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Research Article

IDENTIFICATION OF MOLECULAR MARKERS IN SPECIES OF *ABELMOSCHUS* MEDIK. (MALVACEAE) FOR INTER- AND INTRA-SPECIFIC DISCRIMINATION

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ABSTRACT
Molecular markers such as Random Amplified Polymorphic DNA (RAPD) and Protein banding patterns have often been used to characterize identities and relationships in various crops. These help resolve species/varietal identification and thus have a range of applications in breeding programmes. In *Abelmoschus* however, there is dearth of data on both these aspects. The current study focuses on the utility of these markers among collections of seven species of *Abelmoschus* for inter- and intraspecific discrimination. Results of the study reveal that among the chosen 12 decamer primers, five (OPX-18; UBC-210, 292 and OPAE-03, 15) are more suitable for characterizing the species of *Abelmoschus* included in the study. The seed protein profile though uniform within accessions of a taxon, could identify unique bands characteristic for a particular taxon.

***Key Words*:** *Abelmoschus, RAPD, Seed Protein Profile*

INTRODUCTION
Molecular markers serve as powerful tools in the assessment of genetic variation and in the elucidation of genetic relationships within and among species (Kresovich *et al.,* 1992; Chakravarthi and Naravaneni, 2006). The rapid development of molecular techniques has allowed the analysis of large number of gene loci distributed throughout the plant genome. DNA and protein profiling techniques are the most commonly used techniques for molecular diversity analysis. Among the molecular markers, RAPD markers have proven to be the most useful to characterize identities and relationships of various crops (Aladele *et al.,* 2008), including *Abelmoschus* (Martinello *et al.,* 200). Accumulating evidence shows that variation in protein banding patterns can also help resolve varietal and species identification in several crop plants and has a range of applications in breeding programs (Sammour, 1991; Das and Mukherjee 1995; Cheema *et al.,* 2012).

Genetic diversity analysis at theinter- and intraspecific levels in 21 accessions of species of *Abelmoschus* was the focus of the present investigation. Seven species of *Abelmoschus*, namely *A. eculentus*(L.)Moench and *A. caillei* (A. Chev.)Stevels,*A.angulosus* Wall. ex Wight and Arn., *A. Manihot* (L.) Medik, *A. Moschatus* Medik., *A. Teraphyllus* Roxb Ex. Horrnemand *A. Tuberculatus* Paul and Singh. were collected for the study. A perusal of the literature showed that although these species could be identified morphologically based on the previously proposed taxonomic keys, confusion still persists among some. Therefore the collected taxa were grouped conveniently for the study so that confusing species/ subspecies/ varieties could be clubbed together in groups for the analysis as shown below.

Group I: *A. esculentus* (4 accs*.) with *A. caillei*(*2*accs.)
Group II: *A. moschatus* ssp. *moschatus* (3 accs.) with *A. moschatus* ssp. *tuberosus* (3 accs.)
Group III: *A. angulosus* var. *grandiflorus* (2 accs.) with *A. angulosus*var. *purpureus* (2 accs.)
Group IV: *A. manihot* (3 accs.), *A. tetraphyllus* (1 acc.) and *A. tuberculatus* (1 acc.)

MATERIALS AND METHODS

For the molecular level studies, genomic DNA was isolated from all the collections and characterized using RAPD. Seed proteins were analyzed using SDS PAGE.

Genomic DNA analysis

Genomic DNA was isolated from young cotyledonary leaves, collected from germinated seedlings using the modified CTAB method (Doyle and Doyle, 1987). The DNA so obtained was then quantified using the biophotometer (Eppendorf BioPhotometer Plus, Germany).

*RAPD analysis***:** DNA amplification was done using 12 arbitrary decamer primers of the OPAE, OPX, UBC and OPAN series (Williams *et al.*, 1990). Amplification products were separated on 1.2% agarose gels run at 120V for about 1 h in 1x Tris-borate-EDTA (TBE) buffer containing 0.5 µg/ ml of ethidium bromide. Molecular sizes of the amplification products were estimated using a known molecular weight marker DNA (1 kb DNA ladder). The gels were viewed and photographed under UV in the gel documentation system (Alpha Innotech Corporation, USA) and the banding pattern for each primer was scored by visual observation.

