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Varietal identification in rice (*Oryza sativa*) through chemical tests and gel electrophoresis of soluble seed proteins

P S RAO [1], M BHARATHI [2], K BAYYAPU REDDY [3], K KESHAVULU[4], L V SUBBA RAO[5] and C N NEERAJA[6]

Seed Research and Technology Centre, Acharya N G Ranga Agricultural University, Hyderabad 500 030

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ABSTRACT

Variety identification has great significance from seed production, breeding as well as intellectual property rights point of view to ensure quality seed. Forty varieties of rice were identified on the basis of seed colour (phenol, modified phenol and NaOH), seedling response (GA_3 and 2, 4-D) to chemical tests and electrophoresis of soluble seed proteins (SDS-PAGE). Though no individual chemical test was able to distinguish all the genotypes, different chemical tests in conjunction were useful in identification of varieties. The SDS-PAGE of seed proteins was able to identify all the 40 genotypes of different origin and can be employed effectively for identification of these rice varieties. Dendrogram of the data indicated the wide diversity of the varieties released from Andhra Pradesh with similarity ranging from 0.36 to 0.98. Based on the response of 40 rice varieties to biochemical techniques and total soluble protein profiles through SDS-PAGE, a comprehensive 'seed key' has been developed for rapid varietal identification.

Keywords: Electrophoresis, Protein bands, Relative mobility, Rice, Seed key

Rice (*Oryza sativa* L.) is one of the most important crops in the world and is consumed by more than half the world population. About 20% of the total calorie supply worldwide comes from rice and especially in Asia; more than 2 billion people derive 60–70% of their daily energy requirement. India is one of the countries that took full advantage of the plant type based high-yielding varieties of rice since their introduction in the mid-sixties. Spectacular production growth initially through combined growth of productivity and area and later largely through productivity enabled the country to attain self-sufficiency by the early eighties and sustain the same since then (Siddiq 2000). Several varieties have been developed or in the process of development catering to the different needs of various rice ecologies. Now, the identification of these rice cultivars and lines and determination of their genetic relations is very essential for plant improvement programmes, authentic seed production and protection of farmers' and breeders' rights (Patra and Chawla 2010). Establishing the identity of a variety through registration is critical from the point of Plant Variety Protection (PVP) as well as seed multiplication and subsequent handling. According to Protection of Plant Varieties and Farmer's Rights Act 2001 (PPV&FR) of India, the varieties need to be characterized in detail for establishing their distinctness, uniformity and stability (DUS) before they are introduced in seed multiplication chain (www.plantauthortiy.gov.in).

The most practised approach to varietal identification involves the study of morphological characteristics through grow out test (GOT). For rice (*Oryza sativa* L.), 62 morpho-physiological characteristics have been described under DUS guidelines in India (Anonymous 2007). Though morphological traits are simple and irreplaceable, these descriptors suffer from many drawbacks, such as influence of environment on trait expression, epistatic interaction and pleiotropic effects (Pragya *et al*. 2010). Furthermore, paucity of sufficient number of stable morphological markers for unequivocal identification of increasing number of reference collection of varieties enforces to look for alternatives. Chemical tests are quick, easy and reproducible and adopted in several crops (Biradar Patil *et al*. 2008). Several biochemical techniques were proposed in rice for varietal identification, viz phenol colour reaction (Varier *et al*. 1995, Kumar *et al*. 2005), modified phenol reaction (Kumar *et al*.1995), ferrous sulphate colour reaction, potassium hydroxide reaction and seedling growth response to hormones such as GA_3 and 2, 4- D etc. (Chakrabarthy and Agrawal

[1,2]Associate Professor (e mail: sampalrao@gmail.com, bharathi_koram@yahoo.com) [3]Scientist (e mail: drkaipu2@yahoo.co.in), [4]Associate Professor and Head (e mail: keshava_1972 @yahoo.com);

[5,6]Principal Scientist (e mail: lvsubbarao1990@gmail.com, cnneeraja@gmail.com), Directorate of Rice Research

1990, Biradar Patil *et al*. 2008). One of the biochemical methods more extensively used for taxonomic purposes has been the electrophoretic analysis of the proteins found in seeds and storage organs (Ladizinsky and Hymowitz 1979), electrophoresis analysis is also used to study molecular systematic for identification of genotypes based on proteins and this technique of Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) is commonly used for separation of seed storage proteins (Ullah *et al*. 2010). There have been a substantial number of studies that have used SDS-PAGE to profile seed proteins in rice (Netra *et al*. 2007, Pervaiz *et al*. 2011); wheat; sorghum; safflower; groundnut; soybean; brassica; castor; mustard and tomato and it has been reported that SDS-PAGE profiles are polymorphic and environmental influence on their pattern is limited.

