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Biochemical and RAPD molecular markers for establishing distinctiveness of basmati rice (*Oryza sativa* L.) varieties as additional descriptors for plant variety protection

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India has enacted a *sui generis* legislation as Protection of Plant Varieties and Farmers' Rights Act 2001 (PPV&FR) for protection of plant varieties by registration. Under PPV&FR Act DUS (distinctiveness, uniformity, stability) testing procedure will be performed with morphological descriptors. Use of biochemical and molecular markers in DUS testing for establishing distinctiveness as a complement to morphological descriptors has been attempted in this study. Eighteen traditional and improved basmati 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 Hansraj from KLS 24. A high degree of polymorphism (76.5%) was detected among the 18 basmati rice varieties through 12 random primers used for RAPD marker analysis. UPGMA cluster analysis of RAPD data could distinguish all the 18 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 basmati rice varieties.

Keywords: Basmati, rice, DUS, isozymes, RAPD, discriminative power, *Oryza sativa*

Introduction

Rice (*Oryza sativa* L.) is a staple food for over 60% the world's population[1]. Aromatic rice constitutes a special group of small-, medium-, and long-grain type rice accessions well known for their aroma and/or superfine grain quality. There are many known groups of aromatic varieties such as basmati rice from India and Pakistan and jasmine rice from Thailand. Many farmers in India grow local strains of basmati rice. Besides, basmati rice varieties with higher yield potentials have been developed by incorporating the desirable traits. Thus, there are a number of basmati rice varieties, both traditional and improved types, currently under production whose identity and distinctiveness need to be established by various approaches[2].

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, the testing procedures are determined by International union for the protection of new varieties of plants (UPOV). India has however, enacted a *sui generis* legislation as Protection of Plant Varieties and Farmers' Rights Act, 2001 (PPV&FR) something like UPOV Act. The PPV&FR Act recognizes the plant breeders rights as well as rights involved in commercial exploitation of protected 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. This study was conducted in anticipation, if the morpho-physiological DUS descriptors are not able to discriminate varieties,

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then 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 large number of crops and found useful for the purpose of variety identification and characterization[5]. Though protein/ isozyme markers alone may not be sufficient in resolving the identity of a variety, these can provide useful supplementary information, which in combination with morphological descriptors will provide identification keys. Availability of a large number of polymorphic molecular markers has created an interest in their use for varietal identification. Notwithstanding doubts about reproducibility and intra-varietal uniformity of RAPDs, the technique is found to be highly discriminatory, and having considerable advantages over morphological characteristics currently used in varietal identification[6-8]. The present study was conducted on 18 traditional as well as cross-bred basmati rice varieties using biochemical (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 18 traditional as well as cross-bred basmati 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 grains and expression of white core and culm attitude (for floating rice) were not applicable to the materials under study. The experiments were conducted during two kharif seasons 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 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]

1. Biochemical Characterization

Total Protein Analysis by SDS-PAGE

Total soluble proteins were extracted by hand grinding of 1 g decorticated grains in 2 mL chilled Tris-sucrose homogenization buffer containing 0.1 *M* Tris, 0.4 *M* Sucrose, 10 m*M* KCl, 0.1% v/v β-mercaptoethanol and 1 m*M* 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.

Isozyme Analysis by Native-PAGE

For isozyme analysis, 7-d-old etiolated seedlings were ground with chilled extraction buffer [50 m*M* Tris.HCl buffer (*p*H 7.6) containing 5 m*M* each of β-mercaptoethanol and EDTA] with sample to extraction buffer in the 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), malate dehydrogenase (MDH), esterase (EST), peroxidaxe (POX) and superoxide dismutase (SOD) following appropriate staining protocols.