Data Analysis: The presence of an amplification product (band) in each position was recorded as '1' and absence as '0'. Based on presence /absence of bands, a pair-wise similarity matrix (Pearson's coefficient) was formed. This was used to evaluate the relationship among the studied genotypes and analyzed using MVSP 32 software for cluster analysis (Sokal and Sneath 1973) (Un-Weighted Pair Group Method with Arithmetic Averages (UPGMA)).

Seed protein profiling

Seeds of the different collections included in the study were used for seed protein profile analysis. Due care was taken to minimize heterogeneity by selecting seeds from self-pollinated fruits.

Protein extraction and SDS page: About 100mg of seed sample was homogenized with liquid nitrogen in 1 ml of extraction buffer (0.05 TrisHCl buffer). The sample was centrifuged at 12000 rpm (Eppendorf Centrifuge 5804R, Germany), supernatant collected and stored at -20^{0}C. The isolated protein sample was prepared for SDS-PAGE (Laemmli *et al.*, 1970). Electrophoresis was performed in a 12 % resolving gel and a 4 % stacking gel on the vertical gel apparatus (SciePlas, UK) at a constant voltage of 100 V for stacking gel and 70 V for the separating gel. Further, the gel was stained overnight in Coomassie brilliant blue (0.1%), destained the next day in methanol, acetic acid, water mixture (3: 1: 6) and photographed in the gel documentation system (Alpha Innotech Corporation, USA).

Data Analysis: Relative molecular weight of polypeptide bands were calculated in comparison with the protein molecular weight marker (GeNei) using gel documentation system (Alpha Ease FC Version 4, Alpha Innotech Corporation, USA). A 1/0 matrix was prepared for all the bands scored. The genetic distance between all pair-wise differences in the amplification product for all genotypes was represented as Euclidean or Nei and Li's Distance matrix. UPGMA cluster analysis was performed using the software MVSP 32.

RESULTS AND DISCUSSION

Molecular studies are lagging behind in *Abelmoschus,* except for a few reports using RAPD markers [(4)] and that using Sequence Related Amplified Polymorphism (SRAP) markers (Gulsen *et al.*, 2007). This is probably because of the difficulty in DNA extraction due to mucilage interference. Cotyledons of dark grown seedlings may be used to overcome this difficulty (Kochko and Hamon, 1990).

Group I: Abelmoschus esculentus and A. caillei

*RAPD Analysis***:** Maximum polymorphism was exhibited by OPX-18, followed by UBC-210, 292 and 465. The dendrogram based on the genetic similarity matrix (Pearson's coefficient), recognized all the six collections, the two accessions of *A. caillei* (accs. 2 and 5) were most similar, while collections of *A. esculentus* (accs.6,3,4 and 1) showed greater genetic variability. The greater degree of observed intraspecific variation in *A. esculentus* is probably because it is subjected to continuous selection during

cultivation. The number of bands formed and degree of polymorphism revealed by each of the primers are given (Table 1) (Figures.1a-d, Figure 4a).

Table1: Results of RAPD analysis (*A. esculentus* and *A. caillei*)

Sl.No.	Primers	Number of bands	Number polymorphic bands	Percentage of polymorphism
1	OPX-18	6	5	83.33
2	OPAE-03	6	3	50.00
3	OPAE-15	8	3	37.50
4	UBC- 54	6	4	66.66
5	UBC-210	5	4	80.00
6	UBC 123	8	6	75.00
7	UBC-292	5	4	80.00
8	UBC 465	5	4	80.00
9	UBC-509	2	1	50.00
10	UBC-514	3	2	66.66
11	UBC 620	1	0	00.00
12	OPAN-01	3	3	66.66
	Total	58	38	-
		4.83	3.16	61.31

Table 2: Results of RAPD analysis (*A.moschatus* and *A. moschatus* ssp. *tuberosus*)

Sl.No.	Primers	Number of bands	Number polymorphic bands	Percentage of polymorphism
1	OPX 17	27	5	18.51
2	OPAN 01	25	6	24.00
3	UBC 210	35	11	31.42
4	UBC 509	39	10	25.64
5	UBC 514	26	3	11.53
6	UBC 620	0	0	0
7	UBC 292	28	11	39.28
8	UBC 54	25	2	8.00
9	UBC 123	31	5	16.12
10	OPX 18	14	4	28.57
11	OPAE 15	21	3	14-28
12	OPAE 03	13	3	23.07
	Total	284	63	-
		23.66	5.25	20.03

Seed protein profile

About 15 bands found distributed in all accessions with molecular weights ranging from 12.04 TO 78.68 KDa. Only two polymorphic bands were observed (57.19 and 63.09KDa) and were noted for *A. esculentus* collections. The dendrogram showed that the collections could be grouped into two. The first

one consisted of the *A. esculentus* collections and the second one consisted of *A. caillei* collections (Figure 3a, Figure 4b).

