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Isozyme-based genetic fingerprinting of *Manihot* sp

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Abstract
Many cassava varieties have been released into farmers' fields in Nigeria, but no reliable means in tracking their identity, thus causing multiple naming of these varieties. The methods used for identifying different cultivars of crop plants are based conventionally on the phenotypic expressions of the plant, plant parts, seeds or mode of introduction. The objectives of this study were to establish a reliable means of identifying cassava genotypes in in farmers' fields based on isozyme analysis. Thirty- two cassava genotypes comprising of IITA elite cultivars, local landraces and adapted farmers' varieties were planted at a spacing of 1m x 1m in a randomized complete block design in two replications.. Three morphological clusters were observed at 0.44, 0.497, and 0.497 levels of coefficient of similarity. The stem and petiole colours were observed as dominant traits in cassava classification. There are 3, 8, 9 and 11 loci for SKDH, 6-PGD, AAT and G-6-PDH, respectively, while isozyme markers 6-PGD, AAT and G-6-PDH revealed more polymorphism in cassava classification. These results imply important information for cassava population development and improvement that can be exploited by breeder.

Key words: Isozyme markers, genotypes, classification, morphology and polymorphism

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Introduction

Root crops form an important part of the diet in many areas of the tropics and subtropics. Cassava is dicotyledonous and grown for its edible root tubers. Cassava produces starchy storage organs which provide major contributions to the calorific content of the diet, especially in humid tropical countries. Cassava (*Manihot esculenta* Crantz) is a member of the *Euphorbiaceae.* It is the most cultivated of the *Manihot* species. It is not know in the wildest state but has long been cultivated in Central America and Brazil, Africa, Asia and the Pacific Islands.

Reliable identification of cultivars using methods based on morphological and physiological characters has become increasingly difficult because of the large number of lines being released and the convergence of these lines on a few of the most desirable characters. Many cassava varieties have been released into farmers' fields in Nigeria, but no reliable means in tracking their identity, thus causing multiple naming of these varieties. The methods used for identifying different cultivars of crop plants are based conventionally on the phenotypic expressions of the plant, plant parts, seeds or mode of introduction. These expressions are strongly influenced by the environment in which the plant grows. Such conventional means are gradually being replaced by chemical methods, such as isozyme electrophoresis, which greatly improve the chances of correct cultivar identification. Morphological observation on characters such as height of plants, average weight of tuberous root, girth of tuberous root and other morphological traits have assisted in studies on agronomic performance and classification of cassava cultivars (Mathura *et al*., 1986; Rogers and Appan (1973). However, the combination of morphological characters and isozyme markers will enhance the reliability of cassava cultivar classification.

Since isozymes are mostly expressions of the genetic make-up of the plant, and are therefore little affected by the environmental conditions (Lee and Ronalds, 1967); their patterns are highly reproducible. In order to use isozyme techniques successfully for cultivar identification, a particular crop most exhibit significant levels of isozyme polymorphism among the cultivars. In addition, well-resolved isozyme phenotypes must be obtained from leaf or stem tissue and phenotypes should exhibit little or no variability during development or under diverse environmental conditions. Ramire *et al.* (1987) used starch and polyacrylamide gel electrophoresis for determining isozyme electrophoregrams (pattern) of 16 enzymes of cassava varieties as potential genetic markers. Extracts of five different tissues (root, stem, leaf, petiole and bud) were examined and found that the nodal portions of the shoot gave isozyme patterns with the largest number of bands which, were used for the identifications of the varieties.

The objective of this study is to establish a reliable means of identifying released, local and elite cassava genotypes available in farmers' fields.

Materials and Methods

The experiment was carried out on the field at International Institute of Tropical Agriculture (IITA) experimental station, Ibadan. Ibadan is situated at the northern fringe of the tropical forest with mean annual rainfall of 1,200mm, mean monthly temperature of about 26.6^0C and high relative humidity of about 70%. Thirty- two genotypes comprising IITA elite cassava cultivars, local landraces and adapted farmers' varieties were planted at a spacing of 1m x 1m in a randomized complete block design in two replications. Seven plants per genotype per replicate were planted and five plants per genotype per replication were tagged for record taking, two plots of size $224m^2$ per plot were used. The Plots were ploughed and ridged at a distance of 1m apart. Data collected were based on International Plant Genetic Resources Institute (IPGRI, 1990) evaluation system. The genetic materials used in this study, status and country of origin are indicated in Table. 1.

