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Identification of Duplicates in the Germplasm of Sweet potato (*Ipomoea batatas* (L.) Lam.) using Morphological and Molecular Markers

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Abstract

Fifty accessions of sweet potato from National Active germplasm site of ICAR-CTCRI were selected for morphological and molecular analysis and to identify duplicates. Morphological analysis was performed by using twenty IPGRI descriptors. The data analysed using R package separated the accessions into five principal clusters at a Euclidean distance of 15 and resulted in the identification of three pairs of morphological duplicates (S-236 and S-256, S-203 and S-295, S-772 and S-747). The PCA analysis revealed predominant vine colour and secondary vine colour, abaxial vein pigmentation and petiole pigmentation as the major factors that contributed to the clustering of the sweet potato accessions. Molecular characterization using 11 ISSR primers resulted in 162 polymorphic bands with a mean value of 14.7 bands per primer. The dendrogram based on ISSR markers dendrogram grouped the accessions into six clusters at a Jaccard distance of 0.9. The third principal cluster comprised of 20 accessions which were assembled in 5 sub-groups which indicates high intraclusteral variability. The fourth principal cluster comprised of 15 accessions in 4 groups. In this principal cluster S-236 and S-256 grouped together with 100% similarity. This pair can be considered as true duplicates which were also found similar in morphological characterization. The similarity between the different accessions ranged between 52-100%. The least similar accessions were SD-39 and S-298 (52%). Thus it can be inferred that maximum of 48% variability existed within the selected accessions which can be considered as a low to moderate diversity. Based on morphological and molecular analysis three pairs of duplicates were identified and may be culled out from the germplasm.

Key words: Sweet potato, germplasm, ISSR markers, morphological descriptors, duplicate identification, cluster analysis, R package

Introduction

Sweet potato (*Ipomoea batatas* (L.) Lam.) is one of the most versatile storage root crops in the world. The developing countries alone contribute to 95% of the total sweet potato production (Veasey *et al*., 2008). The largest collection of sweet potato (over 8018 accessions) is preserved in the International Potato Center (Rossel *et al*., 2009). CIP germplasm comprises of collections from so many countries and research centers. One of the major goals of germplasm characterization is to describe accessions and establish their diagnostic characters and identify duplicates. These duplicates are a burden for curators because they do not contribute to the diversity in the collection, but do require gene bank budget for maintenance. Thus, both from a genetic and economic point of view, identification and elimination of redundancies should be an important gene bank objective (van Treuren *et al.*, 2001).

In the sweet potato germplasm collection at CIP, Peru the duplicated accessions represented 66% of the total collection (Rossel *et al*., 2009). It is estimated that 50% of the accessions maintained in the gene banks world-

wide are duplicates (Lyman, 1984). Duplicates between gene banks occur for various reasons. One obvious cause is the exchange of accession between gene banks and acquisition of same accessions by several gene banks. Often material obtained for comparison in national variety testing trial was also deposited in the National gene bank; dividing a collected sample between participating centres in the collection expedition (Knuppfer *et al*., 1997)

Sweet potato germplasm maintained at ICAR-CTCRI consists of 1124 accessions acquired from various parts of the world and from different parts of the country through various sources and donors and collectors. A lot of variability exists in the leaves, stem and storage root characteristics. A lot of morphologically similar accessions also exist in the germplasm. This merging of germplasm often creates the possibility of duplicates in the germplasm. In order to identify the duplicates, it is essential to know about the genetic similarity and variability within the genotypes and genetic diversity studies can assist in duplicate identification and specific selection of parents for plant breeding purposes (Martins *et al*., 2012).

But being an asexually propagated crop which is amenable to mutation as well as it shows high variability in morphological traits, the morphological characterization in sweet potato is quite difficult when compared to other tuber crops (Huaman *et al*., 1999; Woolfe, 1992). The storage root skin colour and more frequently flesh colour are most amenable to somatic mutation in sweet potato (Huaman *et al*., 1999).

So the molecular markers often need to accompany morphological and agronomic characterizations as they wrap the whole genome and are not environmentally influenced (Goulao and Oliveira, 2001). It has been reported that ISSR primers can differentiate between clones as well as closely related cultivars and are widely used because of the low cost, high reproducibility and reliability (Zietkiewicz, *et al.,* 1994, Sabarinath et al., 2018). A preliminary study using ISSR markers has revealed duplicates in a small set of 15 sweet potato germplasm (ICAR-CTCRI Annual Report, 2015). So in this study, morphological characterization complemented with molecular characterization using ISSR primers was carried out to identify duplicates in 50 accessions of sweet potato.

Materials and Methods

Fifty *Ipomoea batatas* accessions collected from various geographic locations in India are conserved in the sweet potato germplasm repository of ICAR-CTCRI, were selected for the present study which includes morphologically similar accessions (Table 1). The plants were raised in the field with six plants per accession per mound spaced at 90 cm x 90 cm spacing. The vines were planted during September 2017 in the lowland. Plants were irrigated thrice a week and weeding and intercultural operations were carried out as per POP. The details of the accessions and their geographical origin are given in Table 1.