2. Molecular Characterization

Molecular characterization was conducted for RAPD analysis using 12 random decamer primers (Operon Technologies, and Sigma Genosys, USA),

Table 1—Basmati rice varieties studied for morpho-physiological characteristics

No.	Variety	Pedigree/Origin
1	Hansaraj-3078	Traditional land race (Uttarakhand)
2	Hansaraj	Traditional land race (Uttarakhand)
3	Lal Basmati	Traditional land race (Purola valley of Uttarakhand)
4	Super Basmati	IRR-662/ Bas 320
5	Yamini	BR4/Pakistani basmati
6	Kastoori	Bas-370/Cr-88-17-15
7	Basmati-386	Selection from Pakistani basmati
8	Basmati-98/69-7-2	Traditional land race (Uttarakhand)
9	KLS-24	Karnal
10	Pusa Basmati-1	Pusa 150/Karnal Local
11	Tapovan Basmati	Traditional land race(Rishikesh) Uttarakhand
12	Taroari Basmati	Pure line selection from Karnal local
13	Type-3	Traditional land race (Dehradun)
14	PSD-17	Pusa Basmati-1/UPRI-95-154
15	PSD-15	Basmati-370/Sadri/Bahral/Muskan-41
16	Pusa Sugandh-2	Basmati restorer line
17	Pusa Sugandh-3	Basmati restorer line
18	Basmati-370	Introduction from Pakistan

which were selected based on previous studies[9,10]. DNA was isolated from 10-d-old etiolated seedlings using CTAB method[11] 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 m*M* each of dNTPs, 1 U *Taq*, 0.15 m*M* $MgCl_2$ and 1X reaction buffer supplied by Life Technologies India Pvt Ltd, and 50 ng primer supplied by Operon Technologies and Sigma Genosys. 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 10 mg/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 was used for cluster analysis to measure the relationships between the varieties[12].

Results and Discussion

The accurate description of basmati 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 60 DUS descriptors studied, 27 were found to be monomorphic, 22 dimorphic and 11 polymorphic. Maximum polymorphism was observed for panicle characteristics followed by grain characteristics. Low level of polymorphism especially for leaf and stem characters was obtained which might be due to the fact that the indigenous basmati cultivars were domesticated in their respective ecological zones with narrow genetic base. The low level of polymorphism was reported in sorghum local cultivars also for the DUS descriptors[13].

Eight grouping characteristics have been mentioned in the DUS test guidelines for determining distinctiveness of the varieties. Three grouping characteristics viz. decorticated grain colour, basal leaf sheath colour and decorticated grain aroma were monomorphic in the varieties under study while decorticated grain length character and decorticated grain shape exhibited similar grouping pattern in basmati varieties. Thus, grouping of varieties was based only on 4 characteristics, viz. time of heading, stem length, decorticated grain length and endosperm content of amylose. On the basis of grouping characters as Gazette notified by Govt. of India in the PPV&FR Act, eight varieties which could be discriminated from the others are: Pusa Basmati-1, Pusa Sugandh-2, Type-3, Basmati-370, Basmati-386, Hansraj-3078, Hansraj and PSD-15 (Fig. 1). Out of the 18 varieties studied 10 remained in groups, which could not be discriminated on the basis of morphological grouping characteristics.

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

SDS-PAGE Analysis of Total Soluble Proteins

The electrophoresis of total soluble seed proteins revealed a total of 24 polypeptide bands, out of which 10 were polymorphic showing a moderate degree of polymorphism. UPGMA cluster analysis was able to individually distinguish following nine varieties: Bas-98/69-7-2, Super Basmati, Kastoori, PSD-17, Taroari Basmati, Tapovan Basmati, Type-3, Basmati-370 and Pusa Basmati-1 (Fig. 2). Hansraj and Hansraj-3078 exhibited 100 per cent similarity and likewise two other pairs *viz.* Yamini and Basmati-386 which shared Pakistani Basmati as a common parent and two basmati restorer lines, Pusa Sugandh-2 and Pusa Sugandh-3. 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[14] and hordeins in barley[15]. But in case of basmati rice from our studies it appears to be of limited use for the establishment of distinctiveness of closely related varieties.

