Indian Journal of Plant Sciences ISSN: 2319–3824(Online)
An Open Access, Online International Journal Available at http://www.cibtech.org/jps.htm
2017 Vol.6 (1) January-March, pp.39-47/Bhat and Riar
Research Article

CHARACTERIZING THE TRADITIONAL RICE *(ORYZA SATIVA* L.*)* CULTIVARS ON THE BASIS OF SEED MORPHOLOGY AND PROTEIN CHARACTERISTICS

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ABSTRACT
Assessment of variability and genetic diversity of traditional rice cultivars of Kashmir (J&K) was carried out by analysing their morphological and protein specific characteristics. Most of the cultivars were found to have white belly type of chalkiness, with white spots occurring in the middle part of the caryopsis on the ventral side. The total protein content varied from 7.24% (*Mushki kandi*) to 8.85% (*Teli zag*) whereas the protein fractions, albumin varied from, 4.64% to 5.38%; glutelin, 81.52 to 82.82%; globulins; 7.51 to 10.82% and prolamins, 5.13 to 7.87%. The bands found in these cultivars were monomorphic and the size of the polypeptides bands as determined by SDS-PAGE was found ranging from 100 to 120 kDa. The number of scorable bands generated by the cultivars varied from 4 to 7 with an average number of bands produced by the cultivar equal to 5. The bands with RF values of 0.248, 0.291 and 0.344 were found present in red coloured *Zag* and sparsely red pigmented *Gull zag* and *Teli zag* rice cultivars, which accounted to the similarity of such cultivars in certain traits. The dendrogram obtained from the data of SDS-PAGE of proteins by using un-weighted pair group method with arithmetic averages (UPGMA) separated the analysed rice cultivars into three clusters containing 14%, 43% and 43% of the cultivars.

Keywords**:** *Rice Cultivars, SDS-PAGE, RF Values, Dendrogram; Genetic Diversity*

INTRODUCTION
Rice *(Oryza sativa L.)*, ranking second, is one of most important cereal crop grown throughout the world and is a major source of nutrition for about 2.5 billion inhabitants. India has the second largest area under rice cultivation and is also the second largest producer of rice after China (Patel *et al.,* 2014). Rice constitutes about 28-54% proteins in the Asian diet, 20% of overall nutritional protein and 27% of the global nutritional energy (Bashir *et al.,* 2007). Rice is also an essential crop that can store and synthesize both main classes of proteins including glutelins and prolamins in its cellular organelles (Muench *et al.,* 1998). Rice protein possess some of the important characteristics including colourlessness, bland taste and can be used as an alternative to other cereal and legume proteins due to its unique nutritional and hypoallergenic properties (Agboola *et al.,* 2005).
In India, rice is the staple food of the people of Jammu and Kashmir and the area under rice cultivation in the Kashmir valley is about 75%. The state is rich in traditional rice cultivars having excellent properties like different colours, aroma and varied dimensional characteristics. The pigmented and aromatic cultivars have been given major focus in recent years due to their excellent nutritional and health benefits (Bhat and Riar, 2015). The selection of particular rice cultivar is a major concern for breeders in order to maintain the desired traits in hybrid varieties. The traditional cultivars being a source of valuable genes can be used successfully for the crop improvement and breeding programs for development of hybrid varieties that posses not only the desired traits present in these cultivars but have higher production capacity together with resistance to varied biotic and abiotic stresses (Masood *et al.,* 2005).
The identification of cultivars with varying intensities in pigmentation or aroma producing capacity is an effective provision for plant breeders, plant protectionists and traders. Among the various methods of cultivar identification, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is an important tool to characterise the rice cultivars (Akbar *et al.,* 2012). The proteins in rice are highly polymorphic, stable and are not influenced by climatic and environmental condition, so the determination

of genetic diversity by SDS-PAGE analysis of seed storage proteins is considered a reliable method as compared to seed and plant morphological characteristics (Zada *et al.*, 2013). The SDS-PAGE analysis of seed proteins is an important technique for assessing the genetic variability among crop germplasm within a short period of time (Netra and Prasad, 2007). The polymorphisms of seed storage proteins determined by SDS-PAGE analysis have been successfully applied in the identification and characterization of several varieties of different crops (Song *et al.*, 1996) and in determining the purity of rice varieties (Anitalakshmi *et al.*, 2014). The present research was therefore, planned to access genetic diversity in seven traditional rice cultivars of Kashmir, since no study has been reported using SDS-PAGE analysis of seed storage proteins of these cultivars.