Table 1. Passport data of the sweet potato accessions used for the experiment

Sl. No	Acc. No.*	Cultivar name	Locality of collection
1	S-236 [a]	Vella	Kasaragode, Kerala
2	S-256 [a]	IB14	Coorge, Karnataka
3	S-295 [a]	ND-135	West Bengal
4	S-298	ND-153	West Bengal
5	S-731 [a]	HT0 -35	Kerala
6	S-1026 [a]	TIS9068 (13)	Nigeria
7	S-203 [a]	RED	Kuppad, Kerala
8	S-625	5X-20	Kerala
9	S-651 [b]	5X-58	Kerala
10	S-729 [b]	HTO-33	Kerala
11	S-733	HTO-40	Kerala
12	S-737	Mut-2	Kerala
13	S-738 [c]	Mut-1	Kerala
14	S-739 [c]	Mut-4	Kerala
15	S-740 [c]	Mut-5	Kerala
16	S-747 [d]	Mut-10	Kerala
17	S-755 [d]	Mut-18	Kerala
18	S-772 [d]	Goa Local	Goa
19	S-776 [a]	Basthar-1	Madhya Pradesh
20	S-1700	OPS1	CTCRI, Trivandrum
21	S-1701	RNK-2015-1	Sirsi, Karnataka
22	S-1650	SP-12-SP (SARSE-12)	Joida, Karnataka
23	S-1702 [e]	RNK-2015-2	Sirsi, Karnataka
24	S-1596	AF2	Assam
25	S-1606	SD-10	CTCRI, Trivandrum
26	S-1703 [e]	RSM-2015-1	Joida, Karnataka
27	S-1704	JAS-9-White	Wayanad
28	S-1705	RSM-2015-4	Joida, Karnataka

29	S-1607 [f]	SV-1-2014	CTCRI, Trivandrum
30	S-1603	SD-11	CTCRI, Trivandrum
31	S-1609	526/7	CIP, Peru
32	S-1401	local	Trivandrum, Kerala
33	S-1652	SP-18-SP (SARSE-18)	Joida, Karnataka
34	S-1653[e]	SARSE-13	Joida, Karnataka
35	S-1706	RSM-2015-5	Joida, Karnataka
36	S-1707	RSM-2015-2	Joida, Karnataka
37	S.Arun [f]	S.Arun	CTCRI,Trivandrum
38	S-1654	SP-7-SP	Joida, Karnataka
39	S-1610	665/4	CIP, Peru
40	S-1655	ASSAM-1-2014	Assam
41	S-1708	RSM-2015-3	Joida, Karnataka
42	S-1709	RSM-2015-6	Joida, Karnataka
43	S-1656	SWARNA-AP	Arunachal Pradesh
44	S-1710	JAS-9-PINK	Wayanad, Kerala
45	S-1657	SARSE-7	Joida Karnataka
46	S-1611	SD-24	CTCRI, Trivandrum
47	S-1612	SD-29	CTCRI, Trivandrum
48	S-1613	SD-39	CTCRI, Trivandrum
49	S-1614	SD-53	CTCRI, Trivandrum
50	S-1615	SD-55	CTCRI,Trivandrum

*Similar superscript symbols represent morphologically similar accessions

For morphological characterization, twenty descriptors as per International Plant Genetic Resources Institute (IPGRI) were used to take morphological observations (CIP *et al*., 1991) of which 18 were vine and leaf characters and two were storage root traits. The observations on leaf and vine were made after 45 days of planting. The storage root characters were recorded after the harvest at 110 days after planting. The frequency distribution of the different characters also was studied.

The morphological data were recorded by phenotypic observation of the selected accessions. Each character and trait were recorded by assigning certain numerical value to them as per the IPGRI descriptor states of sweet potato (CIP *et al.,* 1991). All the recorded morphological data were tabulated in excel sheet for further statistical analysis. 5

To bring out the patterns of similarity and dissimilarity, morphological data were subjected to clustering using R-package (R Core Team, 2013). In this morphometric analysis was performed to group morphologically similar accessions under one group and dissimilar accessions in distant groups based on Euclidean distance. Hierarchical clustering was carried out with complete linkage method of clustering. The clustering of accessions was depicted through a dendrogram after the analysis. Principal component analysis (PCA) (Sneath and Sokal, 1973) was performed to analyse the contribution of traits in separating accessions into different groups and also to compare the clustering of accessions with respect to each cluster in a dendrogram.

For molecular characterization**,** DNA was extracted from fresh tender and young leaf samples using CTAB method of DNA extraction for sweet potato (Borges *et al*., 2009). The quality of the DNA was checked by agarose gel electrophoresis in 0.8% agarose. The extracted DNA was quantified using Thermo Scientific NanoDrop™ 1000 Spectrophotometer. Based on absorbance/OD value, appropriate samples were selected. DNA samples were diluted with nuclease free water to a concentration of 10ng/ìl based on the data obtained from DNA quantification.

