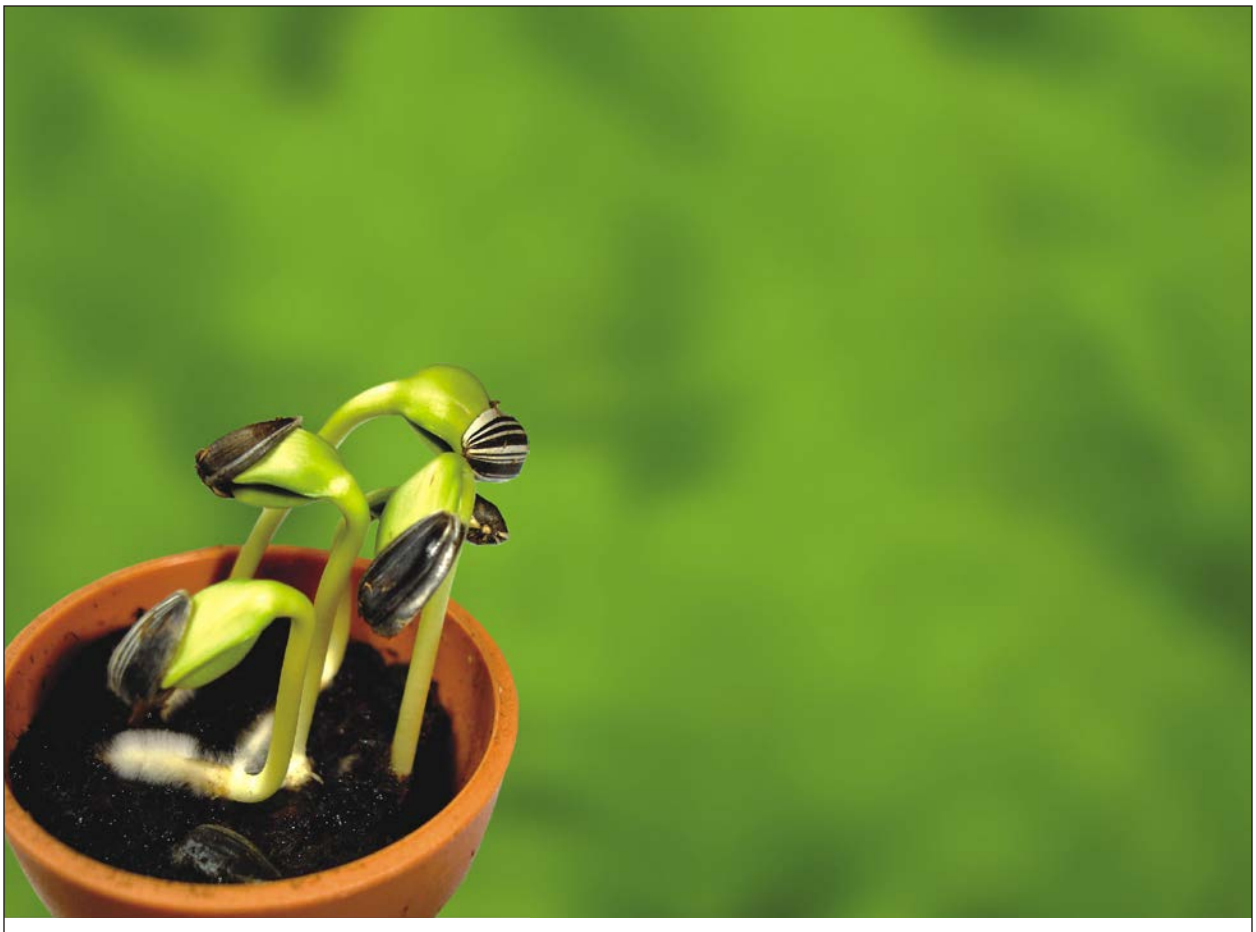

International Journal of Plant Breeding and Genetics

ISSN 1819-3595

Academic Journals Inc.

www.academicjournals.com

International Journal of Plant Breeding and Genetics 4 (2): 89-103, 2010
ISSN 1819-3595

Use of Different Marker Systems to Estimate Genetic Diversity in the Traditional Medicinal Rice Cultivar of Kerala

[1]P. Sanal Kumar, [1]C.R. Elsy, [2]P.A. Nazeem and [2]A. Augustin
[1]Department of Plant Breeding and Genetics, College of Horticulture,
Vellanikkara, KAU P.O., Thrissur-680 656, Kerala, India
[2]Centre for Plant Biotechnology and Molecular Biology, College of Horticulture,
Vellanikkara, KAU P.O., Thrissur-680 656, Kerala, India

Abstract: Njavara the traditional rice cultivar used in Ayurvedic health care system in India is confined to the state of Kerala, India. The efforts for collection, conservation and documentation of this unique type are meager. Hence, the present study aimed in collection of Njavara types from different parts of the state and was subjected to morphological evaluation, isozyme and RAPD analysis for the characterization of ecotypes of this cultivar and identification of markers for distinguishing Njavara ecotypes from other rice cultivars. Among the qualitative characters apiculus colour, lemma and palea colour and seed coat colour showed great variability and hence, can be considered as markers for the identification of Njavara types. The isozyme analysis with alcohol dehydrogenase revealed two unique bands for Njavara ecotypes. A set of eleven RAPD markers were used for DNA profiling of the Njavara ecotypes and the DNA amplification exhibited unique bands with primers OPE 6, OPP 6 and 11. The pooled data for Jaccard's similarity coefficient values revealed that at similarity coefficients 0.33 and 0.41, based on isozyme and RAPD analysis, respectively. The genotypes were grouped into two clusters, one large cluster with Njavara ecotypes and one small cluster with two check varieties. The study revealed no duplicates among the Njavara accessions and also identified unique bands that can be used as markers for identification of the cutivar from other rice varieties.

