 'TKM 9', 'ADT 37', 'CO 46', 'Harsha', 'Kanchana' and 'IR 20', 'PY 1', 'CORH 2' and 'ADTRH 1', 'ADT 43' alone, 'CO 45' and 'ASD 20', 'CR 1009', 'Kairalee', 'CO 43', 'ASD 19', 'Aathira' and 'ADT 39', 'ADT 38' and 'ASD 16', 'White Ponni' and 'Aiswarya', 'IR 50', 'PY 6' and 'MDU 5', whereas 'ADT 40' formed a separate subcluster. In the polyphenol oxidase

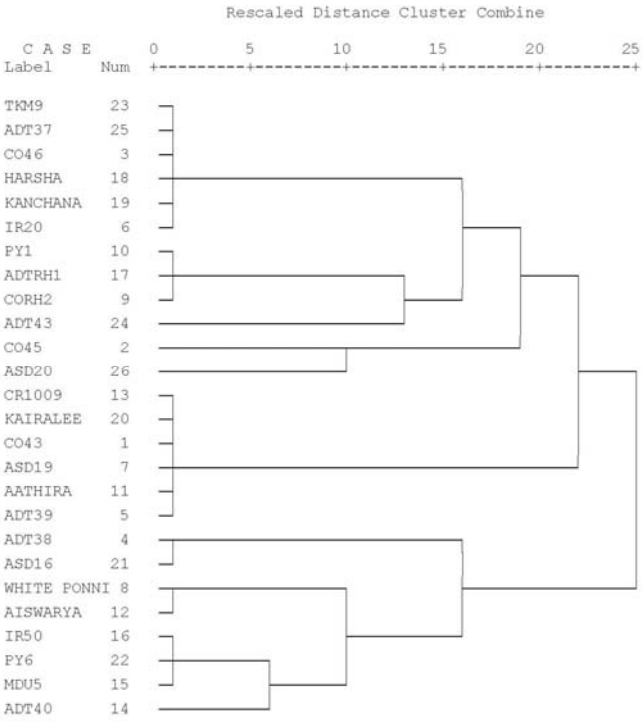

Fig. 3 Dendrogram of 26 rice varieties based on seed peroxidase isoenzyme profile constructed based on Jaccard's Similarity index.

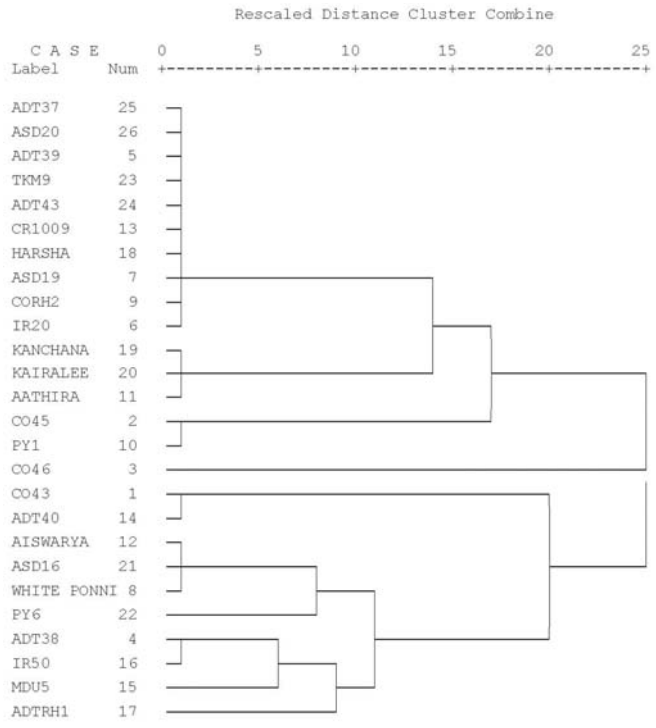

Fig. 4 Dendrogram of 26 rice varieties based on seed polyphenol oxidase isoenzyme profile constructed based on Jaccard's Similarity index.