The diluted DNA samples were amplified for screening using 21 ISSR primers at standardized conditions of temperature and time. The components of PCR (Table 2) and PCR cycle used for amplification are given below.

Table 2. Components of PCR reaction

Components	Stock concentration	Required concentration	Volume for one reaction (20 µl)
Taq buffer (With 25 Mm $MgCl_2$)	10x	1x	2 µl
$MgCl_2$	25 mM	2.5 mM	(From buffer)
dNTP mix	10 mM	0.2 mM	0.4 µl
Primer (ISSR)	10 µM	0.25 µM	0.5 µl
Taq DNA polymerase	5U/µl	1U	0.2 µl
DNA template	10 ng/ìl	40 ng	4 µl
Final volume made up to 20 µl with d H_20			20 µl

PCR was carried out in Proflex Thermocycler with the following programme

Lid	- 105ºC	
Initial denaturation	- 94ºC - 5 min	35cycles
Denaturation	- 94ºC - 30 sec	
Annealing	- 56.3ºC - 1 min	
Extension	- 72ºC - 10 min	
Final extension	- 72 ºC - 10 min	
Hold	- 4 ºC	

The DNA of all the fifty accessions was subjected to molecular characterisation with the selected ISSR primers. The PCR products were resolved in 2% agarose gel along with 1Kb and 100bp ladders to identify the molecular weight of obtained bands and for polymorphism studies. The gel images of resolved PCR products were taken. Scoring was carried out in the form of Binary scoring format via assigning "1" for the presence of a specific band and "0" for the absence of band. The percentage of polymorphism was calculated as given below.

$$\text{Percentage of polymorphism} = \frac{\text{No. of polymorphic bands}}{\text{Total number of bands}} \times 100$$

Using the binary data, dendrogram was generated based on clustering method based on complete linkage using R package and distance measure used was Jaccard distance which gave a clear grouping of 50 accessions. To determine the similarity and diversity between every two accessions, pair-wise distance (similarity) matrices was computed using sequential, agglomerative, hierarchical and nested (SAHN) clustering option of the NTSYS-PC (Numerical Taxonomy and Multivariate Analysis System, Biostatistic, New York, U.S.A., Software Version 2.02 package) (Rohlf, 1998). Morphological and molecular data was compared using Mantel's test (Mantel, 1967).

Results and Discussion

Morphological characterisation

Twenty morphological characters were recorded in 50 sweet potato accessions using IPGRI descriptors. The frequency distribution of the different characters showed variability between the accessions. In plant type 66% of the accessions were semi-compact followed by 20% spreading, 12% non-twining and 2% extremely spreading types. Leaf lobe type distribution showed 54% with five lobed, 18% were 7-lobed,14% 3-lobed, 2% 9-lobed and 12% central tooth with no lateral lobes.

Cluster analysis

Cluster analysis based on 20 morphological descriptors using R package generated a dendrogram which separated all the 50 accessions into five major clusters at a Euclidean distance of 15 (Fig.1). The most distantly placed accessions were SD-29, SD-53, SD-55 and Jas-9-pink of cluster 5. Each of the major clusters were subdivided into many sub-clusters and the pattern of clustering of accessions is described in Table 3. The interclusteral distance is indicated in Table 4. The intraclusteral variation between accessions indicates that, lot of variability exist between the accessions. The accessions in the Cluster I were separated within a distance of 9.49 whereas in Cluster II the accessions were separated within a distance of 14. The accessions within Cluster III were

Table 3. The clustering pattern of different accessions

Clusters	Sub-Clusters	Accessions
Cluster 1	1A	OPS1, Swarna-AP, 526/7, SD-10, ASSAM-1-2014 ,
	1B	AF2, RSM-2015-1, 665/4, RNK-2015-2, SP-18-SP, SARSE-13, RSM-2015-5
	1C	733,729, 651, 772,747, 755,
	1D	625,298
	1E	RSM-2015-2,RNK-2015-1, RSM-2015-6, SP-7-SP
	1F	RSM-2015-4, SARSE-7
Cluster 2	2A	S-1401,776,
	2B	203,295, 731,1026, 256,236
Cluster 3	3A	SD-11, JAS-9 white, Sree Arun, SV-1-14, ,
	3B	SD-39,SD-24, SP-12-SP
Cluster4	4A	737, 740,739,738, RSM-2015-3,SD-29
	4B	SD-53, SD-55
Cluster 5		Jas-9-pink