MATERIALS AND METHODS

Raw Materials Preparation

The seven traditional rice *(Oryza sativa L.)* cultivars including two aromatic (*Mushki budgi* and *Mushki kandi*) and 5-pigmented cultivars (*Teli zag, Zag, Gull zag, Kamad, Kaw quder*) were procured from different cultivating regions and breeding centres of Kashmir (India). Seeds were dried to moisture content of 12 to 12% in a lab drier (Agrosa India Pvt. Ltd.) to ensure optimum yield of head rice. The cleaned paddy grains were subjected to milling using lab rubber de-husker (Agrosa India, Pvt. Ltd). The brown rice samples as required were milled in a pilot scale grinding mill (Agrosa India, Pvt. Ltd.), passed through 60 mesh sieve to get the uniform fine flour. The rice flour was stored at 4^{o}C in air tight containers for further analysis. All the experiments were conducted in triplicate, unless otherwise stated.

Grain Morphological Characteristics

The morphological parameters used in characterizing the cultivars included, pericarp colour, chalkiness position of endosperm and the aroma producing ability.

Chalkiness position of kernels was determined by visualising the milled rice kernels under a stereo-zoom microscope to find the position of opaque portion of starchy endosperm relative to translucent area of the rice kernels and was classified as white belly, white centre and white back based on the orientation of chalkiness in the rice grains. The shape of milled rice kernels were characterised on the basis of their length to width ratio as slender (over 3), medium (2.1-3.0), bold (1.1-2.0) and round (1.0 or less).

Protein Extraction

The whole milled rice grains flour and the proteins from different rice cultivars were isolated by 0.055 M Tris-HCl buffer (pH 6.8) containing 2.3% SDS, 10% Glycerol and 5% β-mercaptoethanol. To 3 mg of the rice flour, 100 µl of prepared extraction buffer was added followed by boiling for 10 min. The homogenate was kept for 4-5 h at room temperature and then centrifuged at 14,000 rpm for 12 min. The protein was collected as a clear supernatant and then stored at 4^{o}C till gel electrophoresis.

Isolation of Protein Fractions

The protein fractions from the rice flour samples were extracted by following the procedure of Turley and Ching (1986). To extract albumin, 500 µl of 10 mmol L+1 Tris+HCl solution at pH 7.5 and 1 mmol L+1 EDTA was added to 100 mg of the rice flours followed by vigorous shaking for one hour. The solutions were then centrifuged at 13,000 rpm for 15 min. The supernatants containing albumin were collected and precipitated with 1.5 ml of cold acetone and stored in a freezer for 12 hr. The albumin samples containing acetone were then centrifuged for 15 min and the acetone as supernatants were discarded to recover the pure albumin fraction.

For the globulin extraction, 500 µl of 10 mmol L+1 Tris+HCl (pH 7.5), 1 mmol L+1 EDTA, and 0.5 mol L+1 NaCl were added to 100 mg of the rice flour followed by shaking and centrifugation at 13,000 rpm for 15 min. The supernatants containing globulin were collected and precipitated with acetone. The solutions were homogenized and stored overnight in a freezer followed by centrifugation so as to discard the acetone in order to obtain pure globulin fraction.

For the prolamin extraction, 500 µl of isopropanol 60% (v/v) was added to the rice flour followed by shaking and centrifugation in order to collect the supernatant containing prolamin. The prolamins were precipitated with acetone, homogenized and stored overnight in a freezer. The homogenised solutions

were centrifuged after thawing to obtain the pure form of prolamin by discarding of acetone as a supernatant.

For glutelin extraction, 700 µl of extraction buffer was added to the flour and shaked vigorously for two hours followed by centrifugation for 15 min. The glutelin in supernatant was collected and precipitated with 1.5 ml of acetone followed by storage in a freezer after homogenisation for 12h. The remaining solution was centrifuged to obtain the purest glutelin pellets after the draining of acetone in the supernatant. The extraction of each fraction was repeated thrice in order to obtain the highest yield.