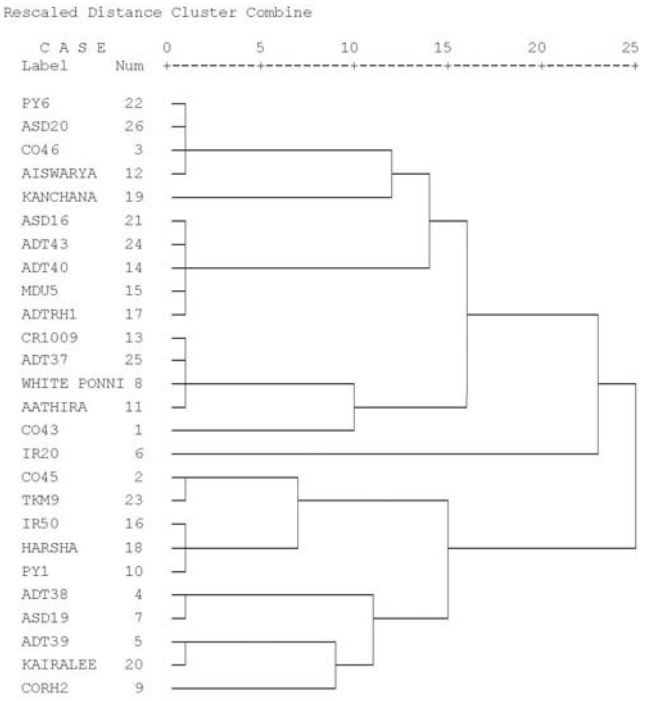

Fig. 6 Dendrogram of 26 rice varieties based on seedling protein profile constructed based on Jaccard Similarity index.

isoenzyme (**Fig. 4**) the number of subclusters were minimum with more number of cultivars in each subcluster. The low level of intercultivar differences for isozymes was reported earlier also (Ainsworth and Sharp 1989; Aldrich *et al*. 1992). Moreover, isozyme marker analyses pose the problems of producing artifacts during electrophoresis. Isozymes, because of their common occurrence and the case of detection, could not be used because of the lack of understanding of the significance of spatial and temporal fluctuations during the ontogeny of an individual. Further, the appearance or disappearance of specific isozyme during development does not a priori reflect gene action. It does not reflect the expression of genetic information (Scandalios 1974). Isozymes can be used as a potential marker, if the genetic cause for the occurrence of multiple forms is well understood. Considering the above points, the study of isozymes in the present study did not establish a clear-cut grouping.

Seedling protein profile (**Fig. 5**) gave few number of subcluster with more number of cultivars in each cluster. The cultivars like 'Harsha', 'IR 50' and 'PY 1' generated more scorable bands (10) where as the cultivar 'IR 20' produced minimum of four scorable bands. In the cluster analysis cultivars like 'CO 45', 'TKM 9', 'IR 50', 'Harsha', 'PY 1', 'ADT 38', 'ASD 19', 'ADT 39', 'Kairalee' and 'CORH 2' formed one major cluster. The remaining cultivars formed another major cluster (**Fig. 6**). In the final level the following sub clusters were formed 'PY 6', 'ASD 20', 'CO 46' and 'Aiswarya', 'Kanchana' alone, 'ASD 16', 'ADT 43', 'ADT 40', 'MDU 5'and 'ADTRH 1', 'CR 1009', 'ADT 37', 'White Ponni', 'Aathira', 'CO 43' alone, 'IR 20' alone, 'CO 45' and 'TKM 9', 'IR 50', 'Harsha' and 'PY 1'. 'ADT 38' and 'ASD 19', 'ADT 39' and 'Kairalee' and finally 'CORH 2' alone.

The total number of bands observed in seed protein profile was approximately 379. Thus the average number of bands produced by the cultivar was 15. The number of scorable bands generated by the cultivar varied from 7 to 22.