Table 3: Results of RAPD analysis (*A. anglosus* var. *grandiflorus* and *A. angulosus* var. *purpureus*)

Sl.No.	Primers	Number of bands	Number polymorphic bands	Percentage of polymorphism
1	OPX 18	7	6	85.7
2	UBC 54	4	3	75.0
3	OPAE 03	8	5	62.5
4	UBC 123	6	4	66.0
5	UBC 514	6	5	83.0
6	UBC 620	1	0	0.0
7	OPAE 15	7	5	71.0
8	UBC 465	6	4	66.0
	Total	45	32	-
		5.63	4.00	63.65

Table 4: Results of RAPD Analysis (*A.manihot, A. tetraphyllus, A. tuberculatus*)

Sl.No.	Primers	Number of bands	Number polymorphic bands	Percentage of polymorphism
1	OPX 18	3	2	67.00
2	UBC 54	5	3	60.00
3	OPAE 03	6	5	83.33
4	UBC 123	6	4	66.66
5	UBC 514	4	3	75.00
6	OPAE 15	4	2	50.00
7	UBC 465	6	5	83.33
	Total	34	24	-
		4.86	3.43	69.33

Group II:Abelmoschusmoschatus and A. moschatus ssp. tuberosus

RAPD Analysis

Cluster analysis performed on RAPD data for the six collections of *Abelmoschusmoschatus* revealed similarity between *A. moschatus*ssp. *moschatus* collections (accs. 1, 2) and also *A. moschatus*ssp. *tuberosus* collections (accs.5 and 6). However acc.3 (*A. moschatus*ssp. *moschatus*) and acc.4 (*A. Moschatus*s sp. *tuberosus*) stood apart suggesting inherent genetic variability (Table 2) (Fig1.e-h, Fig.4c)