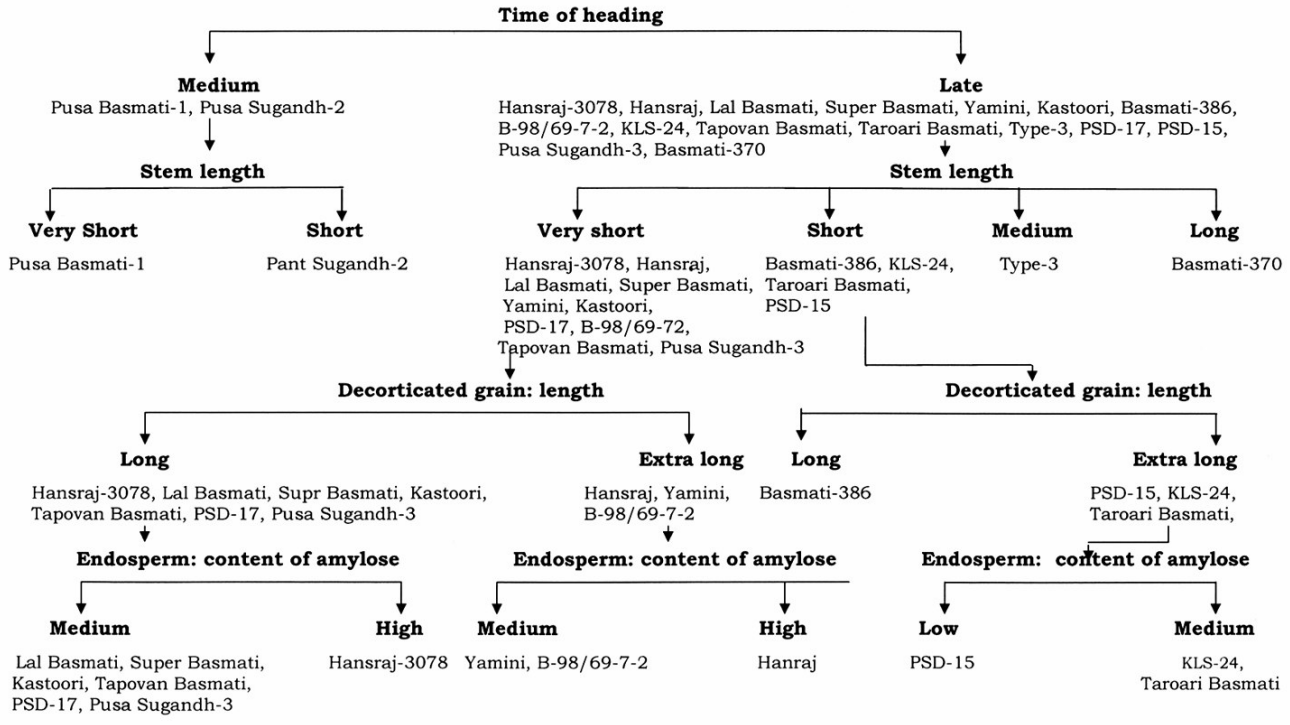

Fig. 1—Grouping of varieties based on the grouping characteristics proposed in the DUS test guidelines.

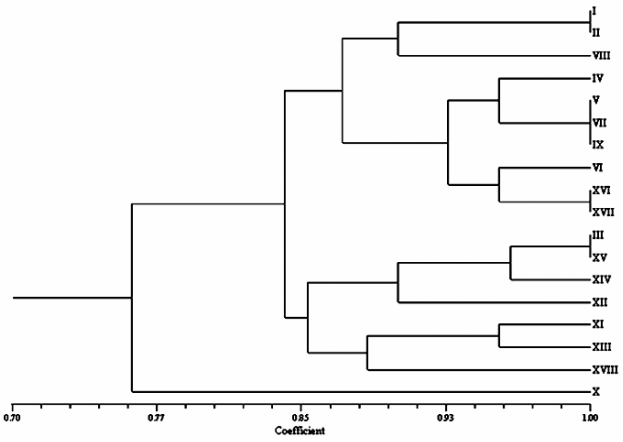

Fig. 2—UPGMA cluster analysis of 18 basmati rice genotypes on the basis of SDS-PAGE of total protein profile (I to XVIII – Names of varieties as mentioned in Table 1).