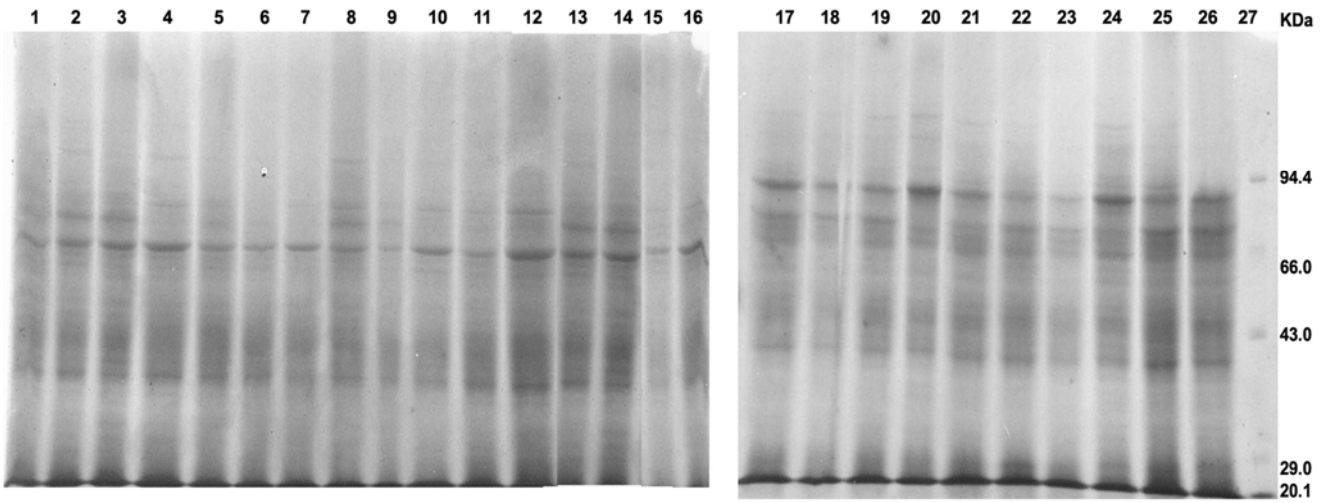

Fig. 5 Seedling protein profile variation in rice cultivars. Lane 1. 'Aiswarya' 2. 'Aathira' 3. 'ASD20' 4. 'CO43' 5. 'CO46' 6. 'Kanchana' 7. 'ADT40' 8. 'ADT37' 9. 'PY6' 10. 'ASD16' 11. 'ADTRH1' 12. 'MDU5' 13. 'CR1009' 14. 'White ponni' 15. 'IR20' 16. 'ADT43' 17. 'TKM9' 18. 'Kairalee' 19. 'Harsha' 20. 'IR50' 21. 'ASD19' 22. 'ADT38' 23. 'ADT39' 24. 'PY1' 25. 'CO45' 26. 'CORH2'.

Table 2 Protein and isozyme profile details of rice cultivars.

Cultivar	Seed protein		Seedling protein		Peroxidase		Polyphenol oxidase	
	TB	PB	TB	PB	TB	PB	TB	PB
CO 43	15	12	6	3	1	0	4	3
CO 45	20	17	9	6	4	3	2	1
CO 46	12	9	6	3	1	0	1	0
ADT 38	7	4	8	5	5	4	6	5
IR 20	22	19	4	1	2	1	3	2
ASD 19	16	13	8	5	1	0	3	2
White ponni	11	8	7	4	5	4	5	4
CORH 2	16	13	8	5	3	2	3	2
PY 1	17	14	10	7	3	2	2	1
Aathira	18	15	7	4	1	0	2	1
Aiswarya	13	10	6	3	5	4	5	4
CR 1009	7	4	7	4	1	0	3	2
ADT 40	16	13	5	2	6	5	4	3
MDU 5	13	10	5	2	7	6	7	6
IR 50	14	11	10	7	7	6	6	5
ADTRH 1	16	13	5	2	3	2	4	3
Harsha	14	11	10	7	2	1	3	2
Kanchana	11	8	5	2	2	1	2	1
Kairalee	18	15	7	4	1	0	2	1
ASD 16	19	16	5	2	5	4	5	4
PY 6	20	17	6	3	7	6	4	3
TKM 9	11	8	9	6	2	1	3	2
ADT 43	18	15	5	2	2	1	3	2
ADT 37	9	6	7	4	2	1	3	2
ASD 20	15	12	7	4	3	2	3	2
ADT 39	15	12	7	4	1	0	3	2
Total	379	305	184	106	82	56	91	65
Polymorphism (%)		80.47		57.60		68.29		71.42

