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Biochemical and molecular markers for establishing distinctiveness of aromatic rice (*Oryza sativa* L.) varieties

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Abstract

Use of biochemical and molecular markers in DUS testing for establishing distinctiveness as a complement to morphological descriptors has been attempted in this study. Twenty indigenous aromatic rice (*Oryza sativa* L.) varieties were studied for morphological descriptors, total soluble proteins and isozymes as biochemical and RAPD molecular markers for determining distinctive features. SDS-PAGE for total soluble proteins and isozyme analysis revealed moderate and moderate to high degree of polymorphism respectively. UPGMA analysis of combined isozyme data of different enzymes could discriminate all varieties except Bindli from Tilakchandan and two related varieties of Tilakchandan. In general neither morphological descriptors nor biochemical markers could discriminate especially related indigenous varieties of a particular group. A high degree of polymorphism was detected among the twenty aromatic rice varieties through 9 random primers used for RAPD marker analysis. UPGMA cluster analysis of RAPD data could distinguish all the twenty rice varieties. It can be concluded that in situations where the morpho-physiological DUS descriptors are not able to establish distinctiveness of a variety then biochemical and molecular markers may be used as additional or complement descriptors for resolving distinctiveness of indigenous related varieties.

Key words: Rice, DUS, isozymes, RAPD

Introduction

India being one of the secondary centres of origin of rice diversity is blessed with a rich diversity of around 1,20,000 accessions of landraces, farmer's varieties and wild relatives [1]. Aromatic rices constitute a small but special group of rices which are considered best in quality. Majority of the Indian indigenous aromatic rices are small and medium grained but may surpass basmati types in many of the quality characteristics [2]. There are a number of indigenous aromatic rice varieties currently under production whose identity and distinctiveness need to be established by various approaches. Varietal registration has attained a critical importance all over the world including India. Testing for distinctiveness, uniformity and stability (DUS) is an essential component of variety registration procedure. In Europe testing procedures are determined by International Union for the Protection of New Varieties of Plants (UPOV). India has enacted a *sui generis* legislation as Protection of Plant Varieties and Farmers' Rights Act, 2001 (PPV&FR) similar to UPOV Acts. The PPV&FR Act recognizes the Plant Breeders Rights as well as rights involved in commercial exploitation of protected varieties. The Act recognizes the farmers as breeders who bred new varieties as well as conserved the traditional varieties. Like UPOV, under PPV&FR Act a variety must fulfill the criteria of DUS and novelty (if new) so as to get protection under this Act [3]. There are 62 morpho-physiological characteristics for rice which are species specific and, recommended procedures for conducting trials are given in the guidelines [4]. As per the DUS guidelines, only morpho-physiological descriptors are used. However, serious problems may arise for establishing distinctiveness of variety only on morpho-physiological DUS descriptors as the number of candidate varieties are growing with decreased variability as well as expansion of reference collections [5]. In such situations, biochemical and molecular markers can be considered as additional descriptors for establishing the distinctiveness of a variety.

Biochemical markers, especially the electrophoretic profiles of isozymes and proteins have been widely used for identification of crop varieties. Electrophoretic methods have been standardized for a

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large number of crops and found useful for the purpose of variety identification and characterization [6]. Though protein/isozyme markers alone may not be sufficient in resolving the identity of the variety, these can provide useful supplementary information which in combination with morphological descriptors provide identification keys. Availability of a large number of polymorphic molecular markers *viz*., RAPD, RFLP, SSRs has created an interest in their use for varietal identification. Though RAPDs are dominant in nature but this technique has been used quite satisfactorily in discriminating genotypes and is having considerable advantage over morphological characteristics as currently used in varietal identification [7-9]. The present study was conducted on 20 indigenous aromatic rice varieties using biochemical (*viz.* total soluble proteins and isozymes) and molecular (RAPD) markers as additional markers to morphological descriptors for establishing the distinctiveness of a variety.