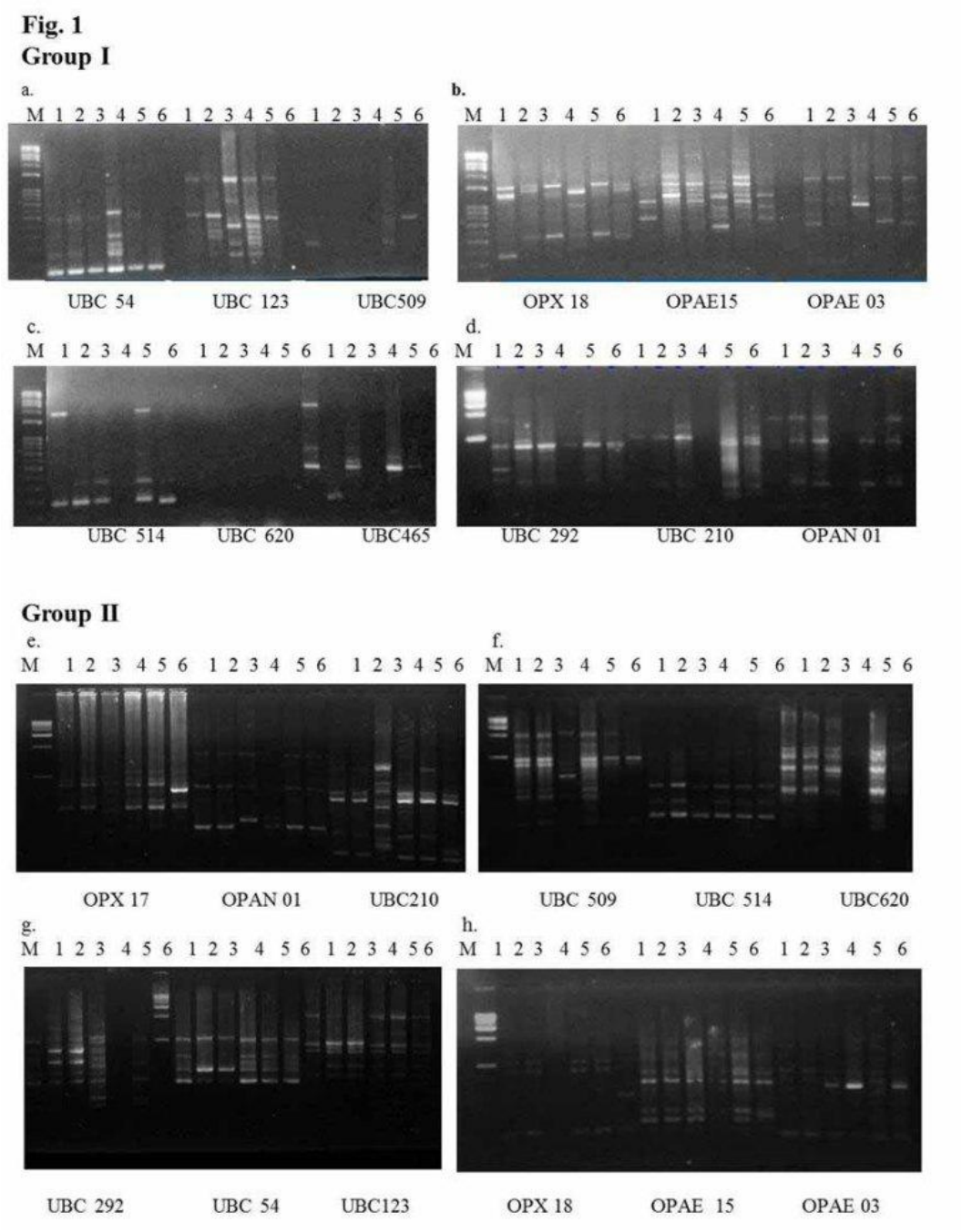
Seed Protein Profile

The SDS banding pattern of seed protein produced 18 bands distributed in all accessions with molecular weights ranging from 12.04 to 97.40 KDa. One band of 29 KDa was found to be characteristic for ssp. *moschatus.* This could be used as a genetic marker to separate the taxa. The data subjected to UPGMA, revealed two principal clusters (Text fig.18). The first cluster consisted of the *A. moschatus* collections and the second one consisted of *A. moschatus ssp. tuberosus* collections (Figure 3b, Figure 4d).

Group III. A. anglosus var. grandiflorus (accs.2,3) and A. angulosus var. purpureus (accs.1,4)

RAPD Analysis

Abelmoschus angulosus var. *grandiflorus* collections (accs. 2 and 3) showed greater similarity while collections of *A. angulosus* var. *purpureus* (accs.1 and 4) stood apart. Thus the dendrogram could differentiate the taxa (Table 3) (Figure 2a-c, Figure 4e).

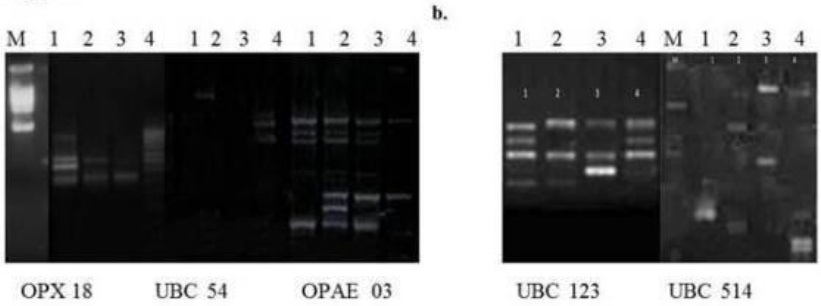


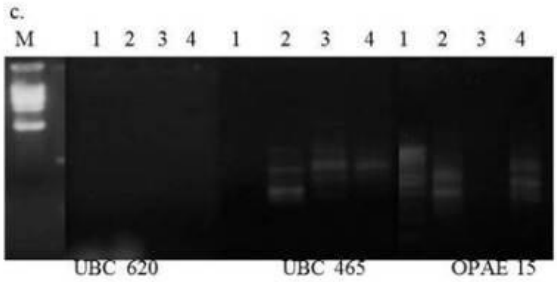
Seed Protein Profile

The SDS banding pattern showed six bands distributed in all accessions with molecular weights ranging from 12.04 to 78.68 KDa. Two bands (48.86 KDa and 63.87KDa) were characteristic for *A. angulosus* var. *grandiflorus* collections. The collections could be grouped in two clusters. The first one consisted of