TB – Total bands; PB – Polymorphic bands

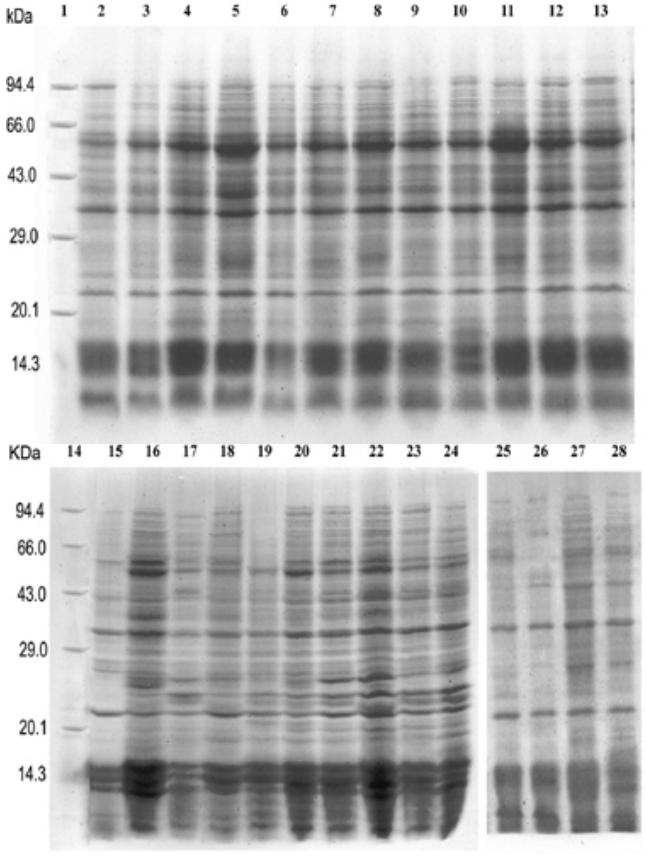

Fig. 7 SDS-PAGE profile of endosperm proteins extracted from seeds of different rice cultivars. Lane 1 and 14. Protein marker 2. 'TKM9' 3. 'CR1009' 4. 'PY1' 5. 'ADT40' 6. 'CO46' 7. 'ADT39' 8. 'CORH2' 9. 'Harsha' 10. 'ADTRH1' 11. 'Aiswarya' 12. 'IR50' 13. 'MDU5' 15. 'ADT38' 16. 'PY6' 17. 'ASD20' 18. 'ASD19' 19. 'CO43' 20. 'IR20' 21. 'Aathira' 22. 'CO45' 23. 'Kairalee' 24. 'ADT43' 25. 'Kanchana' 26. 'ADT37' 27. 'ASD16' 28. 'White ponni'.

Of the 379 bands, 305 (80.47%) bands were polymorphic. The number of polymorphic bands generated per cultivar varied from 4 to 19 (**Table 2**).

Fig. 7 shows the seed protein profile of 26 rice cultivars. The highest of 22 bands were present in 'IR 20' where as minimum of seven bands were present in 'ADT 38' and 'CR 1009'. Putative cultivar specific three bands were obtained in all cultivars. The cluster analysis was performed based on the banding patterned utilizing Jaccard similarity index matrix. The cultivars viz., 'ADT 38', 'CR 1009' and 'TKM 9' formed one major cluster. The remaining cultivars formed another major cluster (**Fig. 8**). The cultivars like 'PY 1' and 'ADT 40', 'MDU 5' and 'IR 50' showed a close relationship with short sub-cluster distance. 'ADT 38' and 'CR 1009' also formed one sub-cluster. In this study denatured protein product of SDS-PAGE was used for characterization of rice cultivars. The advantage of examining denatured proteins was reported by Ferguson and Grabe (1986). They stated that denatured protein is independent from seed vigour and physiological seed activity. The reports of Kapase and Nerkar (1985), Agarwal *et al*. (1988), Rao *et al*. (1990), Dadlani and Varier (1993) and Nerkar and Rao (1993), agreed with the fact that denaturing system provides a simple reproducible technique for cultivar discrimination and identification.