In 1986, ISTA adopted a standard reference method of PAGE for identification of varieties of wheat and barley into its international rules, involving separation of gliadin from wheat and hordein from barley (Cooper 1987). UPOV has recommended SDS-PAGE for analysis of high molecular weight glutenins in wheat (Anonymous 1994a) and hordeins in barley (Anonymous 1994b). Though for rice, molecular markers like SSRs and SNPs are now contemplated for profiling of the varieties; SDS-PAGE profiling is relatively simple, inexpensive, does not need elaborate laboratory equipment or other additional paraphernalia and can be adopted by field laboratories of rice workers for varietal identification and characterization.

In India, Andhra Pradesh is one of the leading states for the production of popular high-yielding varieties of rice (Siddiq 2000). Some of them, viz BPT 5204 (Samba Mahsuri), MTU 7029 (Swarna) are considered as 'Mega varieties' and grown in the major rice tracts of Asia (Mackill 2006). In the context of plant breeder's rights, authentic seed production and rice molecular back crossing breeding programmes, precise varietal characterization is required. Though, a single test/marker is ideal for identification of each rice variety/ genotype, in practice a battery of tests/markers are needed for varietal identification in rice (Pervaiz *et al*. 2011). Therefore, the present study aims at development of seed keys for 40 popular varieties released by Andhra Pradesh using biochemical techniques for seed colour and seedling response and SDS-PAGE profiles of seed proteins.

MATERIALS AND METHODS

Genetically pure seeds of 40 rice varieties, which are in active seed production chain released by Acharya N G Ranga Agricultural University (ANGRAU), Hyderabad, Andhra Pradesh were obtained from the respective breeders of the research stations of ANGRAU (Table 1). The experiment was conducted during 2007–08 at Seed Research and Technology Centre, ANGRAU, Hyderabad. For these forty varieties, a variety specific seed key was developed according to their response to biochemical tests and total soluble seed proteins through SDS-PAGE.

Phenol test

Four replications of hundred seeds each, from each cultivar were soaked in distilled water for 18 hr at room temperature (20 ± 2 ° C). The seeds were then placed in Petri-plates containing filter paper moistened with 5 ml of 1% (v/v) phenol solution and incubated at room temperature for 24 hr. The change in colour of the seed coat in response to phenol reaction was evaluated. The genotypes were categorized into dark brown, light brown and no reaction groups based on the change in seed coat colour (ISTA 1996 and Kumar *et al*. 2005).

Modified phenol test

A similar procedure as phenol test was applied, except in this case, seeds were soaked in 0.5% copper sulphate ($CuSO_4$) for 18 hr instead of distilled water. After 18 hr, seed coat colour was evaluated and the colour reaction was classified as black, dark brown, light brown and no reaction (Kumar *et al*. 1995).

Sodium hydroxide test: Four replications of 50 seeds each from each cultivar were soaked in 5 ml of 2% (v/v) NaOH solution and kept at room temperature for 1 hr. After incubation, the change in colour of the solution and seeds was noted. Based on the intensity of colour reaction the cultivars were grouped into yellow and light yellow.

Gibberellic Acid (G A_3) test: The effect of GA_3 was studied using four hundred randomly selected seeds (100 seeds in each of four replications) placed on two layers of germination paper towels of 24 cm × 14 cm moistened with GA_3 (25 ppm) solution sufficiently apart at the middle of the paper towel. Subsequently, they were covered with another sheet of moistened paper towel and rolled along with untreated set as control. The rolled paper towels were then placed in vertical position in seed germinator at 25º C for seven days and coleoptile length was measured in centimeters. The coleoptile (shoot) growth response to GA_3 was determined on the basis of percentage increase over control. The varieties were classified into three categories, *viz*., high (>100%), medium (> 50- 100%) and low (< 50%) response groups (Chakrabarthy and Agrawal 1990, Biradar Patil *et al*. 2008).