Morphological data collection: Collection of data commenced a month after planting (MAP) for establishment rate, vigour, colour of both unexpanded and first expanded leaf and reaction to African cassava mosaic virus. At 3 MAP, data on leaf lobe and shape, petiole and vein colour, pubescence of young leaves, stipule length, distribution of anthocyanin pigmentation on the petiole, African cassava mosaic virus and cassava bacterial blight were collected based on IPGRI multi-crop passport descriptors (1990). The length and the width of the central lobe, petiole length, leaf area, stem colour, growth habit, African cassava mosaic virus and cassava bacterial blight were assessed at 6 MAP. The height at first and second branching were measured.

The Hoeffer Scientific Instrument model SE700 electrophoresis tank and model PS 2,500 power supply unit were used for electrophoretic analysis. Slab polyacrylamide gels were used which were made up of the following stock solutions (Table 2).

*The separating gel***:** Contains (A = 7.50 ml, C = 15.0ml, distilled H2O = 37.5 ml, E = 0.41 ml and TEMED = 18.75ml) of the stock solutions

*The Stacking gel***:** Contains stock solutions (B = 2.5 ml, D = 5.0 ml, distilled H2O = 12.5 ml, E = 60.0 µl and TEMED = 24. 0 µl)

Sample Preparation and Enzyme Extraction: Samples of young fresh leaves from the field were collected with test tubes placed in an ice container and then taken into the laboratory. The samples were crushed in extraction buffer Solution Trizma base = 1. 211g, Potassium Chloride = 0. 725g, Ethylene Diaminetetraacetic acid (EDTA) = 0.298g, Sucrose = 3.420g, 2M-E (Mercaptoethanol) = 60.0 µl, and distilled H_2O to 100 ml at pH 7.5 with 200 mg of Polyviylpolypyrrolidone (PVPP) of ratio 1:5 (W/V)) per sample. After crushing, the sample was centrifuged at 4^0 C for 10 min. at 10,000 x g. About 20.0 µl of the supernatant was loaded into each slot. Two standard genotypes were included on each slab to provide reference bands. The stock electrode buffer was Davis 10x Tris/glycine at pH 8.57 at room temperature and 10x concentration. The Hoeffer Scientific Instrument model SE700 electrophoresis tank and model PS 2500 power supply unit were used for electrophoretic analysis. Slab polyacrylamide gels were used which were made up of the following stock solutions.

Table 1: Genotype use, their status and country of origin

S/no.	Genotype of TMS* series	Status	Country of Origin
1	71673	Improved	IITA
2	71693	Improved	IITA
3	58308	Improved	IITA
4	91934	Improved	IITA
5	83672	Improved	IITA
6	81/01623(4X)	Improved	IITA
7	63397	Improved	IITA
8	50395	Improved	IITA
9	30572	Adapted variety	IITA
10	4(2)1425	Adapted variety	IITA
11	88/112-7(3X)	Improved	IITA
12	91934(4X)	Improved	IITA
13	LCN 8010	Land race	Nigeria
14	ODUNGBO	Landrace	Nigeria
15	42025	Improved	IITA
16	40764	Improved	IITA
17	30001	Adapted variety	IITA
18	30555	Adapted variety	IITA
19	30337	Improved	IITA
20	61036	Improved	IITA
21	60506	Improved	IITA
22	90853	Improved	IITA
23	60444	Improved	IITA
24	60142	Improved	IITA
25	71173	Improved	IITA
26	61324	Improved	IITA
27	90257	Improved	IITA
28	61677	Improved	IITA
29	Isunikankiyan	Land race	Nigeria
30	4(2)O267	Improved	IITA
31	4(2) 1443	Improved	IITA
32	30211	Improved	IITA

* TMS : Tropical Manihot Species; IITA: International Institute of Tropical Agriculture

For electrophoresis, an initial voltage between 200-300V and a constant current of 60mA for 3 hours at 4^0 C was used. The gels were then stained according to the following recipes:

Staining markers and constituents: AAT: Aspartate amino transferase (E.C.2.6.1.1.): Modified Vallejos method (1983) Tris-HCL pH 8 (0.1M) = 10 ml, H_{2O} (distilled) = 90 ml, Aspartic acid = 0. 30g, a- ketoglutaric acid = 0.15g, pyridoxal -5- phosphate = 0.01g or pinch and Fast blue BB salt = 0.15g