SDS-PAGE Analysis: SDS-PAGE of the extracted protein was carried out on 20% polyacrylamide slab gel under reducing conditions by following the method of Laemmli (1970). 50 µ of protein was loaded along with protein marker on 1.5 mm thick 20% acrylamide gel containing 0.135% by weight of N,N-methyleneacrylamide in 50 mM Tris-HCl buffer (pH 6.8), 0.27% SDS and 20% by weight acrylamide. The gel was polymerized with 17 microlitters Tetramethylenediamine (TEMED) and 70 microlitters ammonium per sulphate (APS). The electrophoresis was carried on a constant voltage of 80V for 7 h. The gel was stained in 0.1% bromophenol blue followed by de-staining in a solution containing methanol, distilled water and glacial acetic acid in the ratio of 4:5:0.7 % (V/V). The Bromophenol blue as a dye was used to show the drift of polypeptides in the gel. The bands generated by the protein were visualised under white light.

Data Analysis: The presence or absence of protein bands in an electrophoregram was scored as 0 for absence and 1 for the presence of protein bands. Based on the outcome of electrophoretic band spectra, Jaccard's similarity index (JSI) was considered for all conceivable sets of protein type electrophoregrams by the following formula (Sneath and Sokal, 1973).

$$S = \frac{W}{A + B - W}$$

Where, W represented the number of bands of common mobility, A, the number of bands in A-type and B, represented the number of bands in B- type. Cluster analysis was done on the similarity matrix based on Jaccard's similarity index by the UPGMA method.

Evaluation and Documentation of Electrophorograms of PAGE

The electrophorograms of the gels were prepared by calculating the distance of each band from the point of loading. The gel was documented by means of Biovis gel documentation software from which the Rf values of the bands were recorded. The Rf value of each band was calculated by the following formulae:

$$\text{Rf} = \frac{\text{Distance travelled by protein band}}{\text{Distance travelled by the dye}}$$

Apart from these, intensity, position, presence or absence of bands were measured and recorded to distinguish the cultivars.

Statistical Analysis

The data presented in the tables represented the average of three observations, (±) standard deviation which were subjected to one way analysis of variance (ANOVA) by Duncan's test ($p<0.05$) using Statistica-7 (M/s. Stat Soft Inc., OK, USA). Correlation was performed of rice characteristics using XLSTAT, software.

RESULTS AND DISCUSSION

Seed Morphological Characteristics

The different morphological characteristics used in distinguishing the rice cultivars are given in Table 1. The analysed rice cultivars were found to possess both non-pigmented as well as pigmented grains. The aromatic cultivars were brown coloured and the two pigmented *Gull zag* and *Teli zag* were sparsely red coloured having red kernels dispersed in white coloured rice kernels. Chalkiness of rice is considered a varietal factor and is considered an important indicator in determining the quality of rice grains. Most of the cultivars were found to have white belly type of chalkiness, with white spots occurring in the middle part of the caryopsis on the ventral side. *Mushki kandi* was observed to have chalky spots oriented along the edge of the ventral side extending towards the centre of the caryopsis and thus, characterise white core

while *Mushki budgi* with chalky area on the dorsal side was classified into white back type of chalkiness. The awn characterised as thorn-like extension from the keel of the lemma were observed to be present in *Zag, Mushki budgi* and *Mushki kandi* cultivars. The rice cultivars were found to specify their shape in the categories of bold and medium grains.

Table 1: Morphological Characteristics of Traditional Rice Cultivars of Kashmir

Cultivars	Pericarp Colour	Awn	Chalkiness Position	Aroma	Shape
Zag	Red	Present	White belly	Absent	Medium
Gull zag	Sparse red	Absent	White belly	Absent	Medium
Teli zag	Sparse red	Absent	White belly	Absent	Bold
Mushki budgi	Brown	Present	White back	Present	Bold
Mushki kandi	Brown	Present	White core	Present	Bold
Kamad	Brown	Absent	White belly	Present	Bold
Kaw quder	Deep red	Absent	White belly	Absent	Bold