2, 4 - D test: The seedlings were raised in similar manner as in case of GA_3 test, except that the germination paper towels were moistened with 2, 4-D solution (5 ppm). The coleoptile growth response to 2, 4-D was determined on the basis of percentage decrease over control. The varieties were classified into three categories based on their sensitivity, viz highly (>50%), moderately (30–50%) and least (< 30%) sensitive (Chakrabarthy and Agrawal 1990, Biradar Patil *et al*. 2008).

Table 1 Pedigree and source of origin of 40 rice genotypes

Genotype		Pedigree	Year of release	Ecosystem
MTU- HR 2002	APHR-1	IR 58025 A/Vajram	1994	Irrigated medium duration
MTU- HR 2008	APHR-2	IR62829A/MTU9992	1994	Irrigated medium duration
MTU 1001	Vijatha	MTU 5249/MTU7014	1995	Irrigated medium duration
MTU 1010	Cottondora sannalu	Krishnaveni/IR64	2000	Irrigated medium duration
MTU 2077	Krishnaveni	Sowbhagya/ARC5984	1988	Irrigated rainfed shallow land
MTU 5249	Vajram	MTU4569/ARC6650	1986	Rainfed shallow land
MTU 5293	Prathibha	Sowbhagya/ARC6650	1986	Rainfed shallow land
MTU 7029	Swarna	Vasishta/Mahsuri	1982	Rainfed shallow land
MTU 9992 –R		Selection from IR 50		Rainfed shallow land
BPT 5204	Sambamahsuri	GEB 24/TN (1)//Mahsuri	1986	Rainfed shallow land
NLR 145	Swarnamukhi	Cica 4/IR625-23-3-1//TETEP	1991	Irrigated medium duration
NLR 9672	Kothamolagolukulu 72	BulkH-9/Millek Kunning	1979	Rainfed shallow land
NLR 9674	Kothamolagolukulu 74	BulkHG9/Millek Kunning	1982	Rainfed shallow land
NLR 27999	Tikkana	RP31-49-2/BCP2	1988	Rainfed shallow land
NLR 28523	Sriranga	RP5-32/Mahsuri	1991	Rainfed shallow land
NLR 28600	Simhapuri	RP5-32/BulkH-9	1991	Rainfed shallow land
NLR 30491	Bhavani	IR36/TET2508	1997	Irrigated medium duration
NLR 33057	Swathi	IR 36/MTU 4569	1996	Irrigated medium duration
NLR 33359	Sravani	Selection from IR 50	1996	Irrigated short duration
NLR 33365	Penna	NLR9672/IR36	1997	Rainfed shallow land
NLR 33641	Vedagiri	NLR 9692-96/IET 7230	1999	Irrigated long duration
RPW 6-17	Phalguna	IR8/Siam29	1997	Rainfed shallow land
WGL 3825	Kesava	W9L28712/IR36-1996	1997	Rainfed shallow land
WGL 3943	Shiva	Phalguna/IR50	1997	Rainfed shallow land
WGL 20471	Erramallelu	BC5-55/WR708	1991	Irrigated early duration
WGL 22245	Pothana	IR529/WGL12708	1988	Irrigated early duration
WGL 44645	Divya	WGL3022/Surekha	1989	Irrigated medium duration
WGL 47970	Orugallu	Obs677/IR2070-423-2-5	1993	Irrigated rainfed shallow land
WGL 48684	Kavya	WGL27120/WGL7672/Mahsuri/Surekha	1991	Irrigated medium duration
Mahsuri		Taichung 65/Mayang Ebo 680/ Mayang Ebo 680	1972	Irrigated rainfed shallow land
RNR 1239	Rajendra	IJ 52/T(N)I	1976	Rainfed upland
RNR M-7	Early Samba	Mutant of BPT5204	2000	Irrigated medium duration
RNR 1446	Satya	Tellahamsa/Rasi	1987	Irrigated early duration
RNR 18883	Sumathi	Chandan/Pusa basmati	2002	Irrigated rainfed shallow land
RNR 4044				Irrigated medium duration
RNR 99377	Rajavadlu	Rajendra/SR 30	1993	Irrigated late duration
RNR 10754	Tellahamsa	HR12/T(N)1	1971	Irrigated early duration
JGL 384	Polasaprabha	BPT5204/Kavya	2002	Irrigated medium duration
JGL 1798	Jagtial Sannalu	BPT5204/Kavya	2002	Irrigated early duration
RDR 763	Indur Samba	BPT5204/Surekha	1997	Rainfed shallow land

SDS-PAGE

For the extraction of proteins for electrophoresis, seeds were ground to fine powder with mortar and pestle. Sample buffer (400µl) was added to 0.01 g of seed flour as extraction liquid and Bromophenol Blue (BPB) as tracking dye to follow the movement of protein in the gel. The proteins were extracted with the extraction buffer containing the following final concentrations: 0.5 M Tris-HCl (pH 6.8), 2.5% SDS, 10% glycerol and 5% 2- mercaptoethanol.