6-PGD: 6-phosphogluconate dehydrogenase (E.C.I.I.I.44): Modified Vallejos method (1983) Tris –HCl pH7.5 (0.IM) = 50 ml, $MgCl_2$ (IM) = 0.25 ml, 6-P-gluconic acid = 10 mg, NADP (20mg/ml) = 0.38 ml, MTT (10mg/ml) =1.0 ml and PMS (10mg/ml) = 0.2 ml

G-6-PDH: Glucose-6-phosphate dehydrogenase (E.C.I.I.I.49): Modified Vallejos method (1983). Tris- HCL pH 7.5 (0.IM) = 50 ml, $MgCl_2$ (IM) = 0.5 ml, Glucose -6-phospate = 20 mg, NADP (20mg/ml) = 0.38 ml, MTT (10mg/ml) = 1.0 ml and PMS (10mg/ml) = 0.2 ml

*SKDH: shikimate dehydrogenase (E.C.I.I.I. 25)***:** Modified Vallejos method (1983). Tris-HCL pH 7.5 (0.Im) = 50 ml, Shikimic acid = 50 mg, NADP (20mg/ml) = 0.38 ml, MTT (10mg/ml) = 1.0 ml and PMS (10mg/ml) = 0.2 ml

Table 2: Biochemical Analysis and solutions

Solution	Stock solutions: Concentrations
A	Tris - HCL buffer Trizma base = 36.60g + distilled H_2O at pH 8.9 to 100 ml at room temperature
B	Tris – HCl buffer Trizma base = 5.0g + distilled H_2O to 100 ml at room temperature
C	Acrylamide = 28.00g Bis- acrylamide = 0.735g + distilled to 100 ml at room temperature
D	Acrylamide = 10.00g Bis-acrylamide = 2.50g + distilled H_2O to 100ml at room temperature
E	Ammonium Persulphate at 10g/100 ml prepared fresh weekly

The gels were equilibrated with staining buffer for about 10 min. in the incubator at 37^0C. This was replaced with the complete staining solution and incubated in the dark at 37^0C for half an hour or until the bands developed.

Statistical Analysis: The morphological data were subjected to hierarchical cluster analysis in GENSTAT computer programme which drew dendrograms. Genetic analysis: Enzyme loci were evaluated for polymorphism and enzyme activity. The allelic frequencies for each genotype were recorded based on the repeatability and interpretation of the dendogram. To study the genetic relationships among the genotypes, the presence or absence of isozyme bands transformed into a binary character matrix (1 = presence and 0 = absence of isozyme band). Thus, isozymic variability was created and a binary matrix was derived. Pair-wise distance matrixes between genotypes were again derived using the numerical taxonomy and multivariate analysis system (NTSYS-PC), Version 2.1 (Rohlf, 2000) and the Jaccard coefficient of similarity (Jaccard, 1908). Genetic diversity dendogram for the genotypes was created by unweighted pair group method with arithmetic mean (UPGMA) cluster analysis (Sneath and Sokal, 1973; Swofford and Olsen, 1990).

Results

Three clusters of the genotypes were observed at 0.44, 0.497, and 0.497 levels of coefficient of similarity for cluster I, II and III, respectively. Cluster I, II and III had 12, 3 and 17 genotypes, respectively (Fig. 1). As the level increases, more clusters were being formed and the cluster size decreases, at 0.81 level of coefficient of similarity, some of the genotypes become distinctly different from others. At 0.554 levels of coefficient of similarity, TMS 60444 became distinctly different from other genotypes.

Some of the distinct morphological features in cluster I are purple petiole colour, silver green stem and small tuber size are the common features found among the genotypes (Fig. 1 and Table 3). The local genotypes such as Isunikankiyan and Odungbo are among the genotypes in this cluster. The common morphological features among the genotypes in cluster II are light green petiole colour, silver stem colour, easy to remove the root texture and medium size storage root (Table 3). In cluster III, light green petiole colour, dark brown root surface colour with rough texture and white cortext are the common morphological features among the genotypes.

Table 3: Major distinguishing morphological features among 32 cassava genotypes.