Key words: Medicinal rice, Njavara, morphological characterization, DNA markers, isozyme

INTRODUCTION

Njavara is a rice cultivar of Kerala traditionally used in Ayurveda, the most ancient health care system in India. The uniqueness of Njavara rests not only in its widely proclaimed medicinal properties but also, in its extreme short growth duration. Ecotypes of Njavara had been reported from different parts of the state. Earlier studies on black glumed Njavara type in Kerala revealed that though black glumed type is poor yielder, it has good source-sink relationship and high photosynthetic ability. Menon and Potty (1999) revealed variability in qualitative and nutritional traits of this medicinal cultivar. It is necessary to collect all ecotypes, present in different localities of the state, under the popular vernacular

Corresponding Author: P. Sanal Kumar, Department of Plant Breeding and Genetics, College of Horticulture, Vellanikkara, KAU P.O., Thrissur-680 656, Kerala, India

broad name Njavara for evaluation and characterization and there is a strong need that the germplasm of this unique medicinal cultivar be collected, preserved and characterized in detail (Sreejayan and Thomas, 2003). With the characterization of cultivars, it becomes possible to study the level of diversity existing within the species and to establish an index of genetic similarities among different varieties/cultivars. The classification based on phenotypic traits will be far more than the actual variability present in the germplasm (Shivapriya and Hittalmani, 2006). The selection of plant varieties based on morphological marker alone is not very reliable because many characters of interest have low heritability and are genetically complex.

Isozymes and proteins are the commonly studied biochemical markers as they are multiple forms of an enzyme that catalyze the same biochemical reaction but differ in their kinetic properties. Many rice geneticists had used electrophoretic techniques to add isozyme loci to the linkage groups as additional gene markers and to determine the genetic divergence among cultivars and their wild relatives (Thanh *et al*., 2006). The applications of isozymes include the study of genetically defined variation, general variation, multi-locus analysis etc., (Mukherjee and Dutta, 2008). The use of isoenzymes as markers for identifying cultivars or genotypes is recommended as a supplement for molecular techniques due to their low input cost (Sharma and Maloo, 2006).

Molecular markers based on DNA sequence are more varied and reliable. It has been used for a variety of applications including DNA fingerprinting, varietal identification and diversity analysis in rice (Jain *et al*., 2004). The DNA markers offer precise means to measure genetic diversity and affinity among germplasm collections than the morphological and biochemical markers due to their environmental insensivity and abundance in genome (Sharma *et al*., 2006). The PCR based Random Amplified Polymorphic DNA (RAPD) approach has been extensively used for studying DNA polymorphism and marker assisted breeding program (Jena and Mackill, 2008). Random amplified polymorphic DNA, a PCR based DNA marker technology, offers advantages in speed, technically simplicity, random coverage of genome and higher level of polymorphism (Mackill, 2007). The major advantage of RAPD method relies on the fact that it allows the exploration of a much large genomic portion and requires much less DNA and is technically simple and cheaper.

Since, the area under traditional cultivars is reducing at fast pace due to the spread of high yielding and improved varieties, there is every chance that this unique rice cultivar may become extinct in the near future. However, collection and characterization of Njavara rice cultivars remains largely unexplored. Hence, the present study is formulated to characterize and evaluate the seven Njavara genotypes along with two check varieties (Ptb 10 and Karavella) based on morphological characters, isozyme analysis with Alcohol dehydrogenase (ADH), an enzyme of glycolytic pathway and also a set of eleven selected RAPD markers for DNA profiling to detect the genetic diversity within the germplasm collection at morphological and protein-DNA level and to identify the duplicate accessions, if any.

MATERIALS AND METHODS

Plant Material

During 2001-2004, the present study was carried out using seven Njavara genotypes viz., four from the National Bureau of Plant Genetic Resources (NBPGR) Regional Station, Vellanikkara, Thrissur, Kerala, India and three collected from different locations of Kerala along with Ptb-10 and Karavella as check varieties. The details of these genotypes used for the study are given in Table 1.

Table 1: Details of the genotypes utilized in the study

Genotypes	Indigenous Collection (IC) No.	National Indigenous Collection (NIC) No.	Source	
			Village	District in Kerala, India
Njavara types				
N1*	-	18383-A	Chittoor	Palakkad
N2*	-	18430-B	Chittoor	Palakkad
N3*	203771	-	Tellicherry	Kannur
N4*	203767	-	Tellicherry	Kannur
N5	-	-	Kottakkal	Malappuram
N6	-	-	Alwaye	Alwaye
N7	-	-	Thrissur	Thrissur

Genotype	Detail
Local check	
Ptb-10	A short duration traditional variety released from Regional Agricultural Research Station, Pattambi
Karvella	A medium duration traditional variety obtained from Regional Agricultural Research Station, Pattambi

*Njavara genotypes obtained from NBPGR Regional Station, Vellanikkara

Morphological Characterization

The Njavara types along with checks were evaluated under field conditions during the planting seasons of 2002-2003 based on morphological characters. The genotypes were grown in a Randomised Complete Block Design (RCBD) with three replications in plots of 4.0×5.0 m with 10×15 cm spacing. The field experiment was laid out and cultural practices for raising the crop were followed as per the recommendations in Package of Practices (Jose *et al.*, 2002). All the genotypes were evaluated for 28 qualitative characters at different plant growth stages and observations were recorded and scored following the Standard Evaluation System for Rice (IRRI, 1995) in 10 randomly selected plants from each entry per replication.

Isozyme Analysis

Rice seeds of each genotype were placed in separate pertidishes lined with two layers of Whatman filter paper for 24 h at 30±1°C in an incubator for germination. Germinated seeds were pressed with blotting paper to remove water and used for extraction of enzyme.

Protein Extraction and Electrophoresis

For extraction of alcohol dehydrogenase, 500 mg of the sample was taken and homogenized in a pre-cooled mortar, along with 0.5 M Tris-HCl buffer (pH 7.4) containing 5 mM 2-mercaptoethanol. In general, 0.1 mL of buffer was used per seed. The samples were ground at 4°C by keeping and pestle in an ice tray. The homogenized samples were centrifuged at 10,000 g for 30 min in a Kubota high speed centrifuge at 5°C. After centrifugation, the supernatant was collected in eppendorf tubes, labeled and used for running the gel. Fresh samples were used for the assay though enzyme extracts can be stored at sub-zero temperature for one day. The extract (100 μg protein) along with tracer dye [Bromophenol blue (1% solution) in 0.125 M Tris HCl buffer (pH 6.8) and 20% glycerol] was loaded onto graded polyacrylamide gel (7.5-10%) and electrophoresis carried out at 4°C. After electrophoresis, the gels were taken out washed, incubated 0.5 M Tris-HCl buffer (pH 7.1) for 5 min at 37°C and then stained in the reaction mixture containing NAD+ - 50 mg, NBT 30 mg, PMS 2 mg, Ethanol 3 mL, Tris-HCl buffer (0.5 M, pH 7.1) -15 mL and water 15 mL (Shaw and Koen, 1965).

