Suitability of seed esterases for establishing distinctness, uniformity and stability of pearl millet genotypes

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Plant morphological characters have been the universally undisputed descriptors applied for testing distinctness, uniformity and stability (DUS) of crop varieties. However, these descriptors, other than being limited in number, make the process time-consuming and also less reliable owing to its interaction with the environment in which the variety is grown and subjectivity in decision-making. The potential of biochemical descriptors such as seed esterases has been well established for diversity analysis and varietal identification. However, their suitability as DUS test characters merits investigation. In the present study, seed esterases were used to examine distinctness of 45 pearl millet genotypes comprising 14 hybrids and their parental lines. Among 45 genotypes studied, 36 could be differentiated from each other and 11 were grouped into four categories at 2.29×10^{-3} probability of identical match by chance. The utility of this marker to study uniformity and stability was analysed using two genotypes, 841B and D-23. Analysis of single-earhead seeds from 100 individual plants, which are morphologically similar, revealed no variations within a variety in 841B and one variant in D-23, which is attributed to the residual heterozygosity. The possibility of esterase markers for testing the DUS of new pearl millet genotypes to give protection under the Protection of Plant Varieties and Farmers' Rights Act, 2001 has been discussed.

Keywords: DUS test, pearl millet, plant variety protection, seed esterases.

IN India, pearl millet is an important dry-land cereal crop grown over an area of about 9.39 m ha. More than 70 genotypes, of which about 60 are hybrids, have been released in the last 35 years and their number will increase in future due to the ever-changing breeding objectives to meet the current and future demands of the producers and consumers. Apart from this, the Government of India under the obligation of the TRIPS agreement has recently passed the Protection of Plant Varieties and Farmers' Rights Act, 2001 (PPV&FR Act) to encourage public/private investment in research and development of new plant varieties by giving protection to the new plant genotypes against unauthorized multiplication of seeds or propagating materials for a specified period. The purpose of the Act will be achieved only when the new varieties are given proper protection under it.

The new pearl millet genotypes will be protected under the PPV&FR Act after confirming the distinctness, uniformity and stability (DUS) of new genotypes through DUS testing[1]. The DUS of the new genotype will be established by growing the new and existing varieties side by side and comparing them with respect to a set of morphological characters throughout the plant growth period. This will be time-consuming and expensive, requiring large areas of land and skilled personnel often making subjective decisions. Added to this, many of the characters used are multigenic or quantitative and their expression is altered by environmental factors, which essentially require replication of observations. It also demands costly cold storage facilities to store, and seeds of all protected and extant varieties. Further, the number of characters may not be sufficient for discrimination of all the extant and new varieties. There are thus compelling reasons to find more rapid and cost-effective procedures to augment this approach.

Biochemical markers assay variation in the polypeptides of proteins and isozymes based on size and/or charge differences. Such differences remain unaffected across different seasons, locations and agronomic practices. Varietal identification and differentiation therefore become more reproducible and objective. The characteristics of a new variety based on electrophoretic patterns of proteins/isozymes can therefore be compared with those commonly known in any part of the world for establishing its uniqueness. The working group on Biochemical and Molecular Techniques (BMT) of the International Union for the Protection of New Varieties of Plants (UPOV) has in fact identified isozymes markers that could be used as complementary characteristics in maize and soybean DUS testing[2,3], and protein markers in wheat and barley[4,5]. However, it is necessary that prior to the use of such markers in DUS testing, it would be essential to evaluate the uniformity and stability of the markers as used for distinctness. In pearl millet, when compared to other isozymes, esterases were found to have good resolution and wide variability in the entire *Pennisetum* gene pool[6]. Hence this marker was extensively used for diversity analysis,

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varietal characterization and seed purity testing in pearl millet[7–11]. In this background the present study was undertaken to examine the suitability of seed esterase marker for ensuring distinctness, uniformity and stability during DUS testing.