Materials and methods

A total of 20 indigenous aromatic rice varieties were studied (Table 1) for 60 of 62 DUS morpho-physiological characteristics as notified by PPV&FR Authority [4]. Two characteristics *viz*., polished grain: expression of white core and culm attitude (for floating rice) were not applicable to the material under study. The experiments were conducted during the two *kharif* seasons of 2005 and 2006 in randomized block design with 3 replications. Each replication consisted of 3 rows of 6 m length with 30×20 cm spacing. Among the 60 morphological characteristics studied, 46 were visually assessed and 14 were measured. The observations were recorded at specified stages of crop growth period when characteristics under study had full expression. Characterization of varieties was done according to eight morpho-physiological grouping characteristics reported in the DUS test guidelines for rice [4].

Table 1. Aromatic rice genotypes studied

1	Kalanamak- 3131	11	Tilakchandan- 3048
2	Kalanamak- 3213	12	Sakkarchini
3	Kalanamak- 3216	13	Lalmati
4	Kalanamak- 3327	14	Lal Ram Jawain
5	Bindli- 3173	15	Govind Bhog
6	Bindli- 3255	16	Badshah Bhog
7	Bindli- 3192	17	Dubraj
8	Bindli-3193	18	Chini Kapoor
9	Tilakchandan	19	Kalajira
10	Tilakchandan- 3047	20	Gopal Bhog

1. Biochemical characterization

a) *Total protein analysis by SDS-PAGE:* Total soluble proteins were extracted by hand grinding of 1gm decorticated grains in 2 ml chilled Tris-sucrose homogenization buffer containing 0.1 M Tris, 0.4 M Sucrose, 10 mM KCl, 0.1 % v/v β-mercaptoethanol and 1 mM each of $MgSO_4$, EDTA and PMSF. The homogenate obtained was centrifuged at 12,000 rpm for 30 min and the supernatant was further used for electrophoresis in a 12% SDS polyacrylamide gel.

b) *Isozyme analysis by Native-PAGE*: For isozyme analysis, seven days old etiolated seedlings were ground with chilled extraction buffer (50 mM Tris.Cl buffer (pH 7.6) containing 5 mM each of β-mercaptoethanol and EDTA) with sample to extraction buffer ratio of 1:2 w/v. The homogenate obtained was centrifuged at 12,000 rpm for 30 min and the supernatant obtained was further separated on a 7% polyacrylamide gel using an anionic system and stained for five enzymes *viz.* alcohol dehydrogenase (ADH) [10], malate dehydrogenase (MDH) [11], esterase (EST) [12], peroxidaxe (POX) [13] and superoxide dismutase (SOD) [14].

2. Molecular characterization

Molecular characterization was conducted with 9 RAPD primers (Operon Tech, USA and Sigma Genosys, USA), which were selected based on previous studies [15, 16]. DNA was isolated from 10 days old etiolated seedlings using CTAB method of Doyle and Doyle [17] with some modifications. PCR was performed in a 0.2 ml reaction tube in volume of 25 µl, consisting of 100 ng genomic DNA, 2.5 mM each of dNTPs, 1 U *Taq*, 0.15 mM $MgCl_2$ and 1X reaction buffer supplied by Life Tech. India Pvt. Ltd., and 50 ng primer. The amplification reaction was carried out in a Eppendorf thermocycler, which was programmed for pre-denaturation at 94°C for 5 min, followed by 44 cycles of denaturation at 94°C for 1 min, annealing at 36°C for 1 min and extension at 72°C for 2 min. The final cycle allowed an additional 7 min period of extension at 72°C. The whole reaction mixture was loaded on to a 1.5 % agarose gel made in 1X TAE buffer containing ethidium bromide at 10mg/ml and the DNA fragments were visualized under UV light and photographed.

Statistical analysis

Varietal profiles generated from total proteins, isozymes and RAPD analysis were scored according to the

presence (1) or absence (0) of bands and the data entry was done into binary matrix as discrete variables. Jaccard's coefficient of similarity was measured and a dendrogram was generated using Unweighted Pair Group Method with Arithmetic Average (UPGMA). The computer package NTSYS-PC version 1.80 [18] was used for cluster analysis to measure the relationships between the varieties.