Seed proteins were separated by carrying out electrophoresis in the discontinuous buffer system through vertical slab type SDS-PAGE using 11.25% polyacrylamide gel according to the method of Laemmli (1970). The gels were later stained in 0.04% Coomassie Brilliant Blue in methanol, acetic acid and distilled water (45:10:45 v/v) for 8–10 followed by destaining in the same solution without Coomassie Brilliant Blue with occasional shaking till the gels became clear.

Protein extraction: For the extraction of the total soluble proteins, seeds were dehulled and powdered (100 mg) and defatted using defatting solution (chloroform, methanol and acetone in 2:1:1). Defatting was performed for 24 hr with at least three solvent changes and the ground material was air dried and transferred into 1.5 ml microfuge tubes. The powdered seed sample was extracted with 0.4 ml of sample buffer (containing 5 ml of 1M Tris- HCl *p*H 6.8, 1 ml of glycerol, 1 ml of absolute alcohol and 43 ml water) and kept overnight at room temperature. The homogenate was centrifuged at 15 000 × g for 20 min. at 4ºC and the supernatant was collected. 20 µl of SDS-β mercaptoethanol mixture, 10 µl of 0.25% bromophenol blue and one pellet of sucrose were added to 50 µl of supernatant. The samples boiled for 5 min. and cooled were used immediately for electrophoresis.

Electrophoresis

Electrophoresis was performed with a model of A E - 6290 Resolmex Slab gel unit with 16 cm × 16 cm glass plates separated by 0.75 mm spacers following modified method based on the Laemmli (1970) protocol.

The resolving gel (12%) was de-aerated and poured to a height of 11.5 cm and over layered with distilled water. After polymerization, the water was decanted and the stacking gel solution (4.5%) was poured. A 12 well comb was inserted into a depth of 1.5 cm in stacking gel. An aliquot of 20 µl of sample was loaded into each well. Electrophoresis was performed with a constant current of 30 mA (at fixed voltage) at 20ºC maintained with circulating cooling water. The electrophoresis was stopped when the tracking dye reached the bottom of the resolving gel after 150 min. Bovine Serum Albumin (BSA) of 66.2 kDa was added as molecular weight marker. Then the gels were stained with a staining solution with 0.25% (w/v) Coomassie brilliant blue R 250 in 50% (v/v) methanol and 10% (v/v) acetic acid. Gels were stained overnight followed by two rinses with distilled water and were destained in 25% (v/v) methanol, 7% (v/v) acetic acid destaining solution, until the bands were clear. The gels were preserved in 10% (v/v) acetic acid and glycerol solution. Three electrophoretic runs were performed for checking the repeatability of the protein profiles.

Gel analysis and data processing

The protein profiles were recorded by placing gel on a transilluminator and the migration values for each of the bands were measured from the point of loading.

Relative mobility (R_m) of each band was calculated as:

$$R_m = \frac{\text{Distance traveled by protein sample (cm)}}{\text{Distance traveled by tracking dye (cm)}}$$

The band intensity was assessed visually and was recorded using a five- step system, i e absence of a band, faint, light, medium and dense or heavy intensity of bands.

Scoring of bands: Protein bands were traced on to the graph by visual observation of the gels and mean Rm value calculated for each genotype and the banding patterns were measured as qualitative and quantitative variation (Wouters and Booy 2000).

Qualitative variation: When a particular band as designated by its Rm value was present in the electrophoregram of one cultivar but absent in that of another, the variation was referred to as qualitative variation.

Quantitative variation: When a particular band was observed in the electrophoregram of two or more different cultivars, but differs in band size or staining intensity.

Based on the qualitative and quantitative polymorphism of protein profiles, the similarity was calculated using the Jaccard's coefficient (S_J) as follows:

$$S_J = n_{AB}/n_A + n_B - n_{AB}$$

Where n_{AB} is the number of bands common for A and B, n_A is the total number of bands in A, n_B is the total number of bands in B. Cluster analysis was performed by means of the unweighed pair group method using arithmetic averages (UPGMA).