Materials and methods

Plant materials

For the present investigation, genetically pure seeds of 14 pearl millet hybrids, their parents (A and R lines) and maintainer (B) lines were obtained from the respective breeders (Table 1) in the pearl millet breeding stations located in different regions of India. The seeds were multiplied by selfing (B and R lines) and crossing (A × B to produce seeds of respective A lines and A × R to produce seeds of respective hybrids) true-to-type plants in the field. Ten seeds of each genotype from both the generations (seeds received from pearl millet breeders and those that were multiplied) were used in the study separately to analyse the suitability of the esterase marker to establish distinctness and to examine the seasonal effects on the expression of the marker.

Suitability of esterase marker for assessing the uniformity was analysed in two cultivars, viz. the maintainer line 841B and restorer line D-23 of popular hybrid Pusa 23 (841A × D-23). The reason for selecting 841B instead of 841A was that the chance for contamination due to outcrossing during seed multiplication was comparatively less in the former. Single-earhead seeds of hundred morphologically true-to-type plants of each cultivar (841B and D-23) were produced by selfing. Thus, selfed earheads were harvested separately and individual earheads were threshed to obtain seeds of single earheads. Ten seeds in bulk as well as five individual single seeds from each single earhead were used separately to study the usefulness of seed esterases for uniformity testing.

α-Esterases analysis

Seed esterases from dry seeds were extracted by grinding ten seeds in 750 µl of extraction buffer (100 mM Tris-HCl, pH 7.5) using a mortar and pestle, kept over ice. The finely ground paste was then transferred to an eppendorf tube and subjected to centrifugation at 14,000 rpm for 15 min at 4°C. The supernatant was electrophoresed to separate the seed esterases in acrylamide gel (8.76% separating gel in Tris-HCl buffer, pH 8.8 and 4.5% stacking gel in Tris-HCl buffer, pH 6.7) using Tris-Glycine buffer (pH 8.3) at 4°C. The esterase profiles were developed by staining the gel according to Vallejos method[12]. Each electrophoretic analysis was replicated ten times. The relative mobility (Rm) of each band was calculated using the following formula:

$$\text{Rm} = \frac{\text{Distance travelled by the band}}{\text{Distance travelled by the tracking dye}}.$$

The bands in each cultivar were identified by their Rm values. Based on the presence or absence of a particular band in each cultivar, distinctness between the particular cultivars was established.

For analysing the suitability of seed esterases for uniformity testing, esterases were extracted from individual seed according to the method of Kumar *et al.*[13].

Statistical analysis of electrophoretic data of seed esterases

The esterase bands were scored for their presence (1) or absence (0) in each cultivar. Data entry was done into a

Table 1. Pearl millet hybrids and parental lines included in the study

Hybrid	A Line	B Line	R Line	Source
GHB235	81A	J2296	81B	GAU, Jamnagar
X-7	L111A	PT1890	L111B	TNAU, Coimbatore
PUSA322	841A	PPMI301	841B	IARI, New Delhi
PUSA605	841A	PPMI69	841B	IARI, New Delhi
PUSA23	841A	D-23	841B	IARI, New Delhi
RHRBH8609	RHRB1A	RHRBI138	–	MPKV, Rahuri
RHRBH8924	RHRB5A	RHRBI458	–	MPKV, Rahuri
GHB316	405A	J2290	405B	HAU, Hissar
–	842A	–	842B	HAU, Hissar
HHB67	–	H-77/833-2	–	HAU, Hissar
HHB60	81A	H-77/833-2	81B	HAU, Hissar
ICMH423	841A	ICMP423	841B	ICRISAT, Patancheru
ICMH356	ICMA88004	ICMR356	ICMB88004	ICRISAT, Patancheru
HHB94	ICMA89111	G-73-107	ICMB89111	ICRISAT, Patancheru
ICMH451	81A	ICMP451	81B	ICRISAT, Patancheru
–	ICMA91222	–	ICMB91222	ICRISAT, Patancheru

binary data matrix as discrete variables. Jaccard's similarity coefficient was used to compute pair-wise genetic similarity values. A dendrogram was generated based on similarly coefficients using Unweighted Pair Group Method with Arithmetic (UPGMA) mean. The computer package NTSYSpc was used for cluster analysis[14]. The average similarity index for all pair-wise comparisons ($\bar{X}_D$) was calculated and used to estimate the probability of electrophoretic profiles of esterases of two cultivars being identical by chance, employing the formula $(\bar{X}_D)^n$, where $\bar{X}_D$ is the average similarity index and n the average number of bands per cultivar.