Results and discussion

The accurate description of indigenous aromatic rice varieties is crucial for registration under PPV&FR Act. The identity/profile of a rice variety is to be established by using a set of morphological characteristics prescribed in the DUS test guidelines on rice. Out of the 46 visually assessed DUS descriptors studied, 24 were found to be monomorphic, 17 characteristics were dimorphic and 5 characteristics were polymorphic, while out of 14 measurable descriptors, one was monomorphic, 6 were dimorphic and 7 characters were polymorphic.

Eight grouping characteristics have been mentioned in the DUS test guidelines for determining distinctiveness of the varieties. Two grouping characteristics of basal leaf sheath colour and decorticated grain aroma were monomorphic in the varieties under study. Grouping of varieties was thus based on 6 characteristics viz. time of heading, stem length, decorticated grain length, decorticated grain shape, decorticated grain colour and endosperm content of amylose. Distinctive profiles were obtained for seven varieties viz. Govind Bhog, Lal Ram Jawain, Badshah Bhog, Lalmati, Dubraj, Sakkarchini and Kalajira (Fig. 1). The rest of the thirteen varieties remained in three different groups.

Thus, grouping characteristics and DUS descriptors of morpho-physiological nature which were mentioned in DUS guidelines could establish distinctiveness for some of the varieties but these alone were not sufficient for establishing the distinctiveness of especially related varieties or similar indigenous varieties grown in a particular niche. Hence for establishing distinctiveness of a particular variety biochemical and molecular markers were considered.

SDS-PAGE analyses of total soluble proteins

The electrophoresis of total soluble seed proteins revealed a total of 35 polypeptide bands which were classified in to two zones- A and B. Polymorphic bands were observed only in zone A which comprised of 18 bands. UPGMA cluster analysis was able to individually distinguish varieties Gopal Bhog and Bindli 3173 while other varieties fell in different groups e.g. Chini Kapoor and Badshah Bhog; Lalmati, Shakkarchini and Govind Bhog; all the similar Bindli varieties in one group and also similar varieties of Tilakchandan and Kalanamak. Some specific polypeptide bands were present in particular varieties and similar varieties of one particular group, e.g. Tilakchandan can be discriminated from Bindli group. But Tilakchandan and Kalanamak varieties could not be discriminated as they fall in one group (Fig. 2).

Electrophoretic analysis of total soluble proteins is widely recognized as a technique for cultivar identification and even UPOV has recommended SDS-PAGE for analysis of high molecular weight glutenins in wheat [19] and hordeins in Barley [20]. But, here it appeared to be of limited use for the establishment of distinctiveness of closely related varieties of indigenous aromatic rice.

Analysis of isozymes profiles

Native-PAGE for analysis of isozymes of five enzymes *viz*. ADH, MDH, EST, POX and SOD was conducted. ADH and SOD revealed monomorphic banding pattern for all the aromatic rice varieties under the study. However, for the other three enzymes a moderate degree of polymorphism was revealed. Some unique isozymic bands were observed in the varieties. Esterase banding pattern identified Badshah Bhog with unique band (EST 6). Likewise MDH-2 was present in all the varieties of Tilakchandan and Bindli alongwith Dubraj but absent in all other varieties. MDH-4 was present in all the varieties except for Lalmati and Dubraj. Absence of POX-4 in Kalanamak 3327 and POX-6 in Bindli-3193 while present in all other varieties was very specific. Using the combined isozymic biochemical data, UPGMA cluster analysis was able to discriminate all the varieties under the study except Bindli 3192 which showed similarity to Tilakchandan. Two other related varieties of Tilakchandan also showed 77% similarity (Fig. 3). This can be explained by the fact that isozyme markers are known to limit the estimates to only a part of the coding region of the genome.

UPOV has included isozyme markers as additional characteristics in case of maize [21] and soybean [22]. The use of biochemical markers thus can be an additional marker for varietal characterization in case of disputes between the varieties.