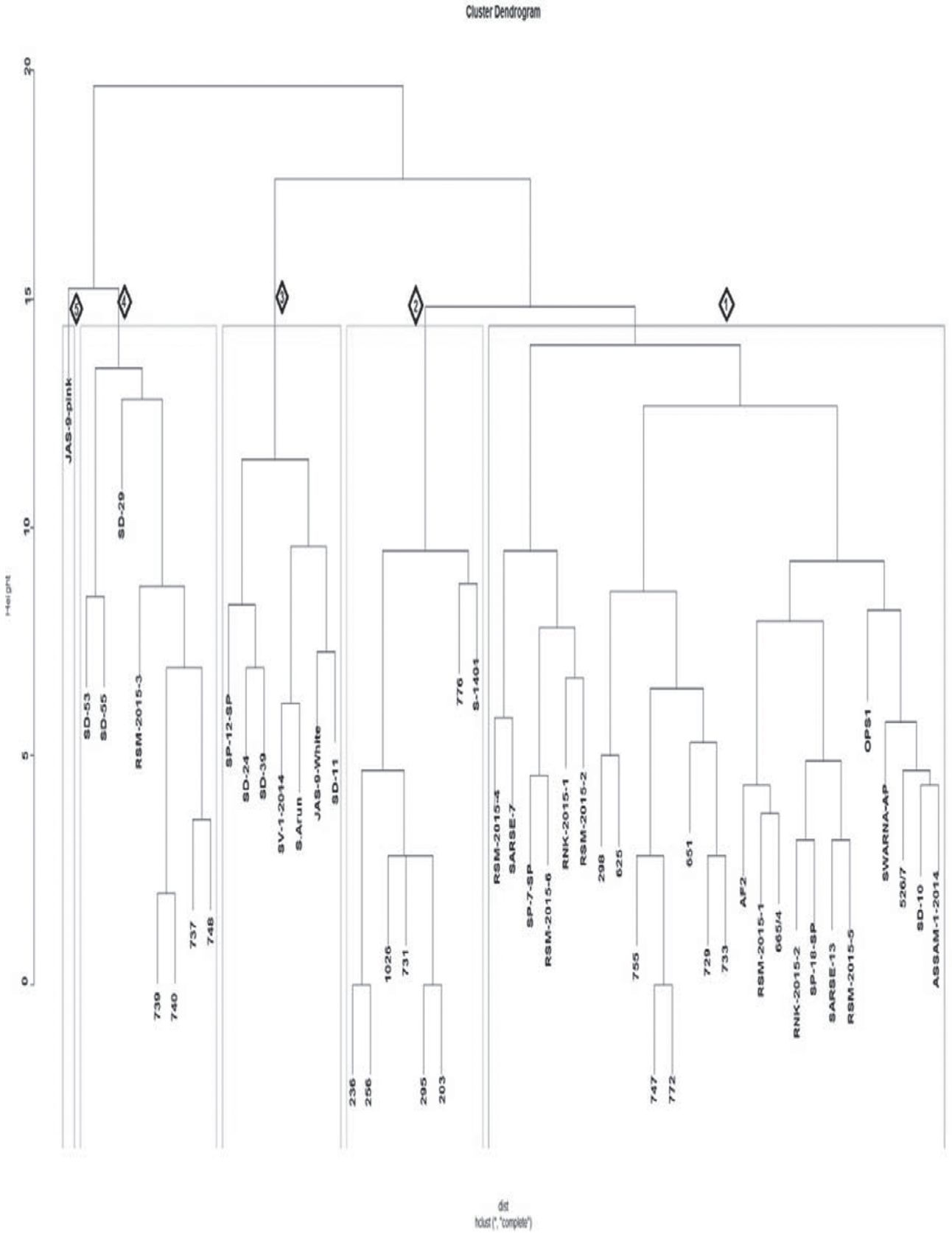

Fig. 1. Dendrogram based on morphological characterization

Table 4. Interclustral distance within the dendrogram of accessions based on morphological characterisation

Cluster	I	II	III	IV	V
I	0.00	14.83	17.72	17.63	19.13
II	14.83	0.00	17.91	15.58	19.67
III	17.72	17.91	0.00	15.77	15.23
IV	17.63	15.58	15.77973	0.00	18.76
V	19.13	19.67	15.23	18.76	0.00

separated within a distance 13.49,whereas in Cluster IV, the distance was 11.49 and zero within Cluster V. Three pairs of accession, S-772 and S-747 in major cluster1, S-236 and S-256, and, S-203 and S-295 in major cluster 2 were the most similar with Euclidean distance of 0 which indicated 100% similarity. Thus, these pairs can be considered as duplicates. All the accessions in this study were grouped within the Euclidean distance between 0 and 15. Karuri *et al*. (2010) evaluated 89 sweet potato accessions morphologically and the dendrogram based on morphological data separated the accessions into two major clusters at a distance of 6.98.

Principal component analysis (PCA)

Principal component analysis of 20 morphological traits in 50 sweet potato accessions indicated high Eigen values for the first three principal components (PC) which accounted for the 66.21% of the variability among the characters studied (Table 5). The first principal component accounted for 31% of the variability which is due to predominant vine colour, secondary vine colour, abaxial vein pigmentation and petiole pigmentation. Predominant vine colour, petiole pigmentation, immature leaf colour and shape of central lobe were the traits included in both PC1 and PC2.