Analysis of isozyme profiles by Native-PAGE

Isozymes of five enzymes, viz. ADH, MDH, EST, POX and SOD were analysed by Native-PAGE. SOD revealed monomorphic banding pattern for all the basmati rice varieties under study. However, for the other four enzymes a moderate to high degree of polymorphism among the basmati rice varieties was revealed. Some unique isozymic bands were observed in the varieties. Super Basmati is the only variety which showed the presence of two bands ADH-1 and ADH-2 (Fig. 3 a,b). Hansraj-3078 could be distinguished from the Hansraj by the absence of MDH-4 in the latter. Similarly, Pusa Sugandh-2 showed the presence of MDH-2 and MDH-4 while its most similar variety Pusa Sugandh-3 showed the absence of these bands (Fig. 3 e,f). Lal Basmati and Kastoori showed a unique dark band EST-3 which was absent in all the other varieties (Fig. 3 c,d). POX-3 was present as a dark band in Pusa Sugandh-3 unlike Pusa Sugandh-2 where it was present as a light band.

Using the combined isozymic biochemical data, UPGMA cluster analysis was able to discriminate all the varieties under the study except Hansraj which showed 100% similarity to KLS-24 but showed only 93.3% similarity to its derived variety Hansraj-3078 (Fig. 4). 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[16].

UPOV has included isozyme markers as additional characteristics in case of maize[17] and soybean[18]. The biochemical markers thus can be used as additional markers for varietal characterization in the case of disputes between the varieties.

RAPD Analysis

The RAPD bands generated from 12 random primers showed 76.5% polymorphism. Unique bands were amplified from different RAPD primers, which

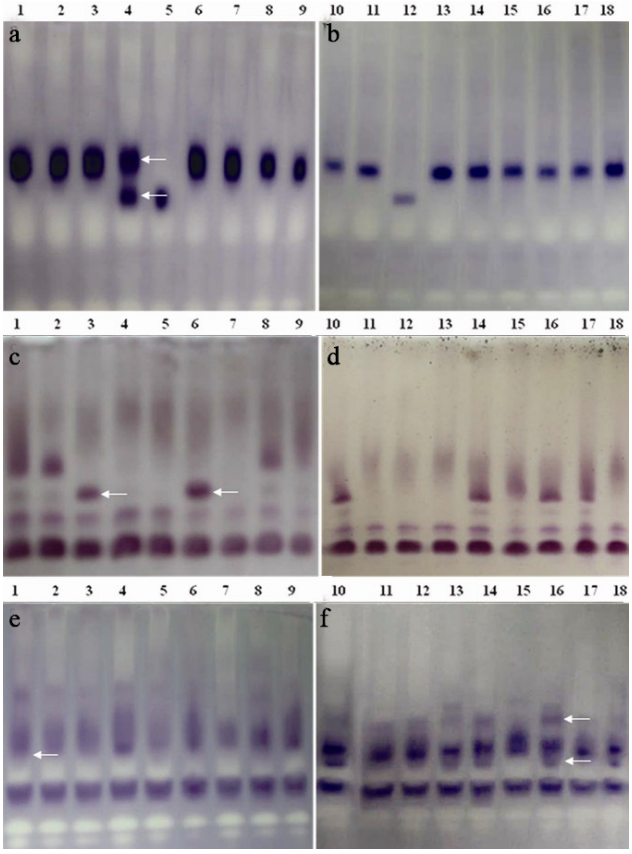

Fig. 3—Isozyme banding patterns of 18 basmati rice varieties. a & b-Alcohol dehydrogenase enzyme; c & d- Esterase enzyme; e & f -Malate dehydrogenase enzyme. 1- Hansraj-3078; 2- Hansraj; 3- Lal Basmati; 4- Super Basmati; 5- Yamini; 6- Kastoori; 7- Basmati-386; 8- B-98/69-7-2; 9- KLS-24; 10- Pusa Basmati-1; 11- Tapowan Basmati; 12- Taroari Basmati; 13- Type-3; 14- PSD-17; 15- PSD-15; 16- Pusa Sugandh-2; 17- Pusa Sugandh-3; 18- Basmati-370.