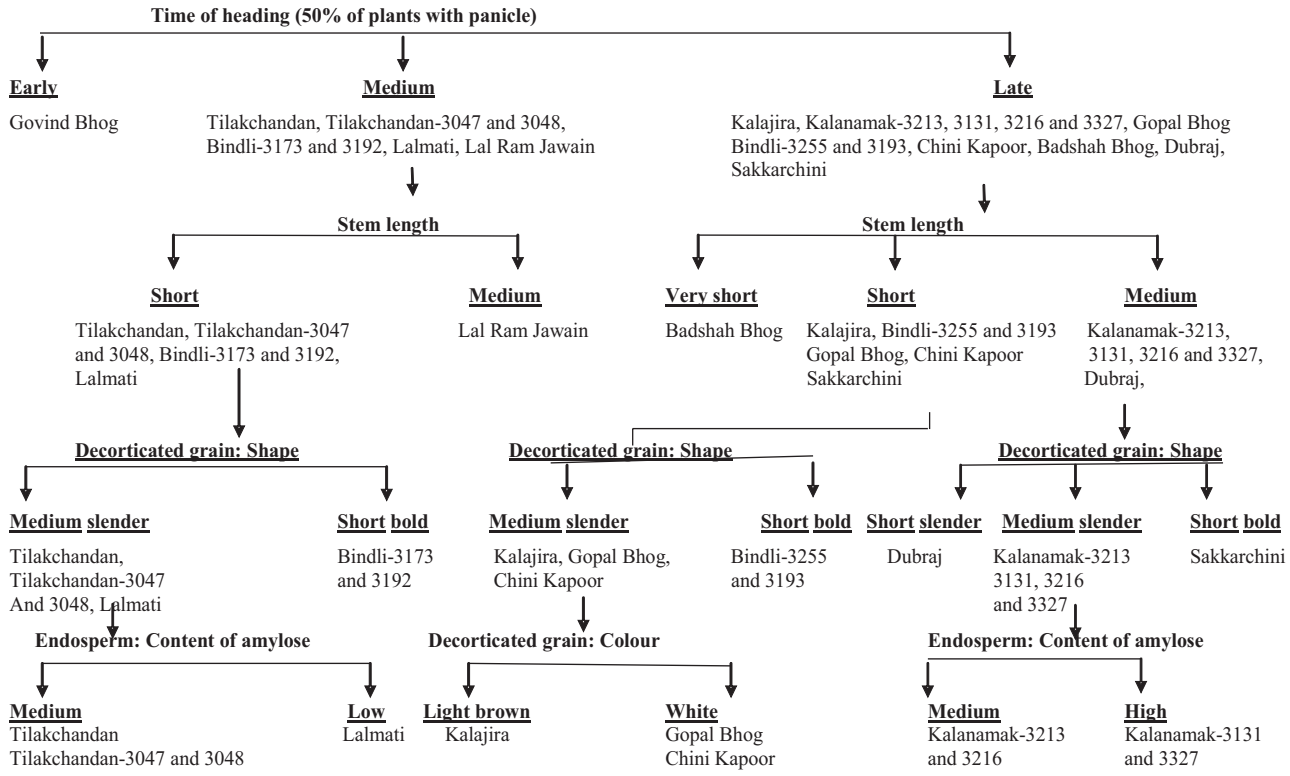

Fig. 1. Grouping of aromatic rice varieties based on the grouping characteristics proposed in the DUS test guidelines

RAPD analysis

The RAPD bands generated from 9 random primers showed high polymorphism. Unique bands were repeatedly amplified from different RAPD primers (Fig. 5), which could identify the varieties – Bindli 3173 (OPD-08), Lalmati (OPD-08), Bindli 3193 (OPD-06), Badshah Bhog (OPD-06), Govind Bhog (OPH 19), Dubraj (OPD-08, OPH 19 and ADG 4) and Sakkarchini (OPH-20). Cluster analysis based on molecular data was able to distinguish eighteen varieties out of twenty. Kalanamak 3213 and Kalanamak 3216 fell in one group and showed 75% similarity (Fig. 4). The clustering obtained through this molecular profiling roughly conformed to the

Table 2. Random primers with their codes used in the study

Primer	Sequence
(5' to 3')	Code
CACAGGCGGA	OPK-19
GTGTGCCCCA	OPD-08
GGGAATTCGG	OPD-06
CTGACCAGCC	OPH-19
GGAAGTCGCC	OPH-04
GGGAGACATC	OPH-20
CCCGCCGTTG	ADG-4
CCGCCCAAAC	OPK-04
GTTTCGCTCC	OPB-1