Results and discussion

In the present WTO regime, establishment of DUS of new plant varieties plays a crucial role in protecting them. The DUS of a new genotype will be established based on the morphological characters. That is time-consuming and has its own disadvantages. Moreover, the number of morphological characters may not be sufficient for discrimination of all the extant and new varieties. Thus the present investigation was undertaken to assess the suitability of seed esterases isozyme for establishing the DUS of pearl millet genotypes.

Establishing distinctness

For protecting the new varieties of the crop species under the PPV&FR Act, it is necessary that the new variety be distinct from the existing varieties, which is established through DUS testing. The new variety is considered distinct if it is clearly distinguishable by at least one characteristic from any other variety, whose existence is a matter of common knowledge at the time of filing the application for protection of the variety[1]. In the present study, maximum polymorphism for seed esterases was observed in the anodal migrating zone of the gel, indicating the suitability of seed esterases in establishing the varietal distinctness. Similar reports have been reported by earlier workers in case of pearl millet germplasm[7–11].

Among 33 inbreds studied, 28 (A, B and R lines) could be distinguished from one another using esterase marker. It also aided in the identification of eight out of 14 hybrids. A total of 19 bands were recorded for the 47 cultivars studied, with a range of 1–9 bands in each cultivar. Esterase isozyme corresponding to band number 3 with Rm value 0.66 was present in all cultivars, except 405A, 405B, 841A and 841B, which in turn were characterized by the presence of a single band having Rm value of 0.76 (in 405A and 405B) and 0.83 (in 841A and 841B; Table 2). The similarity indices between cultivars ranged from 0.000 to 1.000, with an average similarity index of 0.396. The pearl millet hybrids and parental lines could be grouped into three major clusters (Figure 1). The first and second clusters included two cultivars each and the third cluster included 43 cultivars. The third cluster was further subdivided into sub-clusters, with almost all hybrids and their parental lines in the same sub cluster. Among the 47 cultivars, 36 could be identified individually, whereas GHB316, J2290, ICMH356, ICMR356, PUSA322, PUSA23, ICMH423, L111A, L111B, X-7 and PT1890 were categorized into four groups based on the esterase banding pattern. The probability of identical match by chance for the esterase marker was found to be 2.29×10^{-3}, i.e. the probability of obtaining identical profiles between two cultivars was 2 in 1000 cultivars. Thus it is less likely that another variety in the seed production chain will have the same pattern as any one of those characterized in this study. Moreover, as reported by earlier workers[7,15], the seed esterase isozymes do not have association with any of the plant morphological characters; this marker could be effectively used as an additional marker for establishing the distinctness of a variety apart from morphological characters.

Establishing uniformity and stability

The variety registration and protection process requires that the distinctive features of the variety in question