Table 5. Principal component analysis of 20 morphological traits in 50 accessions of sweet potato (Highly loaded variables in combined analysis given in bold face)

Variables	PC1	PC2	PC3
Twining	0.000	0.000	0.000
Plant type	-0.002	-0.124	-0.037
Ground cover	-0.087	-0.019	-0.021
Vine internode length	-0.114	-0.231	-0.038
Vine internode diameter	-0.035	0.008	-0.021
Predominant vine colour	0.362	-0.191	-0.028
Secondary vine colour	0.680	-0.028	0.141
Vine tip pubescence	0.112	-0.247	0.898
General outline of leaf	0.050	0.110	0.159
Leaf lobes type	0.092	0.431	0.130
Leaf lob number	0.037	0.285	-0.022
Shape of central leaf lobe	0.198	0.419	0.168
Mature leaf size	0.004	-0.006	-0.022
Abaxial leaf vein pigmentation	0.343	-0.074	-0.063
Mature leaf colour	-0.002	-0.005	0.006
Immature leaf colour	-0.214	0.235	0.021
Petiole length	-0.121	-0.297	-0.043
Petiole Pigmentation	0.362	-0.234	-0.141
Tuber predominant skin colour	-0.097	-0.006	-0.267
Tuber predominant flesh colour	-0.043	-0.416	-0.028
Eigen values	0.230	0.142	0.115
Percent variation	31.242	19.276	15.699
Cumulative percentage	31.242	50.518	66.217

Karuri *et al*. (2010) in their study of 34 morphological characters found that the shape of the central leaf lobe and the general outline of leaf were the major characters that separated the accessions into two major clusters. In the present study, based on the principal component analysis the major grouping of 50 accessions was based on predominant vine colour, secondary vine colour, abaxial vein pigmentation (Fig.2) and petiole pigmentation. The frequency distribution of these major variable as indicated by PC1 is depicted in pie chart (Fig. 3). The PCA values revealed a very high value for secondary vine colour value which indicates that this character has played a major role in clustering of the accessions studied.

In the present study, three pairs of duplicates S-236 and S-256, S-203 and S-295, and S-772 and S-747 were identified through morphological characterisation. This is a very low value as compared to Huaman *et al*. (1999) where, the identification of duplicates in the Peruvian sweet potato collections in CIP reduced from 1939 to 673 and the duplicate accessions per cultivar ranged from 1 to 99 using only 21 descriptors.

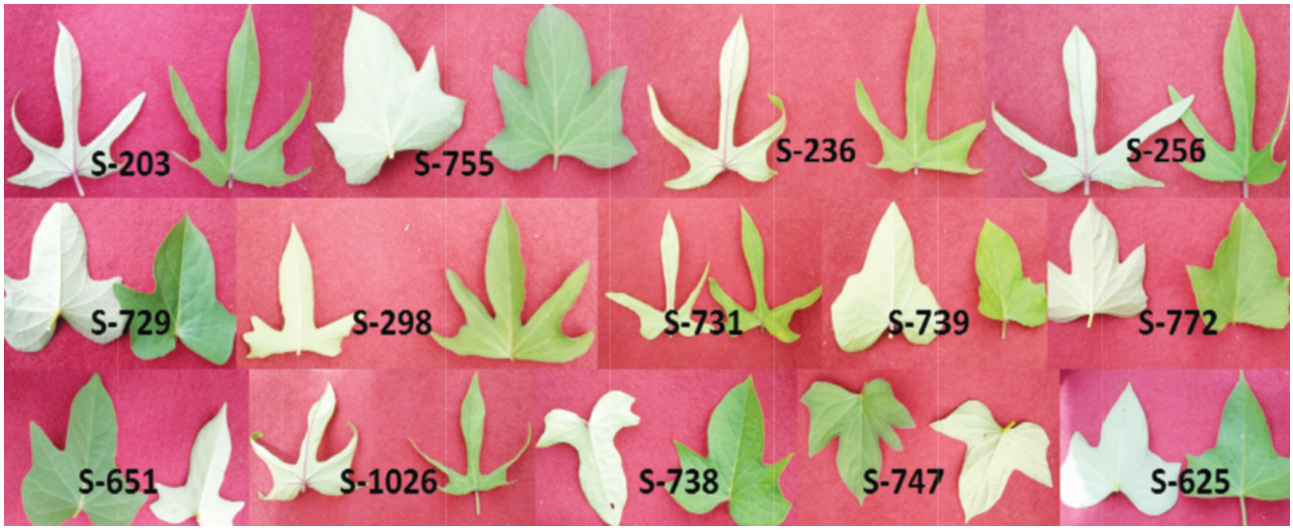

Fig.2. Variability in leaf lobe type (abaxial and adaxial view)