Protein Fractions of Rice Proteins

The total protein and its fractions in the analysed rice cultivars were found to differ significantly from one another (Table 2). The total protein content was found to vary from 7.24% (*Mushki kandi*) to 8.85% (*Teli zag*) and the albumin content from 4.64% to 5.38% with *Zag* having lowest albumin content and *Gull zag* the highest content. It had been reported that glutelin and prolamin content in rice are present in maximum amount and account for about 80–85% of the total protein in rice (Cao *et al.,* 2009). Glutelin has been considered an easily digestible protein that consists of higher content of essential amino acids (Resurreccion *et al*., 1993). The glutelin content in the analysed rice cultivars were found to be higher in *Mushki budgi* (81.52%) and *Teli zag* (82.82%). The protein content had been reported by Kennedy and Burlingame (2003) from 2,869 genotypes of rice to be ranged between 4.5 to 15.9% with an average 8.8% for *O. Sativa*.

Table 2: Percentage of Total Protein Content and its Major Fractions in Traditional Rice Cultivars*

Cultivars	Total Protein Content (%)	Total Protein Relative Fractions (g/100g)			
		Albumins	Globulins	Prolamins	Glutelins
Zag	8.53 ± 0.05^{e}	4.64 ± 0.08^{a}	10.82 ± 0.15^{e}	7.87 ± 0.23^{g}	76.67 ± 0.23^{a}
Gull zag	8.05 ± 0.03^{c}	5.38 ± 0.05^{d}	9.35 ± 0.08^{b}	6.74 ± 0.17^{d}	78.53 ± 0.05^{c}
Teli zag	8.85 ± 0.05^{f}	4.86 ± 0.17^{b}	7.51 ± 0.32^{a}	4.81 ± 0.05^{a}	82.82 ± 0.17^{f}
Mushki budgi	7.56 ± 0.02^{b}	4.75 ± 0.15^{a}	7.52 ± 0.17^{a}	6.21 ± 0.08^{c}	81.52 ± 0.05^{e}
Mushki kandi	7.24 ± 0.01^{a}	5.13 ± 0.03^{c}	10.58 ± 0.05^{d}	7.32 ± 0.15^{f}	76.97 ± 0.08^{a}
Kamad	8.05 ± 0.03^{c}	5.35 ± 0.08^{d}	10.17 ± 0.08^{c}	7.14 ± 0.05^{e}	77.34 ± 0.15^{b}
Kaw quder	8.21 ± 0.05^{d}	4.67 ± 0.15^{a}	10.53 ± 0.17^{d}	5.13 ± 0.17^{b}	79.70 ± 0.08^{d}

*n=3, Data is expressed as means± standard deviation, means with different superscripts in the same column are significantly different (Duncan, $p \leq 0.05$)

SDS-PAGE of Seed Storage Proteins

The electrophoresis of total proteins to serve as genetic markers has been widely accepted by the scientists as reliable technique for distinguishing number of genotypes (Basu *et al.,* 2002). The SDS-PAGE profiling of rice proteins from seven different genotypes were done to investigate the genetic diversity. A total of thirty seven scorable protein bands were found among the seven rice germplasms analysed (Table 3). The results in Tables 3 and 4 showed the varietal differences in the band patterns of different rice cultivars with respect to their relative mobility and relative band intensity of SDS-PAGE

protein profile. The bands found in these cultivars were monomorphic and the size of the polypeptides bands as determined by SDS-PAGE was found to be ranged from 100 to 120 kDa (Figure 1). The number of scorable bands generated by the cultivar varied from 4 to 7 with an average number of bands produced by the cultivars was equal to 5. The bands 1 to 4 were observed to be present to all the analysed rice cultivars, while the band 5 was absent in two cultivars *Mushki budgi* and *Mushki kandi*. The protein band 6 was depicted by only 4 rice cultivars including *Kaw quder, Zag, Gull zag* and *Teli zag*. The protein profile in the rice cultivars were found to vary mostly in the lower protein bands than that of the higher bands as indicated by the Figure 1.