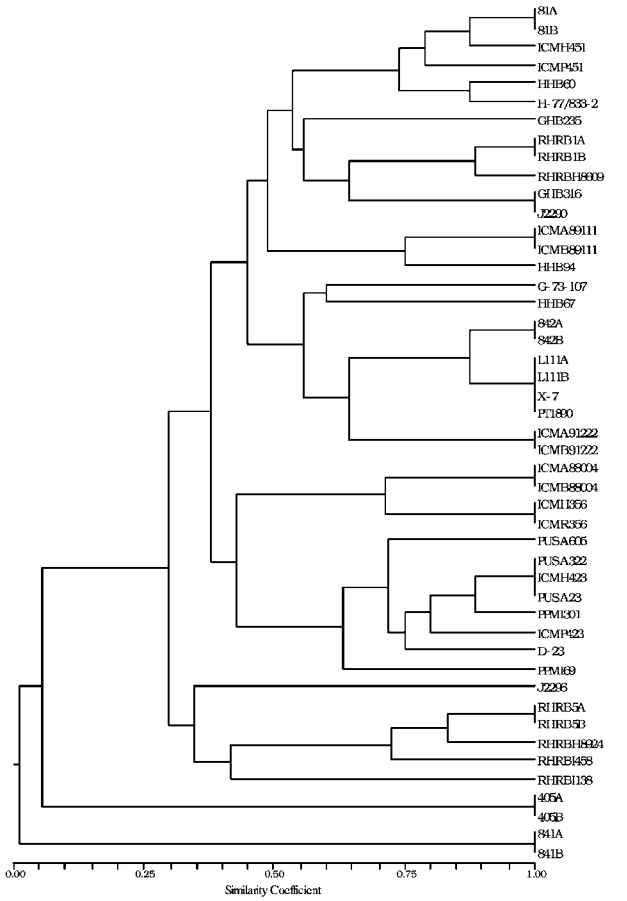

Figure 1. Dendrogram showing polymorphism of seed esterases in pearl millet genotypes based on UPGMA and sequential agglomerative hierarchical nested clustering.

Table 2. Electrophoretic profile of seed esterases of pearl millet genotypes (banding pattern was found similar for both the generation seeds in all genotypes)

	Rm value																		
Genotype	0.59	0.63	0.66	0.70	0.75	0.76	0.78	0.79	0.80	0.82	0.83	0.85	0.86	0.87	0.89	0.90	0.92	0.93	0.96
81A	1	0	1	1	1	1	0	0	0	1	0	1	0	0	0	0	0	0	0
81B	1	0	1	1	1	1	0	0	0	1	0	1	0	0	0	0	0	0	0
GHB235	1	0	1	1	1	1	0	0	0	1	0	0	1	1	0	1	0	0	0
J2296	0	0	1	0	0	0	0	0	0	1	0	0	1	1	0	1	0	0	0
ICMH451	1	0	1	1	1	1	0	0	0	1	0	1	0	0	0	0	0	1	0
ICMP451	1	0	1	1	1	1	0	0	0	0	0	1	0	0	0	0	0	1	0
HHB60	1	0	1	1	0	1	0	0	0	1	0	1	0	0	1	0	0	0	0
H-77/833-2	1	0	1	1	1	1	0	0	0	1	0	1	0	0	1	0	0	0	0
842A	1	0	1	1	0	0	1	0	0	1	0	1	0	0	1	0	0	1	0
842B	1	0	1	1	0	0	1	0	0	1	0	1	0	0	1	0	0	1	0
ICMA88004	1	0	1	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0
ICMB88004	1	0	1	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0
ICMH356	1	0	1	1	1	0	0	1	0	0	0	1	0	0	0	0	1	0	0
ICMR356	1	0	1	1	1	0	0	1	0	0	0	1	0	0	0	0	1	0	0
841A	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
841B	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
PUSA605	1	0	1	1	1	0	0	0	0	0	1	0	1	0	1	0	0	0	0
PPMI69	1	0	1	1	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0
PUSA322	1	0	1	1	1	0	1	0	1	0	1	0	1	0	1	0	0	0	0
PPMI301	1	0	1	1	1	0	1	0	1	0	1	0	0	0	1	0	0	0	0
ICMH423	1	0	1	1	1	0	1	0	1	0	1	0	1	0	1	0	0	0	0
ICMP423	1	0	1	1	1	0	1	0	1	0	0	0	0	0	1	0	0	0	0
PUSA23	1	0	1	1	1	0	1	0	1	0	1	0	1	0	1	0	0	0	0
D-23	1	0	1	1	1	0	0	0	1	0	0	0	1	0	1	0	0	0	0
ICMA89111	1	0	1	1	0	1	0	0	0	0	0	0	1	0	0	0	0	1	0
ICMB89111	1	0	1	1	0	1	0	0	0	0	0	0	1	0	0	0	0	1	0
HHB94	1	0	1	1	0	1	0	0	0	1	0	0	1	0	1	0	0	1	0
G-73-107	1	0	1	1	0	0	1	0	0	1	0	0	1	0	1	0	0	0	0
ICMA91222	1	0	1	1	0	0	1	0	0	0	0	1	0	0	0	1	0	1	1
ICMB91222	1	0	1	1	0	0	1	0	0	0	0	1	0	0	0	1	0	1	1
RHRB5A	0	0	1	1	0	0	1	0	0	1	0	0	0	0	0	1	0	0	0
RHRB5B	0	0	1	1	0	0	1	0	0	1	0	0	0	0	0	1	0	0	0
RHRBH8924	0	1	1	1	0	0	1	0	0	1	0	0	0	0	0	1	0	0	0
RHRBI458	0	1	1	1	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0
RHRB1A	1	0	1	1	0	1	0	1	0	1	0	0	0	0	1	1	0	0	0
RHRB1B	1	0	1	1	0	1	0	1	0	1	0	0	0	0	1	1	0	0	0
RHRBH8609	1	0	1	1	0	1	0	1	0	1	0	1	0	0	1	1	0	0	0
RHRBI138	0	0	1	1	0	1	0	0	0	0	0	0	0	0	1	1	0	0	0
L111A	1	0	1	1	0	0	1	0	0	1	0	1	0	0	0	0	0	1	0
L111B	1	0	1	1	0	0	1	0	0	1	0	1	0	0	0	0	0	1	0
X-7	1	0	1	1	0	0	1	0	0	1	0	1	0	0	0	0	0	1	0
PT1890	1	0	1	1	0	0	1	0	0	1	0	1	0	0	0	0	0	1	0
405A	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
405B	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
GHB316	1	0	1	1	0	1	0	1	0	1	0	0	0	1	0	0	0	0	0
J2290	1	0	1	1	0	1	0	1	0	1	0	0	0	1	0	0	0	0	0
HHB67	1	0	1	1	1	0	1	0	0	1	0	1	0	0	1	0	1	0	0