Nomenclature of the Isozymes

The norms described by Berg and Wijsman (1982) for peroxidase was followed for the nomenclature of the isozymes. The enzyme alcohol dehydrogenase was referred by the abbreviations Adh.

The Relative mobility (Rm) of each band was calculated as:

$$Rm = \frac{\text{Distance of band from origin}}{\text{Total distance run}}$$

Based on relative mobility of each band, the isozyme pattern was schematically drawn.

Numbering of Isozymes and Measurement of Similarity

For numbering, all the isozymes of an enzyme in the species studied were pooled. The slowest moving anodal band was numbered 1 (e.g., Adh-l) faster ones were given the subsequent numbers. The gels were scored for computer analysis on the basis of the presence or absence of the amplified products. If a product was present in a genotype, it was designated as 1 and if absent; it was designated as 0. The alcohol dehydrogenase data were analyzed using NTSYS-PC 2.01e (Numerical Taxonomy and Multivariate Analysis System) computer package using SAHN coefficient. The data were used to generate Jaccards's similarity coefficient for RAPD bands. The Jaccards's coefficients between each pair of accessions were used to construct a dendrogram using the Unweighted Pair Group Method of Arithmetic Averages (UPGMA).

RAPD Analysis

DNA Extraction

Quality of DNA is an important factor that influences the PCR reactions. DNA was extracted from 1 g of tender leaves of 14-day-old etiolated rice seedlings using the method reported by Dellaporta *et al.* (1983) with slight modifications and dissolved in 300 μL sterile double distilled water. Quantification of DNA was done by using a UV-VIS spectrophotometer (Spectronic R Genesys 5). The optical density was measured at 260 nm and the ratio of OD_{260}/OD_{280} was measured to check for the RNA protein^{-1} impurities in the sample.

PCR and Gel Documentation

The eleven different 10-mer oligonucleotide RAPD primers (supplied from Operon Technologies Inc. USA) used in the study were OPE-3, OPE-4, OPE-6, OPE-14, OPE-16, OPP-6, OPP-7, OPP-11, OPP-12, OPP-13, OPP-19. The DNA amplification was performed in 25 μL reaction mixture containing 50 ng genomic DNA, 250 μM each of dATP, dCTP, dGTP and dTTP, 10 p moles of a primer, 0.6 units of Taq DNA polymerase, 2.5 μL of 10X incubation buffer containing 100 mM TAPS [3-tris (hydroxymethyl) methyl aminopropane sulphonic acid], 15 mM $MgCl_2$, 500 mM KCL and 0.1% gelatin and 12.5 μL of sterile Milli-Q water. The amplification was performed in a Thermal Cycler (MJ Research, USA). The cycling conditions were: 1 cycle of 94°C for 3 min followed by 40 cycles of 92dC for 1 min, 37°C for 1 min, 72°C for 2 min, 72°C for 2 min and finally, 1 cycle of 72°C for 5 min after removing the mineral oil, 15 μL aliquots of amplified products mixed well with 4 μL of loading dye was loaded in a 1.2% (w/v) agarose gel for electrophoresis stained with ethidium bromide. The gel was taken from electrophoresis unit and viewed under UV light in a UV transilluminator. The ethidium bromide stain intercalates between the nitrogen bases of DNA and fluoresces in orange color under UV light. The image of the gel was monitored and stored in a gel documentation system (Alpha Imager-2000, Alpha Infotech, USA). These images were used to score the DNA bands for analysis. For each primer, the PCR products were sequentially numbered as 1, 2, 3 and so on. The gels were scored for computer analysis on the basis of the presence or absence of the amplified products. If a product was present in a genotype, it was designated as 1 and if absent, was designated as 0.

Data Analysis

The RAPD data were analyzed using NTSYS-PC 2.01e (Numerical Taxonomy and Multivariate Analysis System) computer package using SAHN coefficient. The data were used to generate JACCARDS's similarity coefficient for RAPD bands. The JACCARDS's coefficients between each pair of accessions were used to construct a dendrogram using the Unweighted Pair Group Method of Arithmetic Averages (UPGMA).

The RAPD-PCR reactions were setup with the 9 rice accessions using different 10-mer primers that were selected based on preliminary screening done by amplifying rice DNA. The amplifications were carried out twice to check for reproducibility. Occasionally, the intensity of some bands was reduced or increased slightly, but the total number of bands obtained with a primer remained the same. A negative control without the rice genomic DNA template was kept for amplification along with nine genotypes with each primer. This was to confirm the quality of the primer and to avoid scoring of bands that may arise due to the primer dimmers or possible contaminants (Fig. 6).

RESULTS

Morphological Characters

Njavara genotypes exhibited least variation among themselves with respect to most of the qualitative characters and in general, they have exhibited glaborous and green leaves, green basal leaf sheath, white and 2-cleft ligule, light green collar and auricle, erect leaves, intermediate flag leaf angle, erect culms, light gold internode, well exserted panicle with heavy secondary branching, difficult threshability, white stigma, straw colored sterile lemma, highly fertile and awnless spikelets, non waxy endosperm, non scented grains and low incidence of pest and diseases. Regarding lemma and palea pubescence glaborous glumes were observed for all genotypes except for N1 that exhibited hairs on upper portion of glumes. With respect to panicle type, genotype N3 and N4 exhibited open panicle and rest of the genotypes showed intermediate panicle type. Regarding panicle axis, all genotypes exhibited droopy panicle except, the genotypes N2, N3 and N4, which had straight panicles. Grains of most of the genotypes were awnless except for N6, which exhibited long and partly awned grains (Fig. 1). Variability was noticed for apiculus colour among Njavara genotypes.