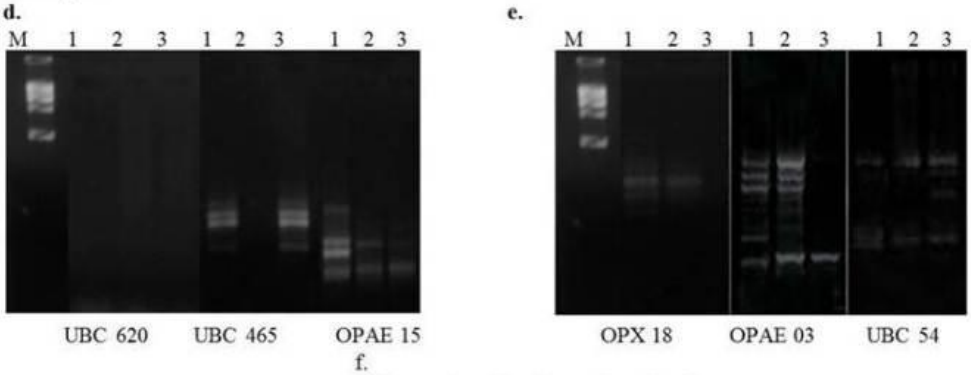
the *A.angulosus var. grandiflorus* collections and the second, *A. angulosus*var. *purpureus* collections (Figures3c, 4f).

Fig. 2
Group III





Group IV



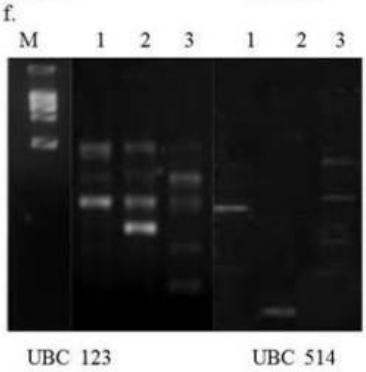
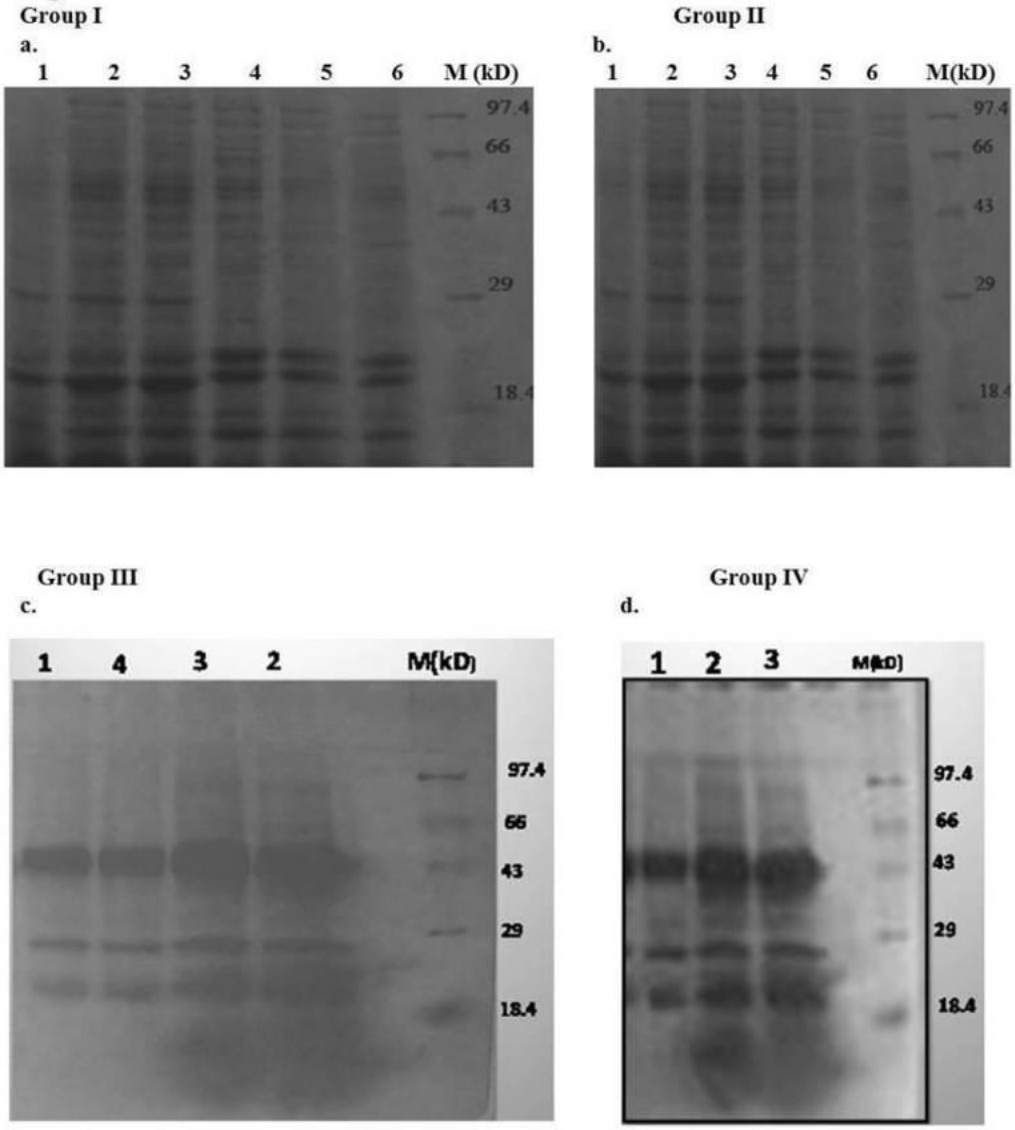