The correlation of all tests including phenol, modified phenol, NaOH, GA_3, 2, 4-D and total soluble proteins were compared using the Mantel matrix correspondence test (Mxcomp module of NTSYS-pc (Rohlf 1998).

RESULTS AND DISCUSSION

Varietal identification and discrimination acquires an increased importance in the context of plant breeder's rights, plant patents and registration of rice genotypes for proprietary purposes. Since rice genotypes are handled as seed most of the time, seed based analysis is more convenient for characterization rather than phenotypic characters. Seeds can be considered ideal protein sources as they represent a fixed physiological stage, being a conservative unit in terms of protein composition and little affected by environmental variations (Ladizinsky and Hymowitz 1979). In the present study, seed keys were developed for forty genotypes of rice varieties released by Andhra Pradesh based on chemical tests of seed colour; seedling response and SDS-PAGE profiles of seed proteins.

Seed colour tests

Out of forty varieties subjected to phenol test, 18 varieties showed dark brown, 16 varieties showed light brown colour and six varieties showed no reaction. Black colour was not observed in any varieties. Phenol color reaction which is an index of polyphenol oxidase activity has been reported to be associated with intravarietal diversity and have been also be used in ascertaining the varietal purity (Chauhan and Nanda 1984). Under modified phenol test, one variety MTU 7029 showed black colour reaction, while 18 varieties showed dark brown, 15 varieties showed light brown reaction and six varieties showed no reaction. MTU 7029 with its japonica background showed black colour reaction to modified phenol test, which is typical of japonica type. For both tests, five varieties showed no reaction. Phenol reaction being highly specific and stable provided a good index for distinguishing rice varieties. The reaction involves melanin formation by oxidizing phenol via orthoquinones and hydroxyquinones. As it is monogenically controlled response, localized in seed coat, it was considered an important diagnostic character for identifying rice varieties in the present study (Jaiswal and Agrawal 1995). Based on colour development in colourless solution of 2% NaOH, the genotypes were classified into two groups. Seven varieties turned the NaOH solution to dark yellow and 33 varieties to light yellow. Though individually the responses to biochemical techniques are of limited value, when these three techniques are used in conjunction with each other, almost any number of rice varieties could be distinguished from each other as confirming the observations of the earlier studies.

Seedling tests

Response of coleoptile length to GA_3 @ 25 ppm was determined on the basis of per cent increase over control. Out of 40 varieties, five varieties had high response (>100%), 30 had moderate response (> 50–100%) and five varieties showed low response (< 50%) to GA_3. The coleoptile growth response to 2,4-D @ 5 ppm was determined on the basis of per cent decrease over control, out of forty varieties four showed high sensitivity values (>50%), 30 showed moderate sensitivity (30–50%) and six varieties showed low sensitivity to 2, 4-D (< 30%). The study of seedling characteristics with their response to GA_3 and 2, 4-D suggested differential response as reported and thus could distinguish the varieties of the present study (Gupta and Agarwal 1988, Nethra *et al*. 2007).

Protein profiles with SDS-PAGE

A total of 47 bands were observed in 40 varieties of the present study. The total number of bands present in different genotypes ranged from 9 (RNR M 7) to 28 (NLR 9674 and NLR 30491). The banding pattern of these forty varieties was uniform and was not affected by the repeated electrophoretic runs. Though no unique band was observed specific for a variety, all the varieties studied exhibited unique banding patterns (Fig 1). Four bands with Rm 0.08, 0.33, 0.36 and 0.54 values were observed in most of the varieties. The differences in banding patterns were either with total number of bands present, location of bands and intensity of bands or it can even be the presence or absence of four categories of bands namely dense, medium, light, and faint. The overall differential banding pattern of seed proteins indicated qualitative and quantitative variations among the different varieties. These observations suggested that with electrophoretic differences in protein banding pattern of different varieties, specific varieties were identified with the presence or absence of a specific position of band and also the intensity of band, which could be used as genetic marker. Based on the qualitative and quantitative protein banding patterns, a finger print profile unique to each variety was generated for all the 40 varieties. Based on the number of the genotypes involved in the reported studies in rice, the total protein bands using SDS-PAGE ranged from a minimum of 12 to a maximum of 50 (Aliaga-Morell *et al.* 1987, Patra and Chawla 2010, Pervaiz *et al.* 2011, Galani *et al*. 2011).This