Fig. 1: Variability in grain characters of the Njavara genotypes. N1-NIC 18383, N2-NIC 18430, N3-IC 203767, N5-Kottakkal type, N6-Aluva type, N7-Thrissur type. (1) Aluva type, (2) CIN 18383, (3) NIC 18430, (4) IC 203767, (5) Kottakkal and (6) Thirssur type

Table 2: Qualitative characters (scores) of Njavara genotypes and check varieties showing variations

	Njavara genotypes							Check varieties	
Characters	N1	N2	N3	N4	N5	N6	N7	PTB 10	Karavella
Lemma and palea pubescence	3	1	1	1	1	1	1	3	3
Panicle type	5	5	9	9	5	5	5	5	5
Panicle axis	2	1	1	1	2	2	2	2	2
Awn presence	0	0	0	0	0	7	0	0	0
Apiculus colour	8	8	3	3	2	8	2	2	2
Lemma and palea colour	11	11	3	3	1	11	1	3	0
Seed coat (bran) colour	5	5	5	4	5	4	5	4	2

Lemma and palea pubescence: 1: Glaborous, 3: Hairs on upper portion; Panicle type: 5: Intermediate, 9: Open; Panicle axis: 1: Straight, 2: Droopy; Awn presence: 0: Absent, 7: Long and partially awned; Apiculus colour: 2: Straw, 3: Brown/tawny, 8: Black; Lemma and palea colour: 0: Straw, 1: Gold/gold furrows on straw background, 3: Brown furrows on straw, 11: Black furrow/patches on straw background (new type); Seed coat colour: 2: Light brown, 4: Brown, 5: Red

Black coloured apiculus was observed for N1, N2 and N6 whereas, other genotypes exhibited brown/straw colour for apiculus (Table 2). High variability was noticed among Njavara genotypes with respect to lemma and palea colour. Grain of genotypes N1, N2 and N6 exhibited lemma and palea colour of black furrows/patches on straw background whereas, brown/gold furrows on straw background were recorded for the grain of genotypes N3, N5 and N7.

Isozyme Analysis-ADH Pattern in Germinated Seed and Leaf Samples

Germinated seed sample expressed more alcohol dehydrogenase banding pattern of the nine bands resolved (Fig. 2), the isozyme band ADH-3 (Rm = 0.205) was present only in N1. N6 and N7 shared a high molecular weight band ADH-2 (Rm = 0.19). The Njavara ecotypes showed two unique bands viz., ADH-4 (Rm = 0.282) and ADH-8 (Rm = 0.590). The band ADH-1 (Rm = 0.128) was observed in all genotypes except for N6 and N7. The bands ADH-5 (Rm = 0.359) and ADH-9 (Rm = 0.667) were common for both the check varieties and not present in the Njavara ecotypes. The isozyme band ADH-6 (Rm = 0.462) was shared by N1 and N3. The number of alcohol dehydrogenase bands in leaf samples observed was less compared to the germinated seed samples (Fig. 3). Only five bands were resolved for leaf samples. Two bands ADH-3 and ADH-4 with Rm values 0.310 and 0.429, respectively were common for all the genotypes analyzed. ADH-1 (Rm = 0.095) was shared by N6 and N7. The isozyme band ADH-2 with Rm value 0.167 was common for N1, Ptb-10 and Karavella. ADH-5 (Rm = 0.595) was resolved by N1 alone.

Based on the ADH isozyme pattern in leaf, the isozyme bands ADH-3 and ADH-4 was observed for all the rice genotypes under study. The band ADH-1 was specific for N6 and N7 suggesting their similarity. The isozyme band ADH-5 was specific for the genotype N1 and it could be easily identified. The banding pattern of the check varieties was similar exhibiting three bands ADH-3, 4 and 5.

RAPD Analysis

Detection of Polymorphism

A total of 376 scorable bands were amplified with 11 random primers and nine cultivars and these were in the size range of 0.1 to 3.0 kbp. Out of the 376 bands, 295 (78.46%) were found to be polymorphic for one or more accessions. The agarose gels showing polymorphism observed with three primers OPE-6, OPP-6 and OPP-11 are shown in Fig. 4a-c, respectively. Primer OPE 6 gave a RAPD profile without any monomorphic band (Fig. 4a). Among the eleven primers, OPP-19 gave the maximum number of nine bands as reported by with rice genotypes (Fig. 4d).

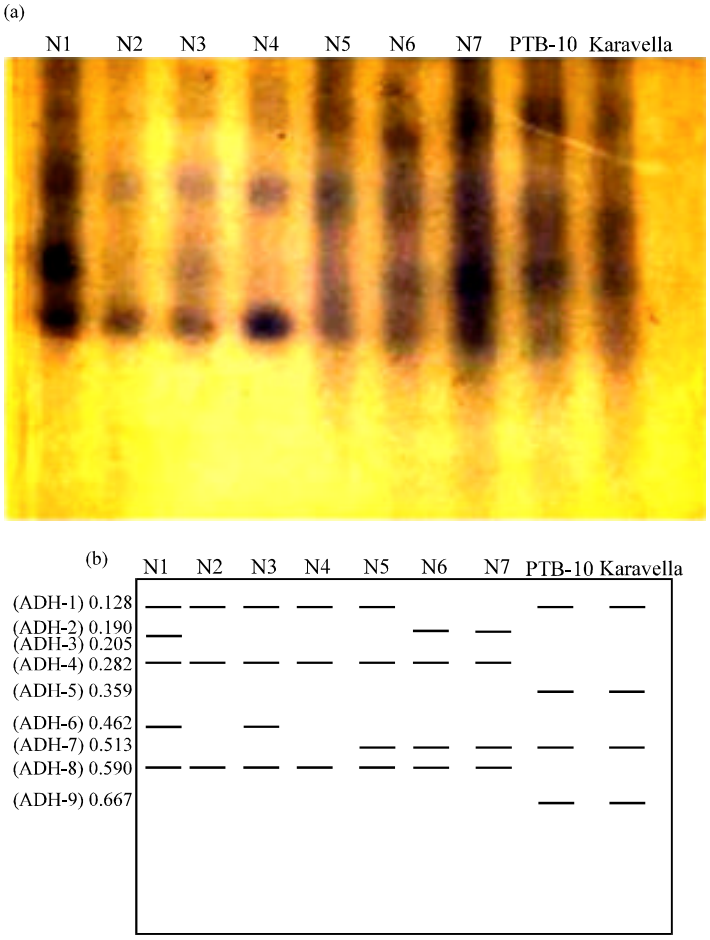

Fig. 2: Alcohol dehydrogenase banding pattern and zymogram in germinated seeds (5 days after soaking) of Njavara ecotypes