Table 3: Relative Mobility (Rf) of Protein Bands of Marker and Rice Cultivar Using SDS-PAGE

Bands	Markers	Cultivars Relative Mobility (Rf) Values						
	Value	***Kamad***	***Kamad***	***Kamad***	***Kamad***	***Kamad***	***Kamad***	***Kamad***
1	0.176	0.136	0.142	0.235	0.238	0.155	0.155	0.155
2	0.263	0.226	0.229	0.285	0.288	0.248	0.251	0.248
3	0.341	0.325	0.279	0.372	0.375	0.291	0.297	0.291
4	0.378	0.529	0.331	0.749	0.752	0.344	0.347	0.344
5	0.421	0.737	0.427			0.551	0.44	0.381
6	0.474		0.486			0.755	0.759	0.762
7	0.539		0.678					
8	0.61		0.749					
9	0.697							
10	0.802							
11	0.938							

The protein profiling in rice by means of SDS-PAGE have been reported an easy and cheap method of determining the genetic diversity (Yousuf *et al.*, 2003). The protein could be visualised as an important tool in studies on genetic variability and in identification of different varieties of rice. The variations in major protein bands were revealed by maximum rice cultivars and variations in minor bands were present in four rice cultivars like *Kaw quder, Zag, Gull zag* and *Teli zag*. The equivalence in the major protein polypeptides bands in the different germplasm had been reported by Ali *et al.*, (2007) to be due to conserved genes coding these proteins. Different researchers had identified varying number of bands in different rice cultivars.

Jiang *et al.*, (2014) had reported 34 protein bands from rice endosperm proteins. The lesser number of protein bands found in the present research could be attributed to the differences in the buffers and techniques used for the extraction of these proteins from endosperm, as proteins depicted highly heterogeneous nature in their solubility in different buffers.

The diversity shown by the extracted proteins in this research would represent their total protein diversity. The total protein content in the rice endosperm had been reported by Shotwell and Larkins (1989) to vary from 4.3 and 18.2% and was divided into four fractions as per their solubility differences *viz* albumins (soluble in water), prolamin (soluble in alcohol), globulins (soluble in salts), and glutelin (soluble in acidic or basic solutions).

The size of the each protein band was determined with an aid of protein marker. All the seven rice cultivars showed monomorphic protein bands with approximate sizes of 46, 30 kDa, 34 kDa, 26, 22, 17 kDa and 13 kDa. The protein bands with sizes of 18-20 kDa, 10-16 kDa, and 30-39 kDa had been identified as albumin, prolamin and glutelin (Borght *et al.*, 2006). Among these protein fractions, glutelin fraction had been found to be the predominant protein in the endosperm of rice kernels (Katsude-Tanaka *et al.*, 2004).

SDS PAGE has been considered an essential tool to assess the genetic diversity and to identify and characterize cultivars of several crop species such as cotton (Rao *et al.*, 1990), sunflower and wheat (Sahoo *et al.*, 2000).