could identify the varieties - Pusa Basmati-1 (OPK-19, OPD-08, OPH-19 and OPB-1), Pusa Sugandh-2 (OPK-19, OPD-06 and OPD-01), Tapovan Basmati (OPD-06), Super Basmati (OPD-06) and Kastoori (OPH-19) (Fig. 5). However, some varieties when compared with most similar varieties on the basis of morphological features or pedigree or derivation from common parent revealed differences in banding pattern. Distinctiveness of Hansraj-3078 from its parent variety Hansraj and the most similar varieties of Type 3 and Basmati-370 by different primers under study has been shown in Table 2. Hansraj from Hansraj-3078 could be distinguished by all the primers except OPH-20 and OPK-10 and likewise Hansraj could be distinguished from most similar varieties Type 3 and Basmati-370 by all the primers except OPD-8 and OPH-20. Similarly,

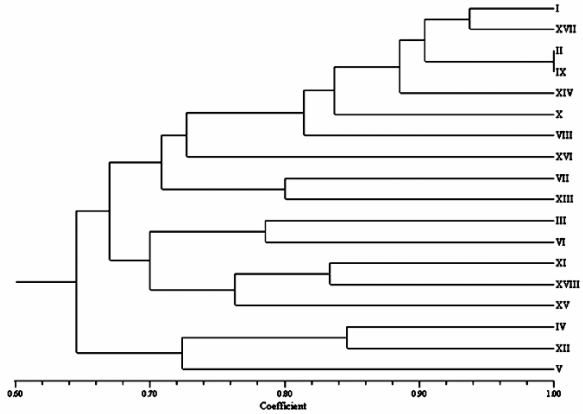

Fig. 4—UPGMA cluster analysis of 18 basmati rice genotypes on the basis of isozyme profile of five different enzymes (I to XVIII – Names of varieties as mentioned in Table 1)