Genetic Similarity/diversity within Njavara Population and with other Traditional Cultivars

The DNA profile with primer OPE 6 revealed that one band with high molecular weight (1.5 kbp) is common for all seven Njavara ecotypes and is absent in check varieties. The N1 and N2 gave uniform electromorphic pattern with two bands (1.5 and 1.2 kbp) and N6 and N7 gave three uniform bands. Amplification with primer OPP 6 produced banding pattern without any monomorphic bands (Fig. 4b). The molecular weight of bands ranges between 0.5 and 1.5 kbp. When compared with the check varieties, all Njavara ecotypes gave one common band with low molecular weight (0.6 kbp). N1, N2, N3, N4, N5 and N6 showed presence of two additional bands (0.94 and 0.80 kbp) in common. Among, the Njavara ecotypes, N4 exhibited one unique polymorphic band (1.2 kbp). N1, N3, N4, N6 and Karavella showed a high molecular weight band (1.5 kbp) in common. Electromorphic banding pattern for primer OPP 11 gave high amplification for all genotypes (Fig.4c). Njavara ecotypes exhibited presence of a distinct band with relatively higher molecular weight (1.5 kbp) compared to the check varieties. Two monomorphic bands (1.8 and 0.9 kbp) were

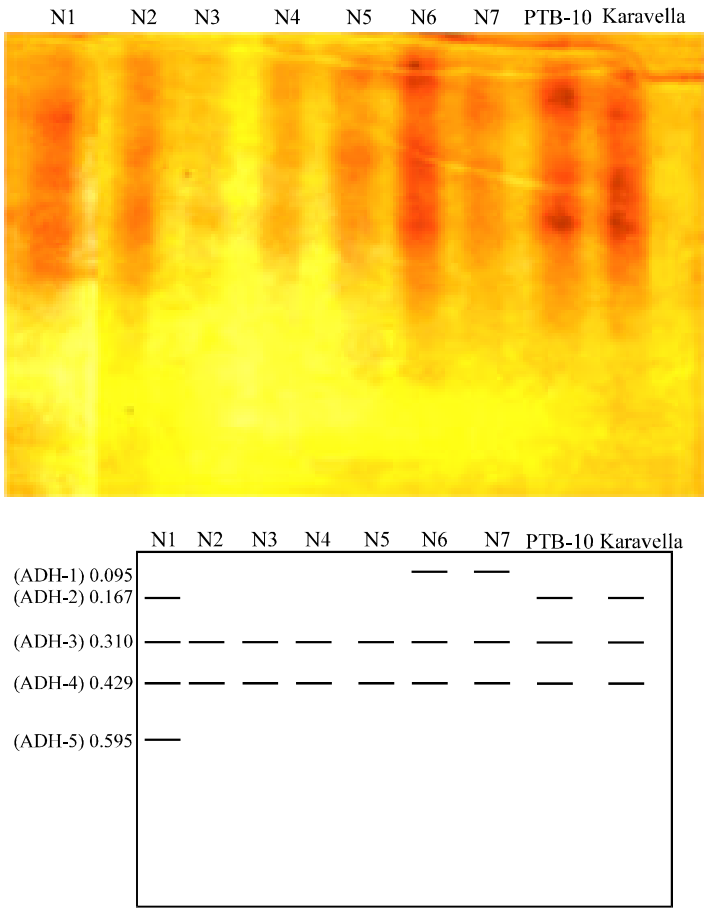

Fig. 3: Alcohol Dehydrogenase banding pattern and zymogram in leaves (30 DAS) of Njavara ecotypes

shown with seven Njavara ecotypes and two check varieties. N4 exhibited maximum number of seven electromorphs. N5, N6 and N7 exhibited uniform banding pattern. These results gave an average of 26.82 polymorphic bands per primer. The remaining 81 bands (21.54%) were monomorphic, i.e., they were present in all the 11 cultivars. Three bands were identified as unique to Njavara ecotypes that made it distinct from the two local check varieties. It is possible to use such unique bands as molecular markers for identification of Njavara types from other rice genotypes.

JACCARDS's Similarity Coefficient

The pooled data of the ADH banding pattern of germinated seeds and leaf for Jaccard's similarity coefficient values revealed that genotypes N2 with N4, N6 with N7 and Ptb-10 with Karavella had highest similarity coefficient of 100%. The similarity coefficient values of Njavara ecotypes with check varieties were low (Table 3). The pairwise Jaccard's Coefficient for the genetic similarities of RAPD bands among nine accessions presented in Table 4 revealed that the Jaccard's similarity coefficient values ranged from 0.29 to 1.00. The least