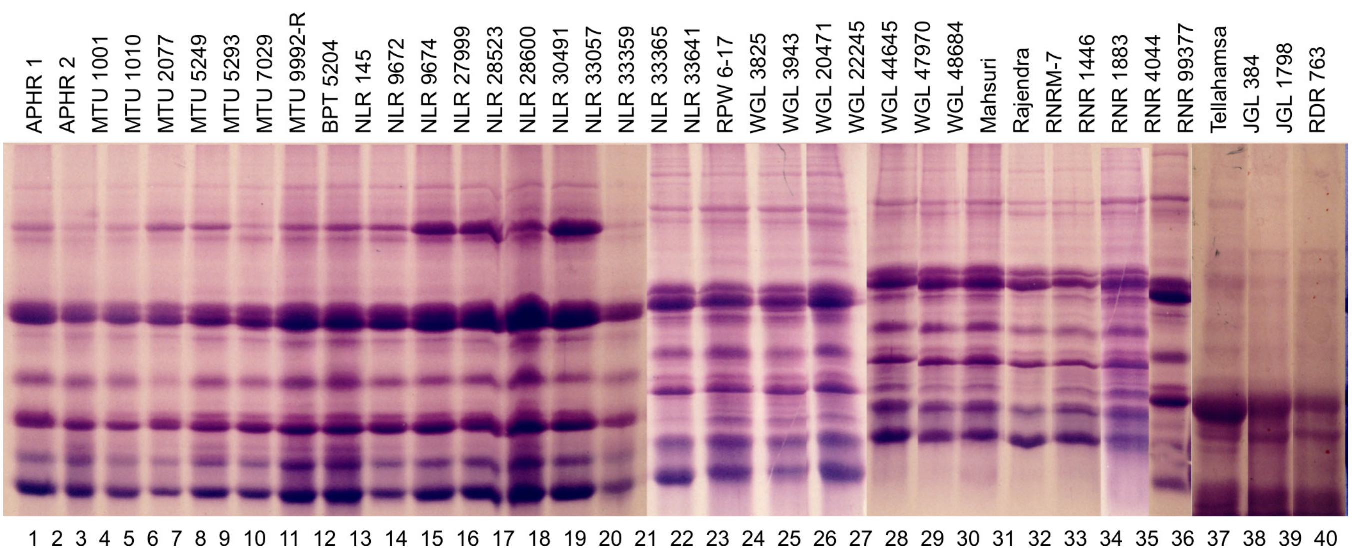

Fig 1 SDS-PAGE profile (banding patterns) of soluble seed proteins in rice varieties

variation in number of polypeptide subunits may be due to gel percentage, preference of major subunits of evaluation and size of the gel. Various groups have confirmed rice varietal identification by SDS-PAGE (Nethra *et al*. 2007, Inamulla *et al*. 2010, Ullah *et al*. 2010).

Genetic similarity

Dendrogram derived from protein profiles separated all the varieties within similarity range of 0.36 to 0.98 (Fig 2). Two major clusters were found with subsequent sub-clustering. No significant association was found between the clustering and the pedigree of the varieties. The similarity values (0.36–0.98) derived from the soluble protein patterns indicated the wide diversity of the rice varieties released from ANGRAU. The genetic similarity indices of rice SDS-PAGE protein profiles ranged widely from a minimum of 0.20 to a maximum of 1.0 based on the genotypes used in the studies (Galani *et al*. 2011).

Correlation between the tests

No significant correlation was found between the tests using the Mantel matrix correspondence test clearly indicating the utility of each technique for varietal characterization used in the present study.

Seed key

Based on the response of 40 rice varieties to biochemical techniques and total soluble protein profiles through SDS-PAGE, a comprehensive key has been developed for all of them for rapid varietal identification (Fig 3). Only those descriptors/characters earlier reported for their high stability and least influence by environment, viz seed colour tests and seedling response tests were considered in the present study for the development of the seed keys along with SDS-PAGE protein profiles (Patra and Chawla 2010).The biochemical techniques can group the varieties to a maximum of four groups, thus if all the five techniques (5^4) were used in conjunction, a set of 625 samples can be analyzed. But the subjectivity of assessment of the colour reaction should be considered. However, SDS-PAGE has high discriminatory power and efficiency over the other biochemical techniques owing to multiple bands per assay. Early studies involving physico-chemical characters of the grain and electrophoretic variants of salt soluble seed proteins for varietal identification of 67 Breeder's seed samples released from various rice stations showed clearly that no single test can be used as 'spot testing' for a rice variety and suggested a combination of tests As observed from the seed keys developed from each variety in the present study, SDS-PAGE could differentiate even genetically close varieties. Seed keys for 29 varieties were developed by morphological characteristics of the grain, seed color tests and seedling response to GA_3 (Gupta and Agrawal 1988).