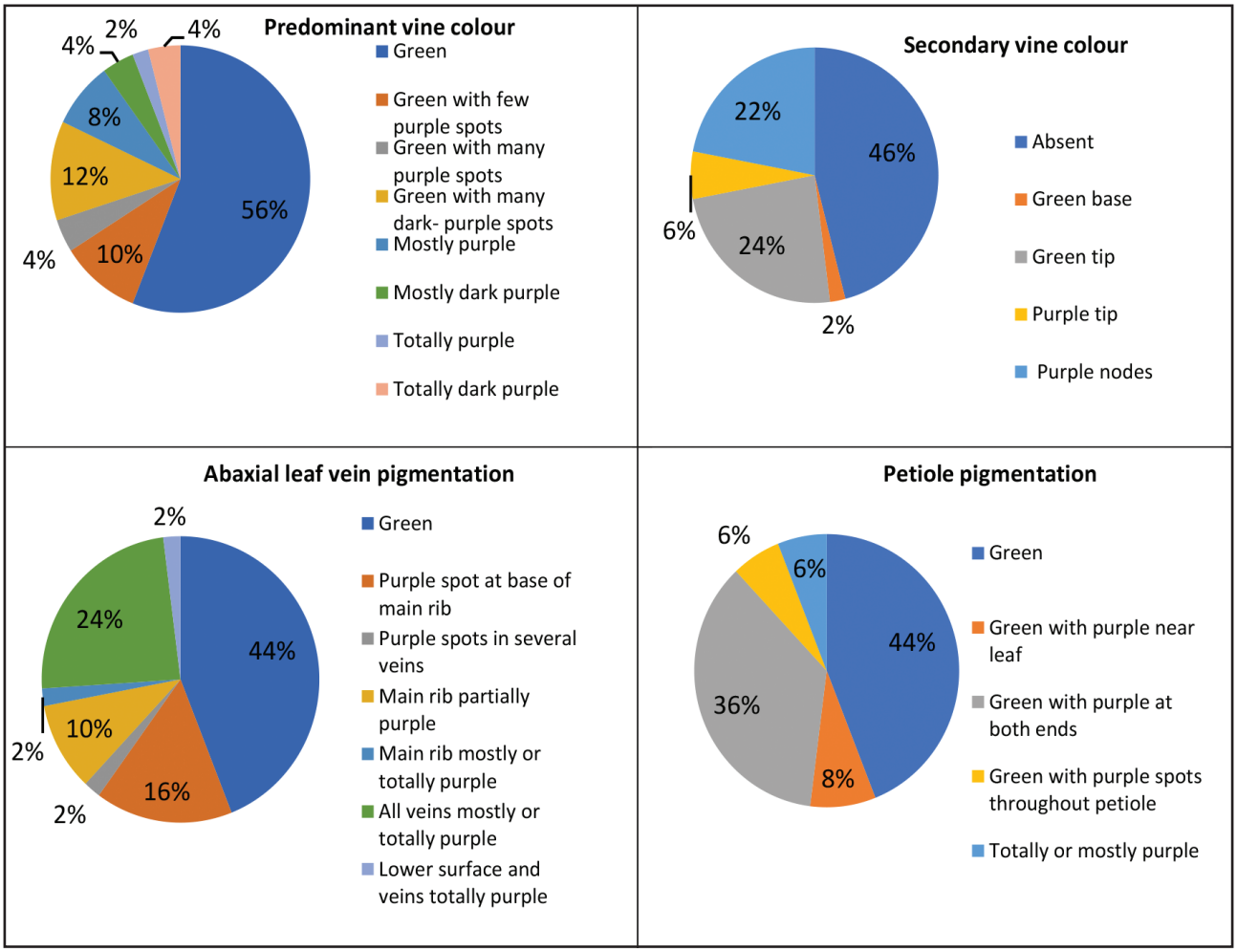

Fig. 3. Frequency distribution of the major variables among the 50 selected sweet potato accessions

Molecular characterization

Among the 21 ISSR primers screened, 11 primers- UBC 807, UBC 808, UBC 809, UBC 818, UBC 825, UBC 827, UBC 847, UBC 860, (ACC)6Y, (GA)9AT and (GA)9AC, gave clear and reproducible bands which were used further for PCR amplification. A total of 162 bands were generated with an average of 14.7 bands per primer. Total number of bands per ISSR primer ranged from 9 (UBC 818) to 18 (UBC 860 and (GA)9AC) (Fig.4). Of the 162 bands obtained all the bands were polymorphic (100%) between the accessions. The percentage of polymorphism is an indication of the discriminating power of primers in separating the accessions. This is high when compared to those obtained in a study conducted in sweet potato by Quiang et al. (2009) where they obtained 13.8 ISSR bands on an average as well as only 89.6% of polymorphism. In the present study, the highest band number of 18 were obtained from UBC 860 and $(GA)_9AC$. The lowest number of polymorphic bands (9) was obtained from the primer UBC 818. So the number of polymorphic bands in the study ranged from 9 to 18 which is high in contrast to that obtained in the study conducted by Moulin *et al*. (2012) among sweet potato accessions where the value ranged from 4 to 11 by using 19 ISSR primers and generated a total of 135 bands which is also low when compared to the present study. In the present study ISSR markers generated 100% polymorphism across 50 accessions and therefore proved as efficient marker system to differentiate various accessions of sweet potato.