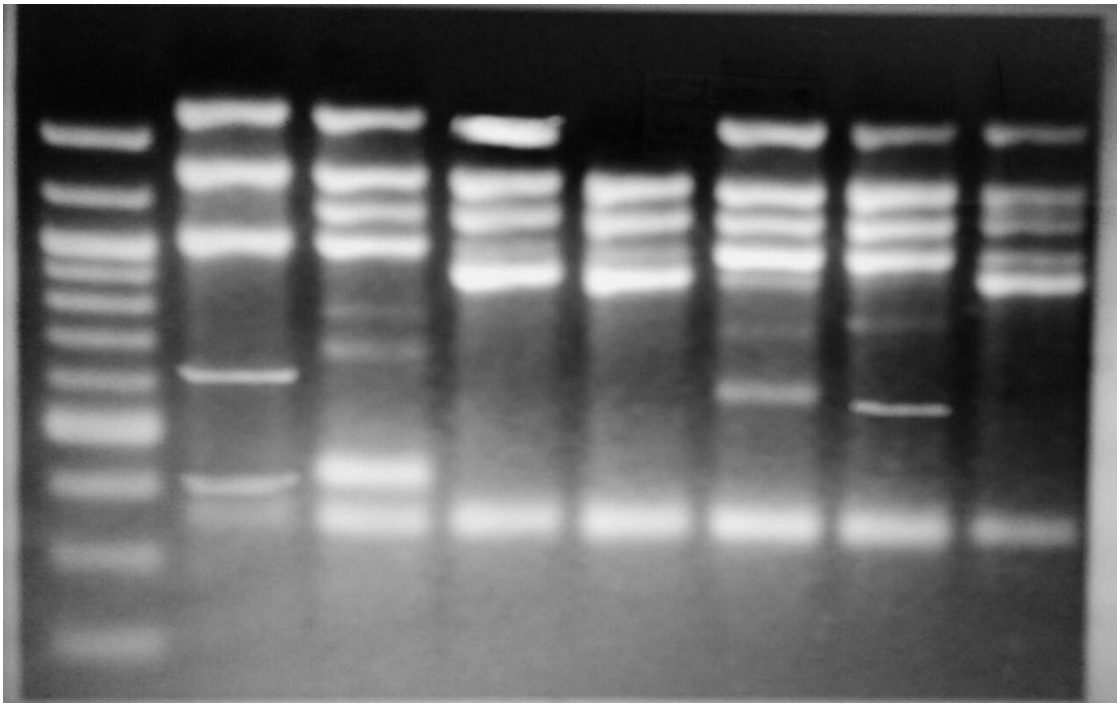

Figure 1: Electrophoretic Bands Produced by SDS-PAGE of Seed Storage Proteins of Traditional Rice Cultivars

Biochemical Studies

The seed proteins extracted from seven rice cultivars after subjecting to SDS-PAGE were used to characterise the cultivars at biochemical level. The bands generated from the protein profiles were scored for the presence (1) or absent (0) of bands and was then subjected to cluster analysis for formation of dendrogram.

The distance travelled by the dye and the protein bands from the top of the running gel was recorded for calculation of RF and refractive index values. Among the seven cultivars, a maximum of eight bands generated were found to have RF values ranged from 0.136 to 0.762 (Table 3) and refractive index values from 1188 to 6601 (Table 4).

The mobility bands with RF value 0.749 were found in *Kaw quder* and *Mushki budgi* at two different refractive index values 4311 and 3940. The protein mobility values 0.737, 0.749, 0.752, 0.755, 0.759 and 0.762 could be designated as fast mobility bands. Among the slowest mobility bands the RF values 0.155 were present in *Zag, Gull zag* and *Teli zag*.

The bands with RF values of 0.248, 0.291 and 0.344 were found present in red pigmented *Zag, Gull zag* and *Teli zag* rice cultivars. The similarity of maximum bands in these cultivars might attribute to the similarity of such cultivars in certain traits.

As these rice cultivars were pure ancestral strains without having any hybridization, they tended to depict the only the monomorphic nature as is evident from the values of RF and refractive index provided. The different bands with different RF and refractive values could serve as biochemical markers for the identification of individual cultivars.

The differences in relative mobility and intensity of the bands could be attributed to difference in solubility of protein or to the lack of separation of several proteins having identical migration rates (Ladizinsky and Hymowitz, 1979).

The separation of seed proteins has been determined by the molecular size of a protein and its net charge in a charged electric field. The position of bands along with its intensity and relative mobility could be used as criteria for the identification of a variety of given species.

Table 4: Relative Intensity (RI) of Protein Bands of Marker and Rice Cultivar Using SDS-PAGE

Bands	Reference RI Values	Cultivars (RI Values)						
		Kamad	***Kaw Quder***	***Mushki Budgi***	***Mushki Kandi***	***Zag***	***Gull Zag***	***Teli Zag***
1	4298	6014	4829	45	4459	4514	3425	3688
2	3574	6022	4002	3003	2969	3699	4337	2777
3	4238	6601	2818	5339	3773	2618	2743	1732
4	2202	2013	4737	3940	3965	3338	4278	1834
5	2400	3606	1299			1695	1188	3921
6	2443		1836			4233	3390	2064
7	2429		4444					
8	5088		4311					
9	3589							
10	3542							
11	3704							