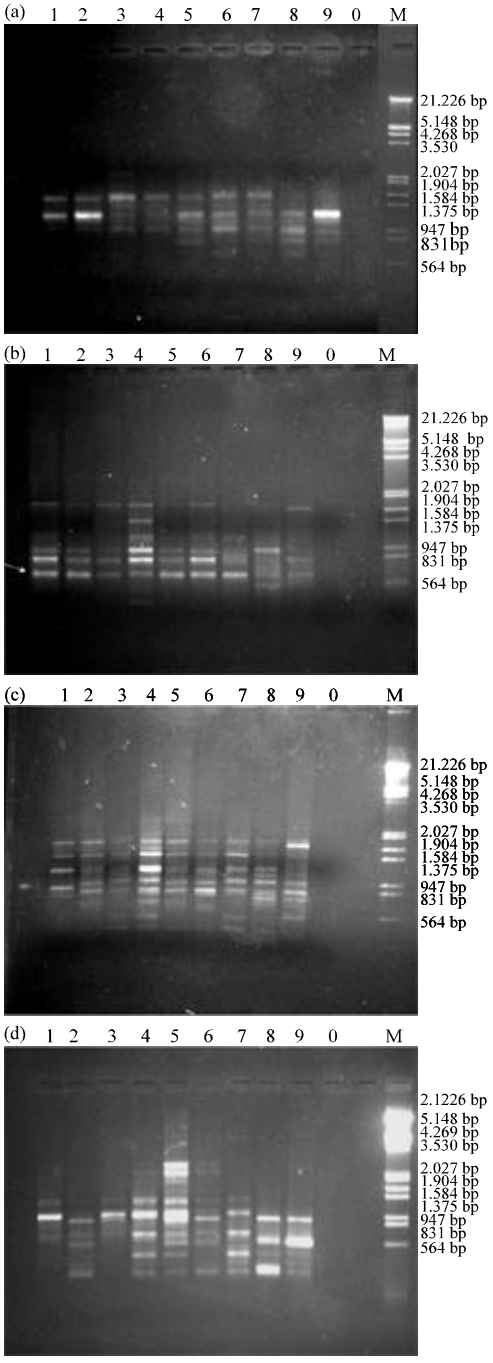

Fig. 4: Amplification products from genomic DNAs of nine rice accessions using (a) primer OPE 6, (b) primer OPP 6, (c) primer OPP 11 and (d) primer OPP 19. The lanes represent, 1-N1, 2-N2, 3-N3, 4-N4, 5-N5, 6-N6, 7-N7, 8-Karavella, 9-PTB 10, 0-negative control without any genomic DNA, M-molecular weight size marker (Eco R1 + Hind III double digest, λ DNA)

Table 3: Similarity index among Njavara ecotypes based on pooled data of alcohol dehydrogenase isozyme pattern

Genotypes	N1	N2	N3	N4	N5	N6	N7	PTB 10	Kara-vella
N1	1.00								
N2	0.56	1.00							
N3	0.67	0.83	1.00						
N4	0.56	1.00	0.83	1.00					
N5	0.50	0.83	0.71	0.83	1.00				
N6	0.33	0.50	0.44	0.50	0.63	1.00			
N7	0.33	0.50	0.44	0.50	0.63	1.00	1.00		
PTB 10	0.33	0.33	0.30	0.33	0.44	0.27	0.27	1.00	
Karavella	0.33	0.33	0.30	0.33	0.44	0.27	0.27	1.00	1.00

Table 4: Genetic similarity index among Njavara ecotypes for 11 selected random primers

Genotypes	N1	N2	N3	N4	N5	N6	N7	PTB 10	Kara-vella
N1	1.00								
N2	0.57	1.00							
N3	0.62	0.67	1.00						
N4	0.60	0.62	0.64	1.00					
N5	0.56	0.67	0.59	0.58	1.00				
N6	0.51	0.67	0.63	0.61	0.60	1.00			
N7	0.56	0.67	0.59	0.64	0.57	0.72	1.00		
PTB 10	0.29	0.44	0.43	0.29	0.44	0.48	0.37	1.00	
Karavella	0.30	0.48	0.42	0.39	0.39	0.49	0.46	0.40	1.00

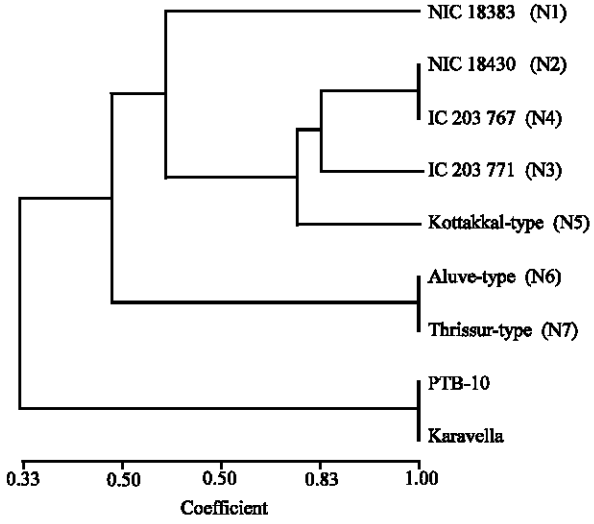

Fig. 5: Dendrogram of Njavara ecotypes from pooled isozyme data using UPGMA clustering

similarity coefficient values were that of N4 with PTB-10 (0.29). The highest value for similarity index was obtained for N6 with N7.

Dendrogram based Grouping of Njavara Ecotypes

The dendrogram, drawn from the pooled data of alcohol dehydrogenase, was divided into two clusters, one large cluster with Njavara ecotypes and one small cluster with two check varieties at 33% similarity (Fig. 5). The cluster with Njavara ecotypes was divided into two at 48% similarity with N1, N2, N3, N4 and N5 in one cluster and N6 and N7 in another cluster. At 57% similarity N1 came in an individual cluster. N2 and N4, N6 and N7 and Ptb-10 and Karavella were clustered separately at similarity coefficient 1.00. The dendrogram

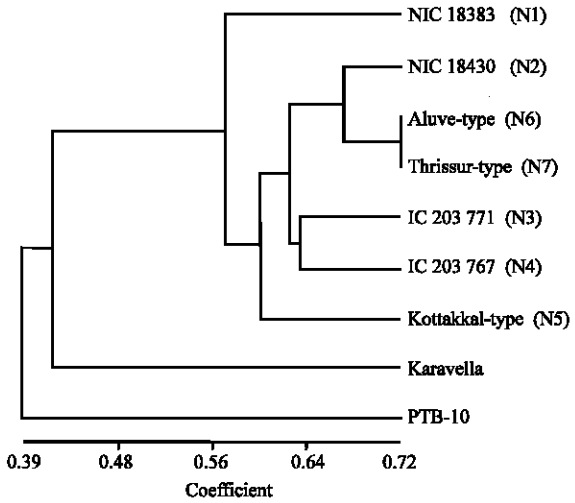

Fig. 6: Dendrogram of Njavara genotypes from pooled RAPD data using UPGMA clustering

revealed the similarity index ranging from 0.33 to 1.00. At a similarity coefficient of 0.33, the dendrogram was divided into two clusters, one large cluster with and one small cluster with two check varieties. Dendrogram of Njavara genotypes from pooled RAPD data using UPGMA clustering is shown in Fig. 6.

At 41% similarity, the dendrogram based on RAPD banding pattern got divided into three clusters; one large cluster with all seven Njavara ecotypes and two individual clusters with two check varieties. The Njavara ecotypes were further subdivided into two clusters at 57% similarity with N2, N3, N4, N5, N6 and N7 in one cluster and N1 in an individual cluster. At 60% similarity, two clusters were obtained one cluster with N2, N3, N4, N6 and N7 and other with N5 in an individual cluster. N3 and N4 came under one cluster at 63% similarity. At 72% similarity N6 and N7 grouped in one cluster.