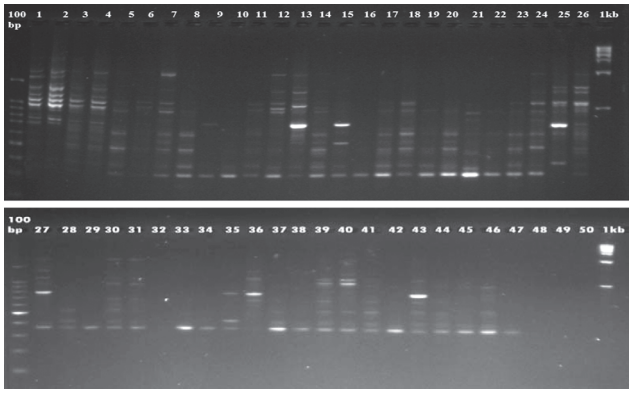

Fig. 4. Electrophoretic pattern of 50 accessions of *Ipomoea batatas in* 2 % agarose gel using UBC (GA)9AC

Cluster analysis

The binary data based on ISSR banding pattern was used to generate UPGMA dendrogram using Jaccards similarity coefficient which separated the accessions into six principal clusters (Table 6) at a coefficient 0.9. (Fig. 5). The third principal cluster comprised of 20 accessions which were assembled in 5 sub-groups which indicates high intra-clusteral variability (Table 7). The fourth principal cluster comprised of 15 accessions in 4 groups. In this principal cluster S-236 and S-256 grouped together with 100% similarity. So this pair can be considered as true duplicates which were also found as

Table 6. Accessions grouped based on dendrogram

Clusters	Sub-Clusters	Accessions
Cluster 1	A	739, SV-1-14, SP-12-SP, RSM-2015-5
	B	747, SARSE-13, S-1401, 651
Cluster 2		RSM-2015-6, SD-24, 776
Cluster 3	A	772, 755, Sree Arun, RSM-2015-4, Swarna-AP, RSM-2015-3, SARSE-7, JAS-10-pink
	B	729,733, 740
	C	Sp-7-Sp, 625, AF2, RNK-2015-2
	D	737, 203, Assam-1-2014, 665/4
	E	SP-18-SP
Cluster 4	A	JAS -9-white, RSM-2015-1 SD-10, RSM-2015-2, RNK-2015-1, OPS1
	B	526/7, SD-11, 236,256,298,295, 1026
	C	738,731
Cluster 5		SD-29
Cluster 6		SD-55, SD-53, SD-39

Table 7. Interclustral distance within the dendrogram of accessions based on ISSR markers

Cluster	I	II	III	IV	V
I 8.30	0.00	8.66	8.18	8.71	8.06
II 8.66	0.00	7.81	7.87	7.87	8.12
III 8.18	7.81	0.00	6.48	5.09	5.91
IV 8.71	7.87	6.48	0.00	5.56	6.70
V 8.06	7.87	5.09	5.56	0.00	4.24
V I8.30	8.12	5.91	6.70	4.24	0.00