Cluster Analysis on the Basis of SDS-PAGE

Cluster analysis was done on the results of SDS-PAGE by using the STATISTICA software in order to determine the genetic variation among the analysed rice cultivars. The outcome of cluster analysis is depicted by the Dendrogram, selected by following the "unweighted pair group method with arithmetic means" (UPGMA) method as the basis for linkage distance. The cluster analysis showed three major clusters with the smallest cluster containing only 1 pigmented cultivar (*Kaw Quder*) and the remaining contains 3 cultivars in each cluster as shown in Figure 2. The three clusters contained 14%, 43% and 43% of the cultivars tested. The second cluster was found to have the red pigmented cultivars wherein the sparsely pigmented (*Teli zag* and *Gull zag*) formed a separate sub cluster at the same genetic distance of 20. The aromatic cultivars were found to be present in the third cluster in which the highly aromatic *Mushki budgi* and *Mushki kandi* were observed to form a separate sub cluster at a genetic distance of 25.

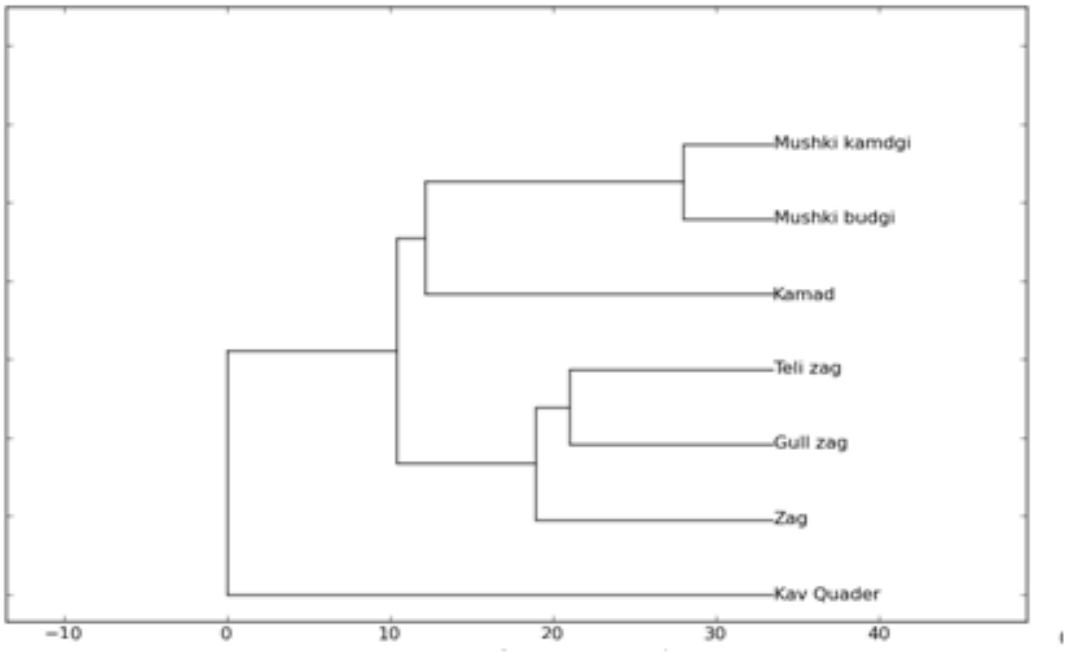

Figure 2: Dendrogram Presenting the Association among Traditional Rice Cultivars Based on SDS-PAGE of Proteins

Conclusion

The research showed SDS-PAGE of proteins is an easy and accurate molecular tool in the evaluation of genetic diversity and identity of rice cultivars. A total of thirty seven scorable protein bands were found in the seven rice germplasms with RF values ranged from 0.136 to 0.762 and refractive index values from 1188 to 6601.

The rice cultivars were found to depict the monomorphic nature of their protein bands as is evident from SDS-PAGE profile and the RF and refractive index values. The different bands with different RF and refractive values could serve as biochemical markers for the identification of individual rice cultivars. The protein profile in the rice cultivars were found to vary mostly in the lower protein bands than that of the higher bands. The position of bands along with its intensity and relative mobility could be used as criteria for the identification of a particular rice cultivar.

ACKNOWLEDGEMENT

We are very grateful to the rice breeding centre Khudwani, (SKAUST), Jammu & Kashmir (India), for providing these rice cultivars to exploit their genetic variation by protein profiling. First author is also thankful to SLIET, Longowal, for providing TEQIP fellowship for assistance of this research work.

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