SDS PAGE is powerful technique, which has been used in the past for identification and characterisation of cultivars of various crop species like cotton (Rao *et al.* 1990) and sunflower (Varier *et al.* 1992; Sahoo *et al*. 2000). SDS-PAGE of high molecular weight glutenins extracted from seed has been recommended by Upov (1994) for DUS testing of wheat cultivars. ISTA (1996) has recommended SDS-PAGE as a standard method for verifying the identity of cultivars of *Pisum* and *Lolium*.

Among the various identification techniques, RAPD (Williams *et al*. 1990) is the most widely used technique. RAPD markers are generated by PCR amplification of template DNA using 8-10 base pair long primers of arbitrary sequence. Polymorphism could be due to single base pair change in primer binding site as well as due to deletions or

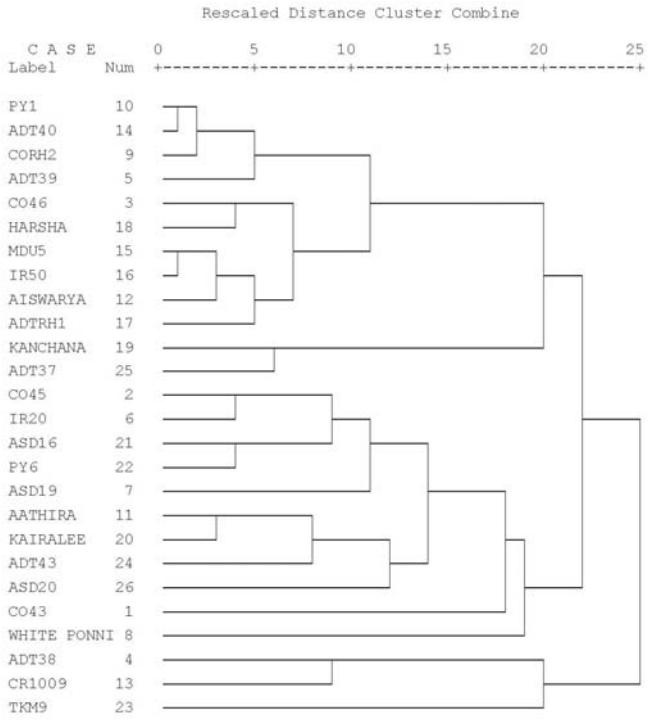

Fig. 8 Dendrogram of 26 rice varieties based on seed protein profile constructed based on Jaccard Similarity index.

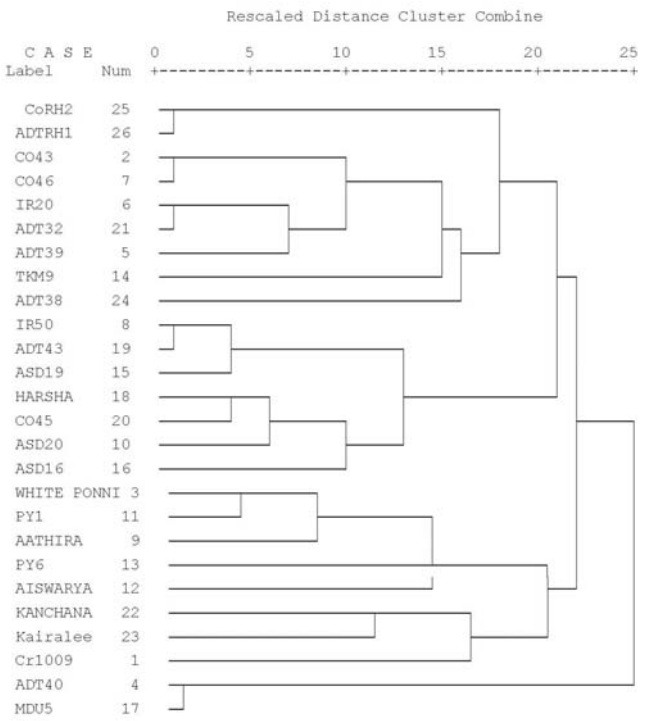

Fig. 10 Dendrogram of 26 rice varieties based on RAPD analysis constructed based on Jaccard Similarity index.