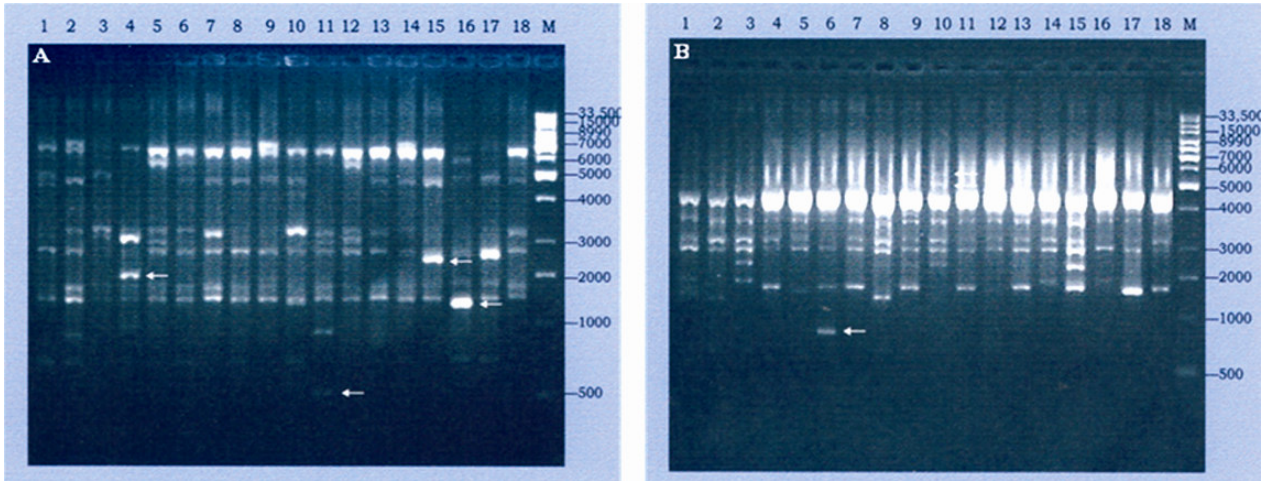

Fig. 5—RAPD amplification patterns of 18 basmati rice varieties by the primers OPD-06 (A) and OPH-19 (B). M - Marker Lane; 1- Hansraj-3078; 2- Hansraj; 3- Lal Basmati; 4- Super Basmati; 5- Yamini; 6- Kastoori; 7- Basmati- 386; 8- B-98/69-7-2; 9- KLS-24; 10- Pusa Basmati-1; 11- Tapowan Basmati; 12- Taroari Basmati; 13- Type-3; 14- PSD-17; 15- PSD-15; 16- Pusa Sugandh-2; 17- Pusa Sugandh-3; 18- Basmati-370.

Table 2—Distinctiveness of Hansraj from its derived variety Hansraj-3078 and most similar varieties

Primer	Derived variety	Most similar varieties	
	Hansraj-3078	Type -3	Basmati-370
OPK-19	D	D	D
OPD-08	D	ND	ND
OPD-06	D	D	D
OPH-19	D	D	D
OPH-04	D	D	D
OPH-20	ND	ND	ND
OPD-01	D	D	D
ADG-4	D	D	D
OPK-04	D	D	D
OPK-10	ND	D	D
OPB-1	D	D	D
OPY-02	D	D	D

Pusa Sugandh-2 and Pusa Sugandh-3 the two restorer lines used in hybrid seed production with derivation from common parent could be distinguished from each other by using the primers OPK-19, OPD-06, OPH-19, OPH-04, OPD-01 and OPB-1.

Cluster analysis, based on molecular data, was able to distinguish all the 18 basmati rice varieties. The clustering obtained through this molecular profiling roughly conformed to the pedigree of the varieties. Basmati-370, Basmati-386 and Yamini with their common origin from Pakistani Basmati fell into a cluster (Fig. 6). Similarly, two cross-bred basmati varieties, *viz*. Kastoori and PSD-15 which had Basmati-370 in their pedigree were grouped into a different cluster. Likewise, KLS-24, Pusa Basmati-1 and PSD-15, which had Karnal Local as a common parent, were grouped together.

Based on the foregoing results, 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 descriptors for resolving distinctiveness of basmati 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 a moderate level of polymorphism and hence could 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.

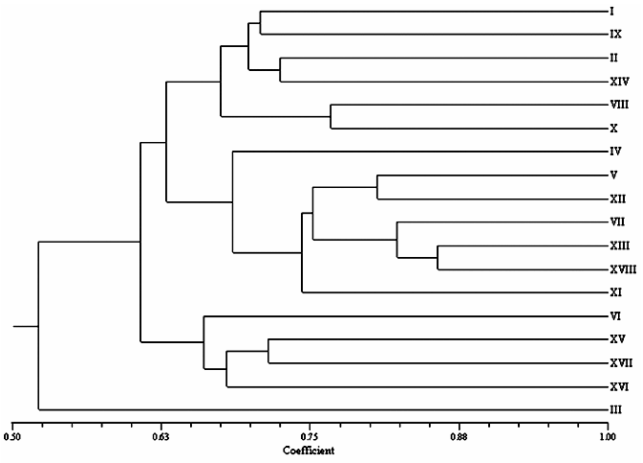

Fig. 6—UPGMA cluster analysis of 18 basmati rice genotypes on the basis of RAPD profile (I to XVIII – Names of varieties as mentioned in Table 1).

Besides, their introduction could offer several advantages. Molecular markers show better resemblance with the pedigree as compared to morphological markers. Present results fully support this statement and are in agreement with the results of a study on maize inbred lines[19]. 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[20]. Further, advantage of molecular markers is their relatively higher discrimination power generated by more balanced distribution of allele frequencies. This could indicate that erosion of variability introduced through breeding is expressed with higher intensity on morphological than on molecular level. Thus, molecular profiling would present a valuable addition to DUS testing procedures in distinguishing closely related varieties in addition to morpho-physiological DUS descriptors.

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