Fig.3



Group IV: A. manihot (acc.1), A. tetraphyllus (acc.2), and A. tuberculatus (acc.3)

RAPD Analysis

Abelmoschus tuberculatus (acc. 2) and *A. tetraphyllus* (acc.3) group together while *A. manihot* stood apart (Table 4) (Figures 2d,e,f; Figure 4g).

Two collections of *A. manihot* could not be included for the RAPD analysis due to technical difficulties

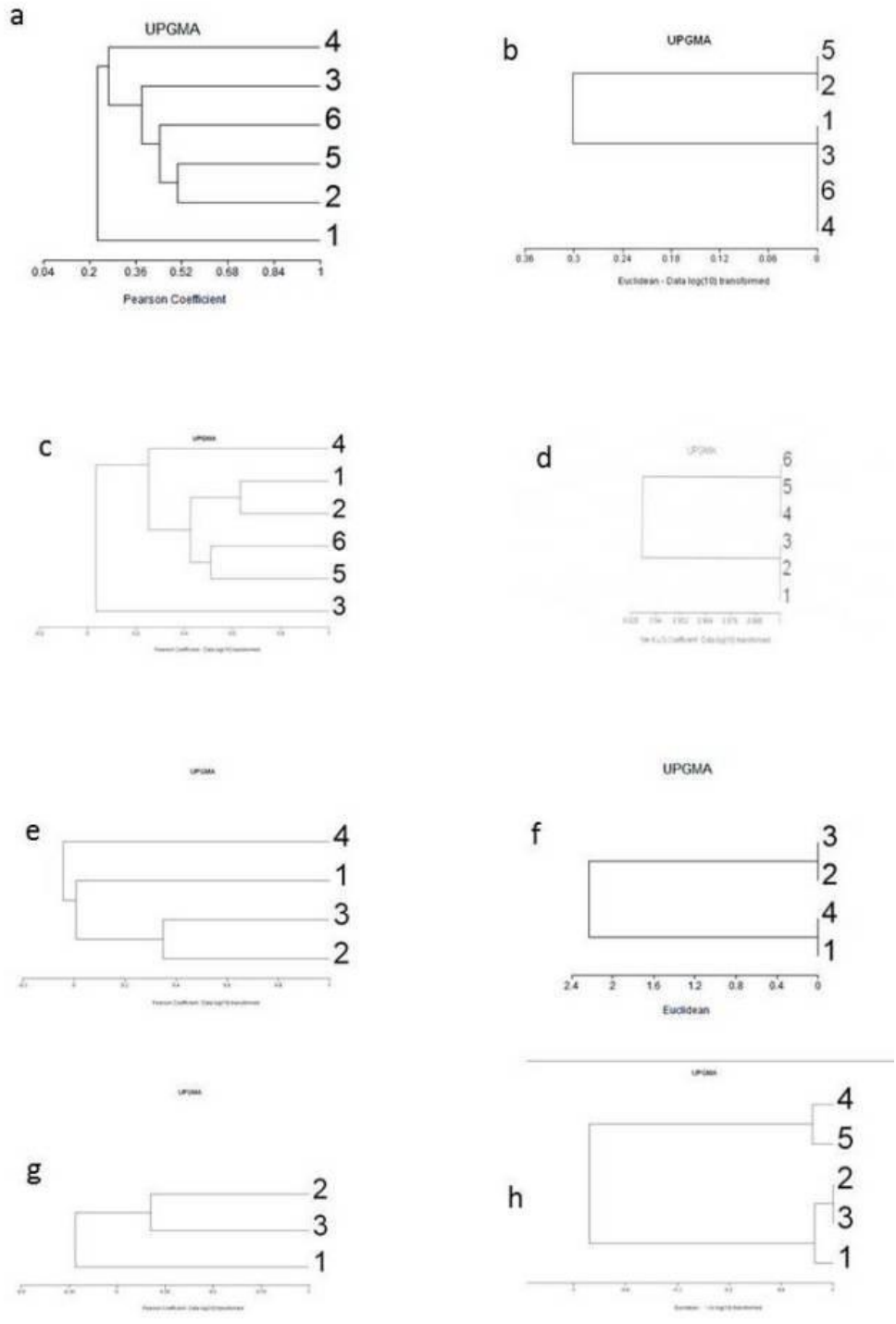
Seed Protein Profile

About five bands noted in all and distributed with molecular weights ranging from 10.204 to 88.68 KDa. A single band at 66 KDa was found to be characteristic for *A. tuberculatus*. Two clusters could be observed, one consisted of the *A. manihot* collections (accs.1,2,3) and the other of *A. tetraphyllus,* and *A.* Tuberculatus(Figure 3d, Figure 4h).

It is essential to develop markers that not only distinguish individuals and accessions, but also reflect the inherent diversity and relationships among collections. All the presently studied groups comprising of accessions and species of *Abelmoschus* reflect the inherent diversity among the accessions. Unique markers could be identified which could possibly be used for future analysis.

Fig. 4

RAPD Analysis **Seed Protein Analysis**



The primer, OPX-18 was found useful for distinguishing Group I (*A. esculentus* and *A. caillei*) and Group III (accessions of *A. angulosus*); UBC primers (UBC-210 and 292) for differentiating Group II (*A. moschatus* group) and OPAE primers (OPAE-03 and OPAE-15) for both Group IV (*A. manihot, A. tetraphyllus* and *A. tuberculatus*) and Group I. However, the collections of a particular species showed less genetic variability among themselves. Previous studies have also shown that molecular markers can be used to identify unique genotypes and associated agronomic traits (Kresovich and McFerson, 1992).