DISCUSSION

The study is conducted to characterize and evaluate the seven Njavara genotypes based on morphological characters, Alcohol dehydrogenase (ADH) isozyme analysis and RAPD markers to detect the diversity within and among the germplasm collection at morphological and protein-DNA level and to identify any possible duplication in the available accessions.

Genetic Variability in Morphological Characters

Among the Njavara genotypes qualitative characters, apiculus colour, lemma and palea colour and seed coat colour showed great variability and hence can be considered as markers for the identification of Njavara types. Njavara genotypes exhibited variations for apiculus colour like straw colour, brown colour and black colour. Genotypes showed a wide array of lemma and palea colour like brown furrows on straw background, gold furrows on straw background and black furrows/patches on straw background. The results are in line with the investigation done by Elsy *et al.* (2004) revealing that the Njavara genotypes exhibited lemma and palea colour variants like straw color, gold furrows on straw background and brown furrows on straw background whereas Sreejayan and Thoams (2003) observed black and

gold and/or gold furrows on straw background colours for lemma and palea in Njavara genotypes as observed in the study. Hence, the morphological studies of Njavara types revealed that the Njavara is genetically diverse and is a composite of distinct morphotypes as reported by Elsy *et al.* (1992).

Variability and Interrelationships based on Protein and DNA Markers

Alcohol dehydrogenase banding pattern in germinated seed showed maximum polymorphism and along with the corresponding similarity indices helps to classify Njavara genotypes into distinct groups. The isozyme bands, ADH-4 and ADH-8, were specific for Njavara genotypes alone and the isozyme bands ADH-5 in leaf sample and ADH-3 in germinated seed sample was unique for genotype N1 hence, could be used as a marker to distinguish Njavara from other traditional rice cultivars. With Njavara genotypes similar results were reported by Elsy *et al.* (2004) showing unique bands with peroxidase and esterase isozymes suggesting the use of those isozymic bands as markers for identifying Njavara ecotypes. The studies conducted by Bimb *et al.* (2004) also revealed similar unique Adh bands while, studying isozyme variation of 24 aromatic and fine rice varieties collected from Nepal. Based on ADH isozyme pattern in leaf and their corresponding similarity, indices with check varieties, Njavara genotypes were classified into four groups. The isozyme band ADH-3 and ADH-4 was observed for all the rice genotypes under study. Similarly, the studies conducted by Grover and Pental (1992) and Dattarwal *et al.* (1999) also exhibited variation in electrophoretic patterns of Alcohol dehydrogenase (ADH) in rice. The isozyme banding pattern for both N6 and N7 with respect to leaf and seed samples was similar showing closeness in geographical area of cultivation. The Njavara genotypes are ideal source of desirable genetic traits that play an important role in improving the nutritional qualities of rice (Menon and Potty, 1999). With the identification of these unique bands, it is possible to mark the specific nutritional characters of this medicinal cultivar to be used in Marker Assisted Selection (MAS) to improve rice grain quality (Zhang *et al.*, 2008).

The RAPD profile of the Njavara genotypes exhibited one monomorphic band each with primers OPE 6, OPP 6 and OPP 11 when compared to that of the check varieties. The unique bands with high molecular weight (1.5 kbp) was exhibited for primers OPE 6 and OPP 11 while, the monomorphic band with primer OPP 6 showed low molecular weight (0.6 kbp). Among the Njavara genotypes, N4 exhibited one unique polymorphic band and hence signifies its use for cultivar identification. From the above discussion, it could be inferred that OPE 6, OPP 6 and OPP 11 produced distinct bands for Njavara genotypes, indicating their significance for identification of this cultivar from other rice genotypes. It is possible to use such unique bands as molecular markers for identification of Njavara types from other rice genotypes. Similarly, genotype specific DNA bands were identified by Shivapriya and Hittalmani (2006) using RAPD markers with land races of rice. The detailed studies will help in associating such markers with the unique medicinal and nutritional properties of this traditional rice cultivar and also will help in the identification of outstanding Njavara genotypes. The use of cost effective DNA markers linked for important agronomic traits and MAS strategies will provide opportunities for breeders to develop better quality rice cultivars (Jena and Mackill, 2008). Moreover, this traditional medicinal rice cultivar is mainly conserved and maintained by local farmers and such unique DNA markers could possibly help in identifying it from closely related rice varieties/cultivars.

The pairwise Jaccard's Coefficient for the genetic similarities revealed that the cultivars under study, in ADH analysis, was divided into two clusters, one large cluster with the Njavara genotypes and one small cluster with two check varieties at a similarity coefficient

of 0.33 while, in RAPD analysis the genotypes were grouped in three clusters; one large cluster with all seven Njavara ecotypes and two individual clusters with two check varieties at 41% similarity. The genotypes showing higher intra-population similarity and lower proportion of polymorphic bands are likely to have less heterozygosity in comparison to those showing less intra genotype similarity and higher proportion of polymorphic bands (Rahman *et al.*, 2007). In other words, the genotypes having higher similarity are more homogenous. The amount of genetic diversity between Njavara types and check varieties is quite high as revealed by the genetic similarity coefficients between them. Similar types of studies have been carried out by Arif *et al.* (2005) using RAPD markers for the classification of aromatic rices.

The Njavara ecotypes classified into five groups at similarity coefficient of 0.72 viz., N6 and N7, N3 and N4 shared common but separate clusters that represent the central region and Northern region of the state respectively, while Njavara ecotypes N1, N2 and N5 were placed in separate clusters. The Njavara types N3 and N4 coming from the same geographical location (Kannur) and almost having similar morphological characters proved their relatedness by being in a single cluster with a similarity index of 0.63. On the other hand, ecotypes N6 (collected from Aluva) and N7 (collected from Thrissur) with distinct lemma and palea color (black patches on straw background and gold color respectively) came in a single cluster represented the central region of the State, while the large cluster comprising of five represents the North and North-Eastern region. This indicates that the five Njavara ecotypes that shared the large cluster may have common parentage. The difference in colour may be due to mutation to one ecotype, even though both evolved from a common ancestral genotype. Adaptability to different environments might have also contributed to their distinct morphology.