The commercial and agronomic necessity to precisely

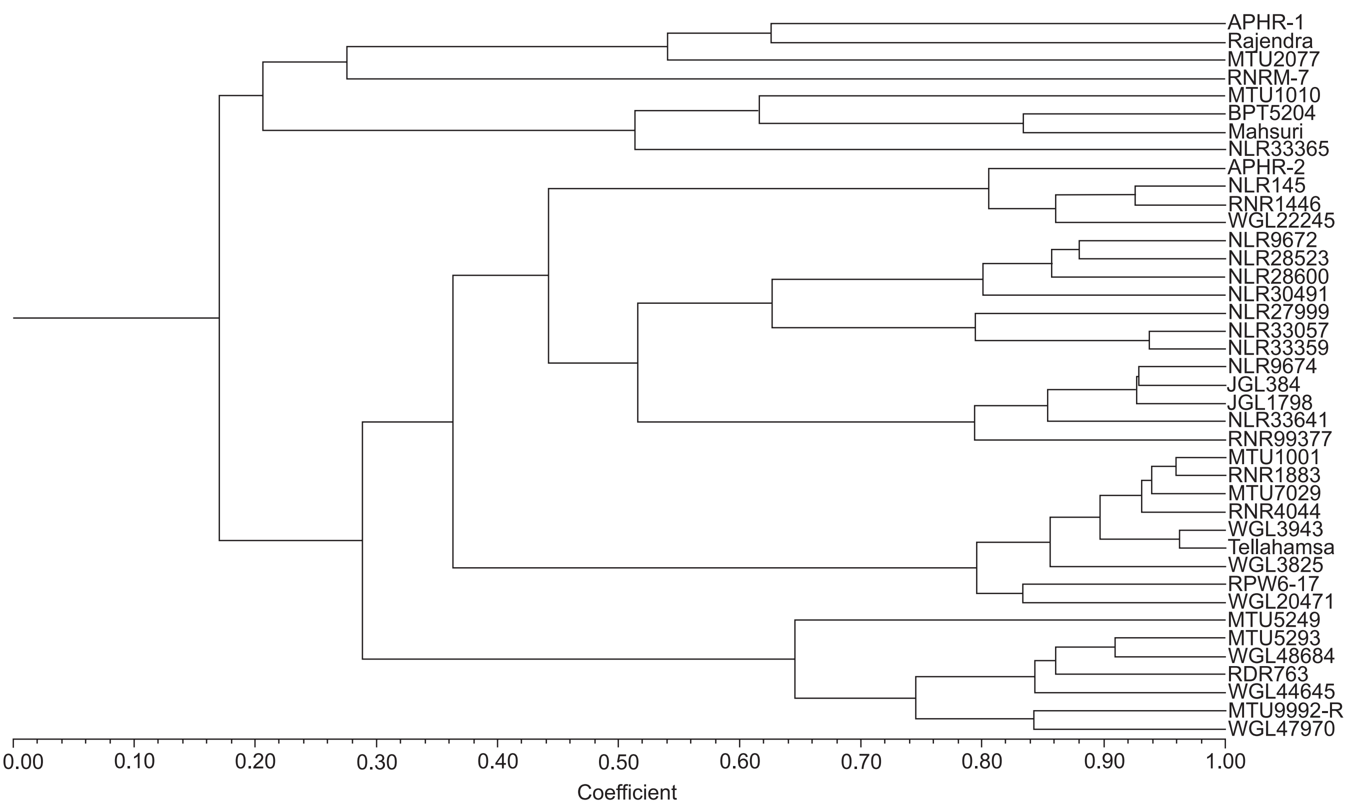

Fig 2 Dendrogram of rice varieties generated using SDS-PAGE of soluble seed proteins