The degree of polymorphism in a sample of amplified DNA may be either due to base substitution or deletion in the priming sites or insertion that renders priming sites too distant to support amplification or due to large size of insertions or deletions that changes the size of the amplified fragment (William *et al.,* 1990). The observed low level polymorphism within species of *Abelmoschus* is indicative of modest levels of genetic variation in the concerned populations (Singh *et al.,* 2010). The observed results may also be due to the inadequacy of the chosen primers.

The cluster diagram relating to the analysis of seed protein electrophoretic profile of all the four groups revealed a higher degree of homology among collections of a particular taxon and was highly consistent. All collections of a particular species showed homology beyond their taxonomic limits and without fail fell into one principal cluster. Thus, the results confirmed the generally accepted opinion that seed proteins have a fixed physiological state, are largely independent of environmental factors and are therefore highly reliable.

Even though the seed protein patterns turned out to be uniform for collections of a particular species, specific bands could be identified, which distinguished the different species/acccesions. Group I: Two bands (57.19 and 63.09KDa) were characteristic for *Abelmoschus esculentus* collections, Group II: One band (29 KDa) was characteristic for *Abelmoschus moschatus* ssp *moschatus* collections, Group III: Two bands (48.86 KDa and 63.87KDa) were characteristic for *A. angulosus* var. *grandiflorus* collections, Group IV: One band (66 KDa) was characteristic for *A. tuberculatus*. The identified unique bands from protein profiles can serve as markers and can possibly be used effectively to study the genetic variation existing in germplasm collections of a species (Javaid *et al.*, 2004).

The study thus attempts to highlight the utility of molecular markers such as RAPD and seed protein profiles in screening the seven species of *Abelmoschus*.

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