CONCLUSION

Even though, a number of genotypes tested here come under the popular vernacular broad name Njavara, no duplicates were observed among the rice accessions. This indicates that it becomes possible to study the level of diversity existing within the cultivar and to establish an index of genetic similarities among different ecotypes and useful for the further improvement of this cultivar. The above results with more detailed study of protein patterns and enzyme activity at different developmental stages could establish specific biochemical markers for genetic characterization and to establish correlation of the biochemical markers with morphological characters. The use unique bands from the study can be used as markers for identification of Njavara types from other rice genotypes.

ACKNOWLEDGMENTS

The authors are thankful for the help provided by National Bureau of Plant Genetic Resources, Vellanikkara in the collection of Njavara ecotypes for the study. Thanks to the Thrissur farmer, Mr. Kochaniyan Warrier for the timely help rendered for carrying out the field experiments. Thanks to the staff members of Department of Plant Breeding and Genetics and Centre for Plant Biotechnology and Molecular Biology for their support during the research work. The work was supported by the Kerala Agricultural University Junior Fellowship [2000-21-15].

REFERENCES

Arif, M., S. Kousar, M.A. Bajwa, A. Arif and Y. Zafar, 2005. Genetic diversity among rice genotypes of pakistan through Random Amplified Polymorphic DNA (RAPD) analysis. Pak. J. Bot., 37: 585-592.

Berg, V. and H.J.W. Wijsman, 1982. Genetics of the peroxidase isozymes in petunia. Theor. Applied Genet., 63: 33-38.

Bimb, H.P., R.P. Sah and N.L. Karn, 2004. Isozyme variations in fine and aromatic rice genotypes. Nepal Agric. Res. J., 5: 59-66.

Dattarwal, S., L.K. Chugh, S. Dhillon and R. Singh, 1999. Alcohol dehydrogenase isoenzymes in relation to identification of basmati rice genotypes. Oryza, 36: 261-262.

Dellaporta, S.L., J. Wood and J.B. Hicks, 1983. A plant DNA minipreparation: Version II. Plant Mol. Biol. Rep., 1: 19-21.

Elsy, C.R., C.A. Rosamma and N.N. Potty, 1992. Njavara- A rice variety with special characters. Oryza, 29: 55-56.

Elsy, C.R., G.S. Reddy and V.V., Radhakrishnan, 2004. Morphological and isozyme characterization of the medicinal rice cultivar Njavara. Proceedings of Compendium of papers of Science Society Interface on Medicinal and Aromatic Rices, March 20-21, Pattambi, Palakkad, India, pp: 131-131.

Grover, A. and D. Pental, 1992. Interrelationships of *Oryza* species based on electrophoretic patterns of alcohol dehydrogenase. Can. J. Bot., 70: 352-358.

IRRI, 1995. Standard Evaluation System for Rice. International Rice Research Institute, Los Banos, Philippines.

Jain, S., R.K. Jain and S.R. McCouch, 2004. Genetic analysis of Indian aromatic and quality rice (*Oryza sativa* L.) germplasm using panels of fluorescently-labeled microsatellite markers. Theor. Applied Genet., 109: 965-977.

Jena, K.K. and D.J. Mackill, 2008. Molecular markers and their use in marker-assisted selection in rice. Crop Sci., 48: 1266-1276.

Jose, A.I., S. Paulose, P. Prameela and B.P. Bonny, 2002. Package of Practices Recommendations: Crops 2002. 12th Edn., KAU., Thrissur, India.

Mackill, D.J., 2007. Molecular Markers and Marker-Assisted Selection in Rice. In: Genomics Assisted Crop Improvement, Varshney, R.K. and R. Tuberosa (Eds.). Springer, New York, pp: 147–168.

Menon, M.V. and N.N. Potty, 1999. Nutritional specificity and quality properties of medicinal rice Njavara. Oryza, 36: 315-317.

Mukherjee, M. and A.K. Dutta, 2008. Evaluation of genetic diversity in five species of Ocimum by SDS-PAGE. Indian J. Genet. Plant Breed., 68: 212-214.

Rahman, S.N., M.S. Islam, M.S. Alam and K.M. Nasiruddin, 2007. Genetic polymorphism in rice (*Oryza sativa* L.) through RAPD analysis. Indian J. Biotechnol., 6: 224-229.

Sharma, S.C. and S.R. Maloo, 2006. Protein electrophoregrams use in soyabean (*Glycine max* (L.) Merrill) cultivar identification. Indian J. Genet. Plant Breed., 66: 79-81.

Sharma, T.R., J.C. Rana, R. Sharma, R. Rathour and P.N. Sharma, 2006. Genetic diversity analysis of exotic and Indian accessions of common bean (*Phaseolus vulgaris* L.) using RAPD markers. Indian J. Genet. Plant Breed., 66: 275-278.

Shaw, C.R. and A.L. Koen, 1965. On the identity of nothing dehydrogenase. J. Histochem. Cytochem., 13: 431-433.

Shivapriya, M. and S. Hittalmani, 2006. Detection of genotype specific fingerprints and molecular diversity of selected Indian locals and landraces of rice (*Oryza sativa* L.) using DNA markers. Indian J. Genet., 66: 1-5.

Sreejayan, V.R.K. and G. Thomas, 2003. Collection and morphological evaluation of Njavara, a traditional medicinal rice (*Oryza sativa* L.), in Kerala, India. Plant Genet. Resour. Newslett., 135: 12-17.

Thanh, V.C., P.V. Phuong, P.H.H. Uyen and P.P. Hien, 2006. Application of protein electrophoresis SDS-PAGE to evaluate genetic purity and diversity of several varieties. Proceedings of International Workshop on Biotechnology in Agriculture, Oct. 20-21, Nong Lam University Ho Chi Minh City, pp: 192-194.

Zhang, J., K. Xiao, Y. Zhang, Y. Dong, J. Xu, Z. Li, L. Luo and M. Matsuo, 2008. Quantitative trait loci analysis for plant morphological traits in rice (*Oryza sativa* L.) under different environments. Int. J. Plant Breed. Genet., 2: 1-8.