insertions that affect the relative position of the primer binding site and thus the size of the DNA fragments amplified. RAPD has several advantages over other fingerprinting techniques. It is fast, simple and requires no radioactive handling facilities or DNA sequence information (Mailer *et al*. 1994: Mailer and May 1999). RAPD detects more polymorphism than isozymes because of the availability of higher number of primers compared to only 20 or so enzyme systems (McDonald and Drake 1990). However, RAPD technique is often critized for its low reproducibility and sensitivity to reaction conditions. Further, the greater discriminatory power of the technique can reveal variations even between individuals of the same variety as a whole (Dulson *et al*. 1998; Sharma and Jana 2002; Rout and Chrungoo 2007). The problems associated with low reproducibility of RAPD profiles can be reduced by standardization of reaction conditions and keeping them constant after standardization. Further, only reproducible bands should be considered for analysis in order to obtain reliable date. In this study bulked DNA preparations were used for RAPD analysis as assaying bulk samples for cultivar identification (Golembiewski *et al*. 1997). It would also be more appropriate to use bulk DNA to overcome the problems of heterozygosity of cultivars with respect to molecular markers. It was observed that in some of the cases polymorphic fragments were not repeatable between amplification with two independent DNA bulks. Dulson *et al*. (1998), have suggested that such fragments are likely to be present in insufficient number of individuals to be amplified from two independent bulk DNA preparations should be included in the analysis. They have recommended the inclusion of 20 individuals per bulked DNA sample and assessment of two bulked samples per cultivar to be prudent to ensure reproducible results. In the present study similar approach was followed except that the number of individuals per bulked sample was increased from 20 to 40 in the cases where polymorphic fragments were not repeatable, only those bands that were consistent (**Fig. 9**) for both the bulked samples have been scored. It was possible to differentiate all the 26 rice cultivars using amplification profiles produced by 10 random primers.

'ADTRH 1' and 'CORH 2' formed one sub-cluster (**Fig. 10**). This may be due to one of the parent (i.e.) male parent 'IR 58025 A' is common for both the hybrid. Similarly, the