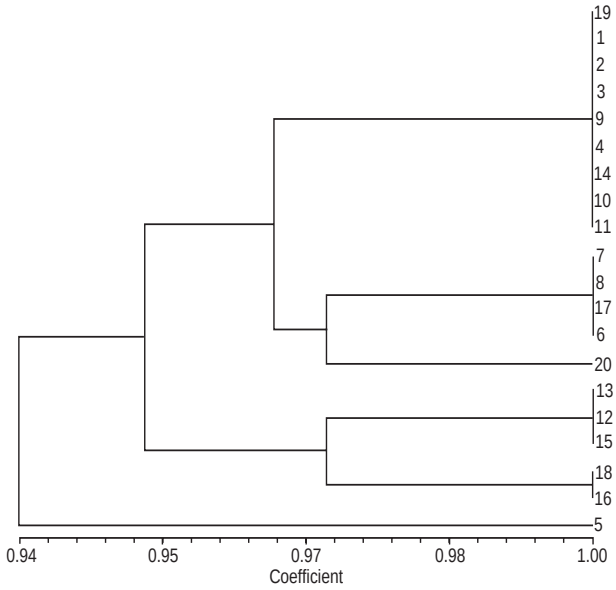

Fig. 2. UPGMA cluster analysis of twenty aromatic rice genotypes on the basis of SDS-PAGE of total protein profile (1-20 – Names of varieties as mentioned in Table 1).

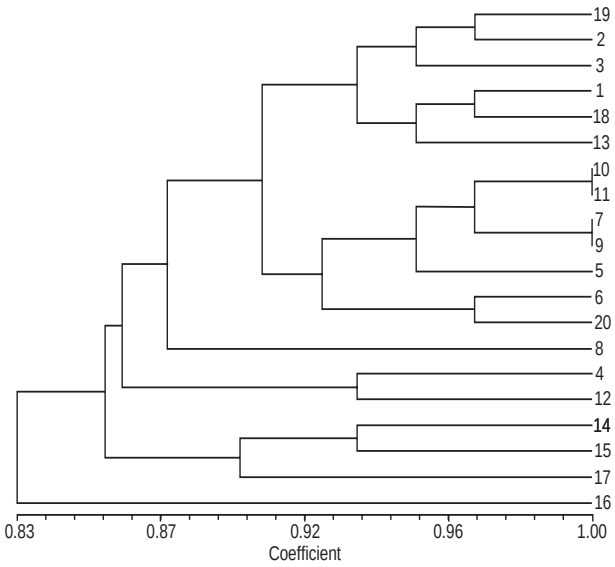

Fig. 3. UPGMA cluster analysis of twenty aromatic rice genotypes on the basis of isozyme profile of five different enzymes (1-20 – Names of varieties as mentioned in Table 1).

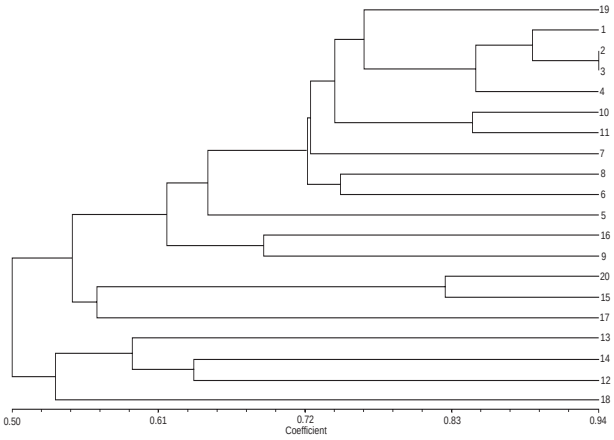

Fig. 4. UPGMA cluster analysis of twenty aromatic rice genotypes on the basis of RAPD profile (1-20 – Names of varieties as mentioned in Table 1).

pedigree of the varieties with varieties of Bindli, Tilakchandan-3047 and 3048 and Kalanamak falling in the same clusters. Kalanamak varieties amongst them showed 70% similarity and this group showed 37% resemblance with Kalajira. All the four varieties of Bindli and Tilakchandan-3047 and 3048 showed 70-74% similarity.