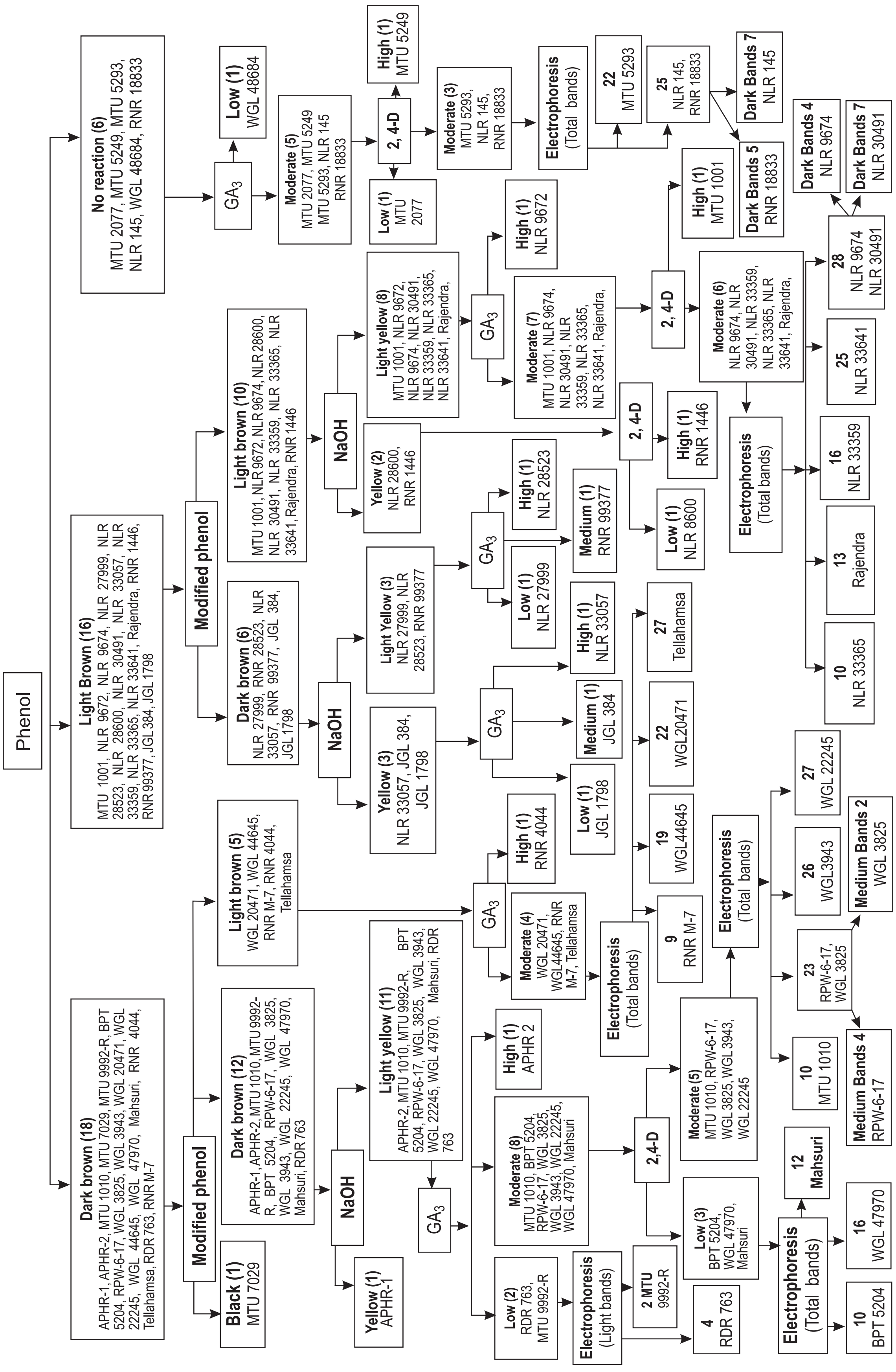

Fig 3 Schematic diagram based on chemical tests and electrophoretic banding pattern for identification of rice genotypes

define cultivated rice varieties is hampered by the great morphological similarity caused by the strong genetic relationships between varieties. DUS descriptors and grouping characteristics mentioned in DUS guidelines are not adequate to distinguish rice genotypes efficiently. Moreover, seed based techniques for varieties are warranted for rapid identification from seed lots. Therefore in the present study, a strategy of using seed based chemicals tests and SDS-PAGE protein profiles for development of seed keys for rice varieties was demonstrated in forty varieties released from Andhra Pradesh.

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