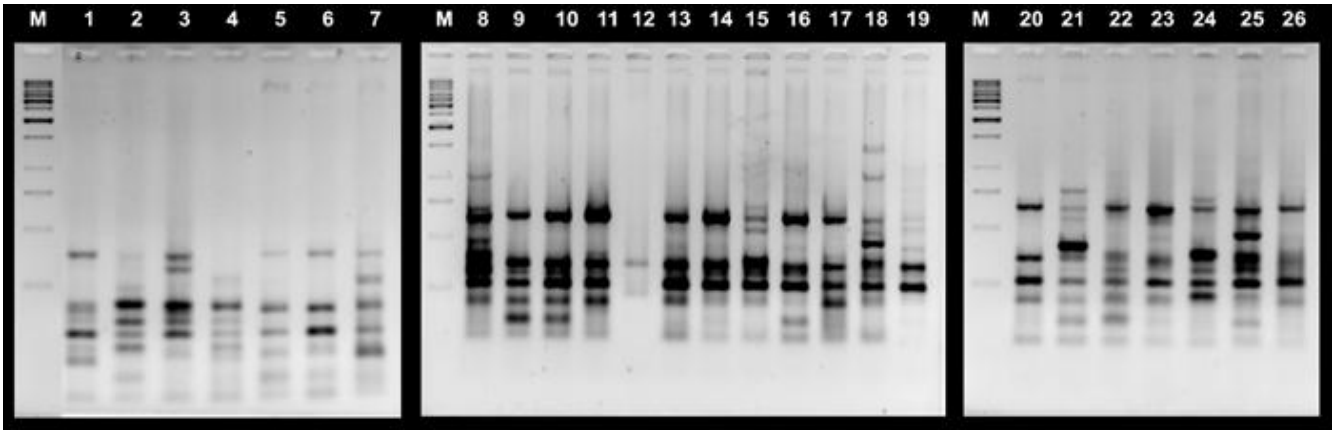

Fig. 9 RAPD profile of genomic DNA extracted from seeds of different cultivars of rice. Lane 1. 'White ponni' 2. 'Aathira' 3. 'Aiswarya' 4. 'ADT40' 5. 'ADT39' 6. 'ADT43' 7. 'IR50' 8. 'ASD16' 9. 'CO45' 10. 'ADT37' 11. 'IR20' 12. 'CR1009' 13. 'ADT38' 14. 'CO46' 15. 'ASD19' 16. 'PY1' 17. 'ASD20' 18. 'Kairalee' 19. 'MDU5' 20. 'CO43' 21. 'Kanchana' 22. 'CORH2' 23. 'TKMM9' 24. 'PY6' 25. 'ADTRH1' 26. 'Harsha'.

cultivars like 'CO 43', 'CO 46', 'IR 20', 'ADT 37' and 'ADT 39' formed one fourth level as major sub-clusters. This may also be due to the fact that in all the above said cultivars one of the parent is 'IR 20'. Within the fourth level sub-cluster cultivars, the cultivars like 'IR 20' and 'ADT 37' showed very close relationship when compared to other cultivars like 'CO 43', 'CO 46' and 'ADT 39'. This may be other parent contribution over the 'CO 43', 'CO 46' and 'ADT 39'.

This study demonstrates the applicability of RAPD technique for finger printing of rice cultivars. The RAPD technique could be useful for the identification of even closely related rice genotypes provided bulk DNA preparations are used and only reproducible markers are scored.

REFERENCES

Agrawal RL (1980) *Seed Technology*, Oxford and IBH publishing company, New Delhi, India, 829 pp

Agarwal PK, Singh D, Dadlani M (1988) Identification of cotton hybrid seeds using PAGE. *Seed Science and Technology* **16**, 563-569

Ainsworth CC, Sharp PJ (1989) The potential role of DNA probes in plant variety identification. *Plant Varieties and Seeds* **2**, 27-34

Ashwani Kumar RK, Chowdhury, Kapoor RL (1993) Varietal identification in pearl millet through morphological characters. *Seed Research* **21 (1)**, 52-54

Cooke RJ (1984) The characterization and identification of crop cultivars by electrophoresis. *Electrophoresis* **5**, 59-72

Dadlani M, Varier A (1993) Electrophoresis for variety identification, Technical Bulletin, IARI, New Delhi, 18 pp

Downey RK (1988) The plant patent dilemma, 25, 1987/88 Annual report. Canadian Seed Growers Association, Ottawa, 42 pp

Faccioli P, Terzi V, Monetti A, Nicola J, Pecchioni N (1995) β-hordein STS markers for barley genotypes identification: comparison with RFLPS, hordein A-PAGE and morphophysiological traits. *Seed Science and Technology* **23**, 415-427

Ferguson JM, Grabe DF (1986) Identification of cultivars of perennial rye grass by SDS-PAGE of seed proteins. *Crop Science* **26**, 169-174

Glaszmann JC, Lacroix TL, Feldmann P (1983) Isoenzyme electrophoresis a research tool for rice varietal improvement. *News Letters in Comm Rome* **82 (2)**, 40-47

Golembiewski RC, Dannerberger TK, Sweeney PM (1997) Potential markers for use in the identification of creeping bentgrass cultivars. *Crop Science* **37**, 212-214

IBPGR-IRRI Rice Advisory Committee (1980) Descriptors for rice (*Oryza sativa* L.). IRRI, Los Baños, Philippines, 350 pp