have to be expressed uniformly among individual plants of the variety and be inherited stably over generations[1]. Thus seed esterase markers used to establish distinctness of a variety must also satisfy these requirements of uniformity and stability. Pearl millet is propagated through seed and involves several stages of seed production, which is carried out by different agencies. Lack of uniformity might arise from residual heterozygosity, cross-pollination or mixing during harvest or post-harvest operations. In the present study, we have selected two genotypes; 841B, which is the maintainer line of 841A, the most widely used female parent in hybrid breeding and D-23, the restorer line of popular hybrid PUSA23.

The esterase profiles developed using bulk seeds collected from the respective breeders and those produced in the field were found similar, leading to the conclusion that the seed esterase markers are stable over generations and unaffected by the environment. For determining the uni-

formity of esterase markers, ten seeds in bulk and five individual seeds from earheads of 100 morphologically similar plants were analysed in the respective genotypes. Electrophoretic banding pattern of esterases obtained using ten bulk seeds from each of the 100 single earheads of 841B, were similar and uniform as that of the pooled sample (i.e. seed sample obtained by mixing 25 seeds from each of the 100 earheads; Figure 2 *a*). In the case of D-23, among 100 earheads analysed, 82 exhibited esterase profiles similar to that of the pooled sample and 18 exhibited a different profile, where two bands with Rm values 0.80 and 0.86 were missing (Figure 3 *a*). This may be because of residual heterozygosity for seed esterase marker in case of D-23. The possible reason for this leftover heterozygosity could be the fact that breeders will not select plants based on biochemical markers during the synthesis of a variety.

In case of single-seed analysis, all the five seeds from the respective earheads showed similar pattern as that of the bulked sample in the case of 841B (Figure 2 *b*). In D-23, when 18 earheads showing esterase profiles with two missing bands were subjected to single-seed analysis, all the five seeds from each head showed an esterase profile similar to that of bulked sample, i.e. with two bands (Rm values 0.80 and 0.86) missing. The remaining 82 earheads showing uniform esterase profiles with all the seven bands present, were also subjected to single-seed analysis. All five seeds from 29 earheads showed profiles similar to those of the bulked sample. However, among five seeds from the remaining 53 earheads, 1–2 seeds showed an esterase profile with two bands (Rm values 0.80 and 0.86) missing (Figure 3 *b*). This confirms the presence of residual heterozygosity in the case of D-23. The reason for not noticing the variants in bulk seed analysis was due to the masking of the off-types/variants because of dilution effect[13]. Variations within cultivars for electrophoretic profiles of different markers have also been reported in other crops[16–20]. This was attributed to breeding methods, where selections were based on morphological characteristics and not on electrophoretic markers. Hence, residual heterozygosity is likely to be left at the respective loci of these markers, leading to intravarietal segregation in subsequent generations.