The relationship between different markers was assessed by correlating the genetic similarity/ dissimilarity matrices of different markers. The similarity/ dissimilarity coefficients of twenty varieties measured through different morphological (visually assessed and measurable characters), SDS-PAGE, isozymes and RAPD data were subjected to calculate the Pearson's correlation coefficient (Table 3). It revealed highly significant correlations for RAPD with SDS-PAGE (r = 0.3977), isozymes with visually assessed characters (r = 0.19148) and visually assessed characters with measurable characters (r = 0.58598). Isozymes also showed significant correlation with measurable characters (r = 0.21876).

Based on the observed results, it can be concluded that in situations where the morpho-physiological DUS descriptors fails to establish distinctiveness of a variety then biochemical and molecular markers may be used as additional descriptors for resolving distinctiveness of indigenous aromatic rice varieties for granting plant variety protection under PPV&FR Act. Among the biochemical markers, the efficacy of total seed protein markers was found to be limited in delineating closely related varieties. Isozyme marker analysis was able to generate

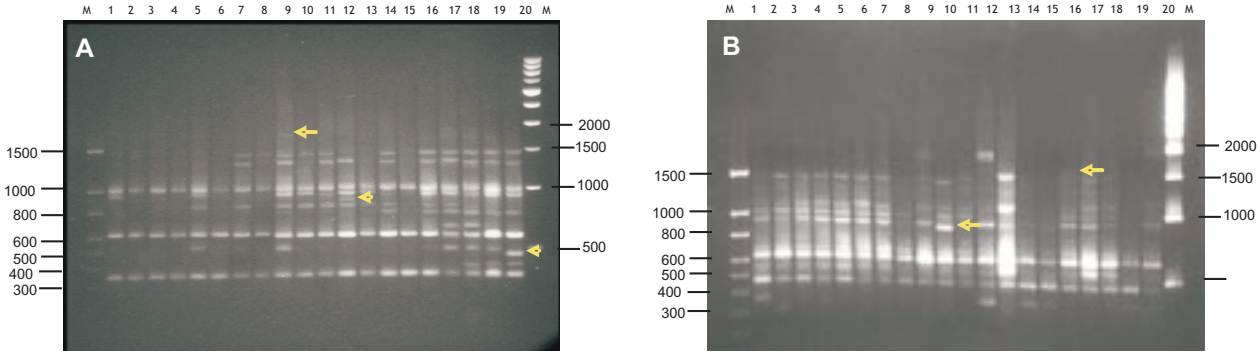

Fig. 5. Rapid amplification patterns of 20 indigenous aromatic rice varieties by the primers OPD-08 (A) and OPD-06 (B)

Table 3. Pearson correlation (Mantle t-test) between genetic similarity/ dissimilarity matrices based on different marker systems. Euclidean distances were used for morphological descriptors and Jaccard coefficients of similarity for molecular markers

	RAPD	Isozymes	SDS-PAGE	Visually assessed characters	Measurable characters
RAPD	-	0.19150	0.39770**	0.03945 ^{NS}	0.01504
Isozymes	-	-	0.17078	0.19418**	0.21876*
SDS-PAGE	-	-	-	0.08391	0.05600
Visually assessed characters	-	-	-	-	0.58598**
Measurable characters	-	-	-	-	-

*,**Significant at 5% and 1% level of probability, respectively

a moderate level of polymorphism and hence can be used as supplementary criteria for characterization. Results of this study provide sufficient evidence that molecular markers would increase the standards of DUS testing, if included. Besides, their introduction could offer several advantages. Molecular markers showed better resemblance with the pedigree as compared to morphological markers and are in agreement with the results of a study on maize inbred lines [23]. Resemblance with the pedigree would nominate molecular markers as good candidates for use in essential derivation procedures, but this relationship is not expected to be perfect one [24]. Further advantage of molecular markers is their relatively higher discrimination power generated by more balanced distribution of allele frequencies. Thus molecular profiling would present a valuable addition to DUS testing procedures in distinguishing closely related varieties in addition to morpho-physiological descriptors.

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