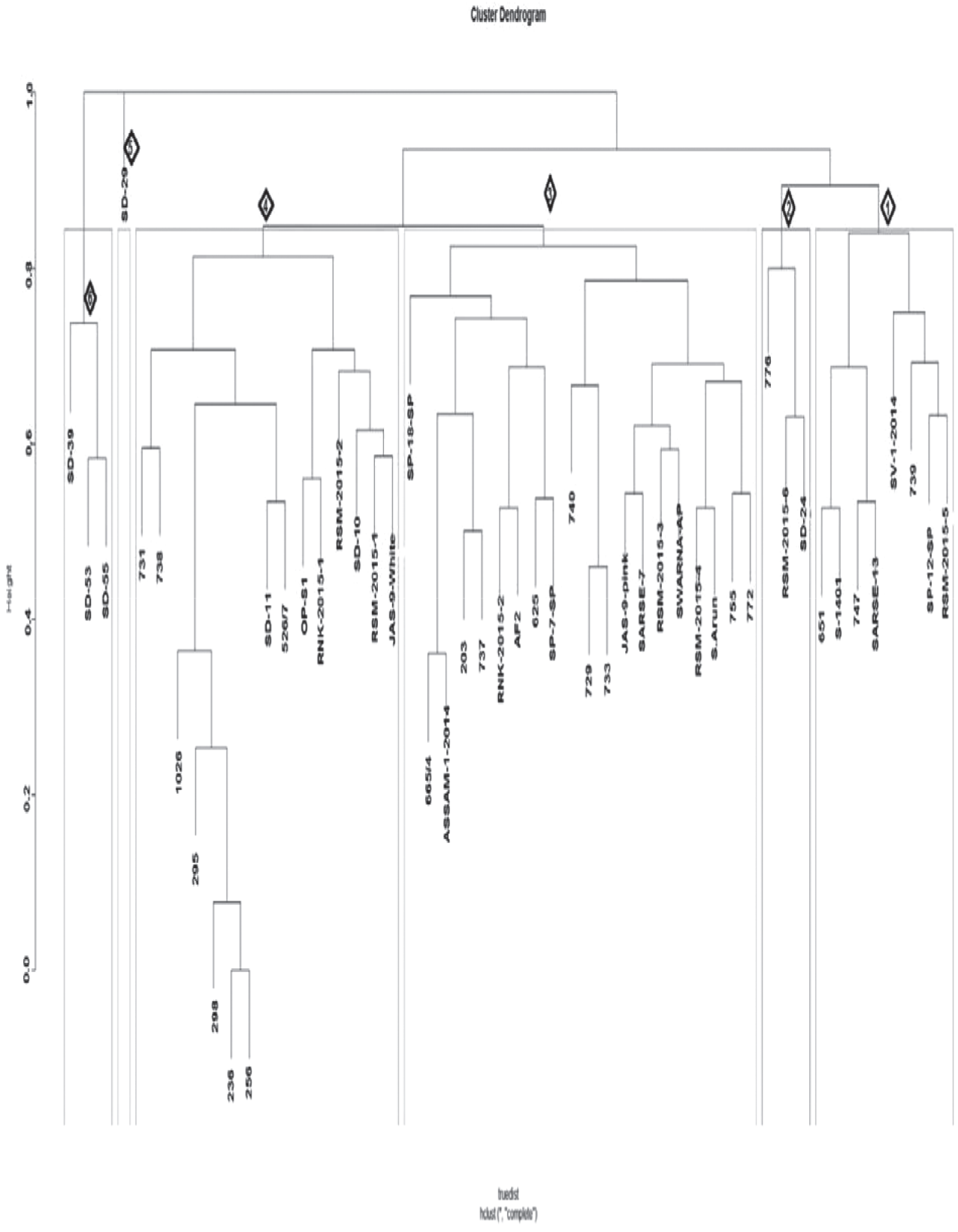

Fig. 5. Dendrogram of 50 accessions based on ISSR markers

duplicates based on morphological analysis also. The accession SD-29 was represented as a single- membered cluster. The intraclustral distance between the accessions in the Cluster I was 8. 77 whereas in Cluster II the accessions were separated within a distance of 7.81. The accessions within Cluster III were separated within a distance 5.2, in Cluster IV,the distance was 6.32 and zero within Cluster V and 3.74 within Cluster VI.

Moulin *et al*. (2012) obtained a dendrogram from a study of 44 sweet potato accessions based on molecular data and found that the clustering and geographical location of the accessions were quite unrelated as accessions were grouped in same cluster regardless of their geographical location. They obtained highly genetically distinct accessions from dendrogram as they did not fit into any of the cluster formed. Similarly the present study also obtained a highly genetically distant accession that existed as an outlier in the dendrogram represented as fifth cluster (SD-29). Similar result was obtained in the dendrogran generated from morphological data also where SD-29 and SD-55 stood as different outliers.

Genetic relationships based on similarity matrix

Pair wise comparison of accessions based on Jaccards similarity coefficient indicated genetic similarity between accessions ranging from 52% to 100%. This means that dissimilarity or variability ranged from 0-48%. This indicates that low to moderate variability exist between the selected accessions. The maximum similarity of 100% was observed between S-236 and S-256 which are genetically true duplicates. A 96% similarity was observed between S-203 and S-236. JAS-10-pink and RSM-2015-2 showed 95% similarity. Similarly S-772 and SP-7-SP showed 95% similarity. The least similar accessions (most dissimilar) were SD-39 and S-298 (52%). A similarity coefficient of 0.53 was observed between Sree Arun and JAS-10-pink, JAS-9-white and 526/7, SP-12-SP and 526/7, ASSAM-1-2014 and S-1026. SD-29 was different from all the remaining accessions by a similarity coefficient of 0.61. Within each principal cluster there was lot of intraclusteral variation.

Mantel's test revealed a weak correlation (r=0.034, P=0.305) between the morphological and molecular data sets. Mantel's test was performed to check whether there is a correlation between morphological and molecular aspects of the accessions. This suggests that the morphological variation may be determined by environmental factors and also by genetic factors as reported for other crops (Ashish *et a*l., 2010). It is frequently observed that the genetic variation determined by molecular markers can produce different results due to analysis in different regions in the genome captured by the respective markers. This may be also due to the number and the choice of measured traits, ISSR primers and to selection pressure which created similar forms but with a different genetic structure (Corrado et al, 2009).

The wide range of intraclusteral groupings between sweet potato accessions in morphological and molecular dendrogram indicated wide range of variability among the accessions. The self incompatibility, out crossing nature and hexaploidy exhibited by sweet potato might have contributed to this high variability. Despite high number of morphologically similar accessions, only two true duplicates viz, S-236 and S-256 were identified in this study which may be due to the limited number of ISSR primers used in the study. In future, more reliable and specific markers may be more suitable to give better results.

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