Thus there is always a chance for the new or purified genotype being non-uniform with respect to seed esterases, even though it is uniform with respect to other plant morphological characters. UPOV, therefore, has appointed a special committee called the 'Biochemical and Molecular Technique Group' to work on the possibilities of using biochemical and molecular markers in DUS testing[21]. One such possibility could be allowing maximum limits for variants based on the genetics of seed esterases, while evaluating the genotype for its uniformity. Utmost care has to be taken while giving protection to the new variety based on its DUS established using biochemical markers. Otherwise, it may lead to problems of piracy during later stages of varietal protection. For example, in case of D-23, one can register the variant D-23 as a new variety by claiming that it is distinct from D-23 with respect to the electrophoretic profile of esterases. In reality, it will be similar to D-23 with respect to morphological characters and performance potential. Hence if biochemical markers are used for granting varietal protection, then the breeders should see that their variety is uniform with respect to the associated biochemical marker, or they should claim the protection stating the proportion of each variant of their variety with respect to a particular biochemical marker, or only such biochemical markers should be used for granting varietal registration that are associated with any of the morphological characters. Thus while achieving the uniformity of the associated marker, the uniformity of the respective biochemical marker will be achieved automatically.

From the present study it has been found that the seed esterase marker is highly polymorphic among pearl millet

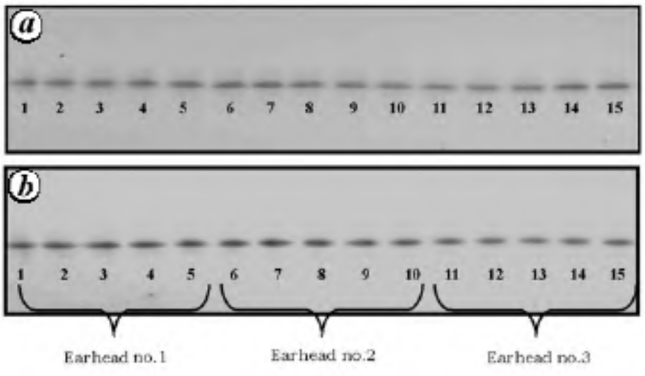

Figure 2. Electrophoretic profile of seed esterases of 841B. ***a***, Lanes 1–15, Esterases extracted from ten bulked seeds of the respective earheads. ***b***, Lanes 1–15, Esterases extracted from individual seed of respective earheads.

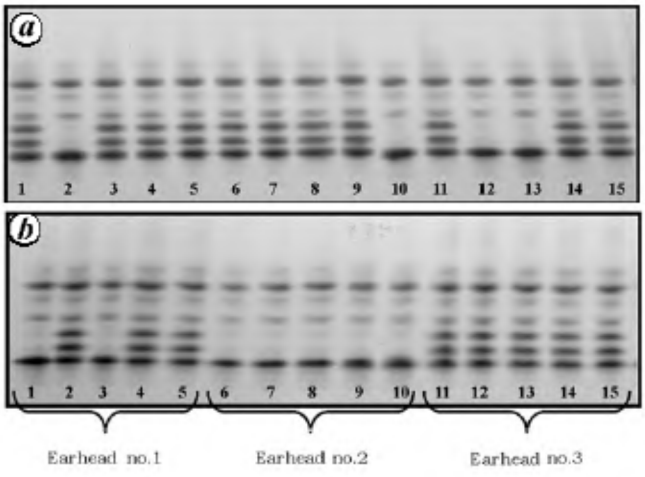

Figure 3. Electrophoretic profile of seed esterases of D-23. ***a***, Lanes 1–15, Esterases extracted from ten bulked seeds of respective earheads. ***b***, Lanes 1–15, Esterases extracted from individual seed of respective earheads.

cultivars, stable over generations, unaffected by environment and not associated with any morphological characters. These attributes which have been tested and confirmed, call for its consideration as an ideal additional descriptor for establishing distinctness and stability of new pearl millet cultivars, which in turn serve the purpose of granting plant variety protection. However, before using this descriptor in DUS testing, its validity for testing the uniformity of genotypes has to be reconfirmed in other pearl millet genotypes.

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