International Seed Testing Association (1996) International rules for seed testing verification of species and cultivars. *Seed Science and Technology* **24 (Suppl.)**, 253-270

Kapse SS, Nerkar YS (1985) Polyacrylamide gel electrophoresis of soluble seed proteins in relation to cultivar identification in cotton. *Seed Science and Technology* **13**, 847-852

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head bacteriophage T4. *Nature* **227**, 680-685

Lorenzetti F, Falcinelli M (1987) Ricerca genetica e attivita sementiera: piante foraggere. *Agricoltura delle Venezie* **41**, 211-220

Mailer RJ, Scarth R, Fristensky B (1994) Discrimination among cultivars of rapeseed using DNA polymorphisms amplified from arbitrary primers. *Theoretical and Applied Genetics* **87**, 697-704

McDonald MB, Drake DM (1990) An evaluation of a rapid and automated electrophoresis system for varietal identification of seeds. *Seed Science and Technology* **18**, 89-96

Murray MG, Thompson WF (1980) Rapid isolation of high molecular weight plant DNA. *Nucleic Acid Research* **8**, 4321-4325

Nerkar YS, Rao TN (1993) Use of seed protein and enzyme polymorphism in the identification of cultivars of cotton. *Seed Research* **1**, 375-394

Rao TN, Nerkar, Patil VD (1990) Identification of cultivars of cotton by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of soluble seed proteins. *Plant Varieties and Seeds* **3**, 7-13

Reddy MM, Gasber EO (1971) Genetic studies of variant enzyme comparative electrophoretic studies of esterases and peroxidases for species, hybrids and amphidiploids in the genus *Nicotiana*. *Botanical Gazette* **132,** 158-166

Rohlf FJ (1993) NTSYS-PC: Numerical taxonomy and multivariate analysis system.Version 2.1. Exeter Publishing, Setauket NY

Rosta K (1975) Variety determination in rice. *Seed Science and Technology* **3**, 161-168

Rout A, Chrungoo NK (2007) Genetic variation and species relationships in Himalayan buckwheats as revealed by SDS PAGE of endosperm proteins extracted from single seeds and RAPD based DNA fingerprints. *Genetic Resources and Crop Evolution* **54**, 767-777

Rutz HW (1990) Seed certification in the federal republic of Germany. *Plant Varieties and Seeds* **3**, 157-163

Sahoo L, Dadlani M, Singh DP, Sharma SP (2000) Characterization of sunflower (*Helianthus annus* L.) genotypes using laboratory techniques. *Plant Varieties and Seeds* **13**, 31-43

Scandalions JG (1974) Isozymes in development and differentiation. *Annual Review of Plant Physiology* **25**, 225-228

Sivasubramanian S, Ramakrishnan V (1978) Identification of rice varieties by laboratory techniques. *Seed Research* **6 (1)**, 71-76

Sharma TR, Jana S (2002) Species relationships in *Fagopyrum* revealed by PCR-based DNA fingerprinting. *Theoretical and Applied Genetics* **105**, 306-312

Smith JSC, Ertl DS, Orman BA (1995) Identification of maize varieties. In: Wringley CW (Ed) *Identification of Food-grain Varieties*, American Society of Cereal Chemists, USA, 238 pp

UPOV (1994) Guidelines for the conduct of tests for distinctness, uniformity and stability: wheat (*Triticum aestivum* L.). International Union for Protection of New Varieties of Plants, Geneva, pp 37-43

Vanangamudi K, Palanisamy V, Natesan P, Karivaratharaju TV (1988) Variety determination in rice – Examination of the hulled grain. *Seed Science and Technology* **16**, 41-46

Varier A, Dodalani M, Cooke RJ (1992) Identification of sunflower hybrids and inbreds by SDS – PAGE of seed proteins. *Seed Research* **20 (2)**, 138-141

Williams JCK, Rubelik AR, Livak KJ, Rafalski A, Tingey SV (1990) DNA polymorphism amplified by arbitrary primer are useful as genetic markers. *Nucleic Acid Research* **18**, 6531-6535