		Distinguishingcharacters												
Genotype of TMS series	Geno.	1	2	3	4	5	6	7	8	9	10	11	12	13
71673	1	LV	LP	LG	LG	P	SG	S	LB	M	S	D	W	S
71693	2	LV	LP	LG	LG	P	SG	S	LB	M	S	D	W	S
58308	3	VV	LP	DG	LG	LG	DB	M	DB	R	M	E	W	S
91934	4	V	HP	GP	LG	LG	LB	A	DB	R	L	D	W	M
83672	5	VV	HP	GP	LG	LG	DB	A	DB	R	S	D	W	S
81/01623(4X)	6	VV	NP	GP	LG	LB	DB	S	DB	R	S	D	W	S
63397	7	VV	NP	LG	LG	LG	LB	S	DB	R	M	E	W	S
50395	8	VV	NP	P	GP	LG	DB	A	DB	R	L	E	W	B
30572	9	V	NP	GP	LG	LG	DB	S	DB	R	M	E	W	M
4(2)1425	10	LV	HP	LG	LG	LG	S	S	W	S	M	E	W	B
88/112-7(3X)	11	VV	NP	GP	LG	LG	DB	S	DB	R	L	E	W	B
91934(4X)	12	VV	HP	GP	LG	LG	LB	A	DB	R	S	E	W	M
LCN 8010	13	LV	HP	LG	LG	P	SG	M	LB	M	M	E	W	M
ODUNGBO	14	VV	NP	LG	LG	P	SG	M	DB	M	L	D	W	M
42025	15	LV	HP	LG	LG	P	SG	S	W	S	S	E	W	S
40764	16	LV	LP	LG	LG	LG	LG	S	DB	R	L	E	W	M
30001	17	VV	P	LG	LG	LG	SG	S	W	S	S	E	W	S
30555	18	LV	NP	GP	LG	P	DB	S	DB	R	L	E	W	B
30337	19	VV	NP	LG	LG	LG	LB	S	DB	R	S	E	W	B
61036	20	VV	NP	GP	LG	P	DB	L	LB	R	S	E	P	S
60506	21	LV	LP	LG	LG	P	DB	L	LB	R	S	E	W	S
90853	22	V	NP	GP	LG	LG	DB	S	DB	R	L	E	W	B
60444	23	V	NP	P	GP	LG	S	L	LB	M	L	E	W	M
60142	24	V	HP	LG	LG	P	S	S	W	S	M	E	W	S
71173	25	V	NP	DG	LG	LG	S	A	LB	S	M	E	W	M
61324	26	V	HP	DG	LG	LG	DB	M	DB	R	S	E	P	M
90257	27	VV	LP	LG	LG	LG	S	A	LB	S	M	E	W	B
61677	28	VV	LP	LG	LG	P	SG	S	DB	R	S	E	P	S
Isunikankiyan	29	VV	LP	LG	LG	P	SG	S	DB	R	S	E	P	S
4(2)O267	30	V	P	GP	LG	LG	LB	L	DB	R	L	E	W	S
4(2) 1443	31	LV	LP	LG	LG	LG	DB	S	DB	R	L	E	W	B
30211	32	LV	NP	LG	LG	LG	LB	M	DB	R	M	E	W	M

Character codes:

1. Vigor: Very vigorous (VV), Vigorous (V) and less vigorous (LV).
2. Pubescence: Highly pubescent (HP), Pubescent (P), Less Pubescent (LP) and Non- Pubescent (NP)
3. Colour of unexpanded apical leaves: Light green (LG), Dark green (DG, Green purple (GP) and Purple (P)
4. Colour of fist fully expanded leaf: Light green (LG), Dark green DG, Green purple (GP) and Purple (P).
5. Petiole colour: Light green (LG), Dark green (DG, Green purple (GP) and Purple (P).
6. Stem colour: Silver green (SG), Light brown (LB), Dark brown (DB) and Silver (S).
7. Storage root peduncle: Absent (A), Short (S), Intermediate (M) and Long (L).
8. Storage root surface colour: White (W), Light brown (LB) and Dark brown (DB)
9. Storage root surface texture: smooth (S) Medium (M) and Rough (R).
10. Storage root length: Short (S), Medium (M) and long (L).
11. Ease of root cortex (Inner skin) removal: Easy (E) and Difficult (D).
12. Colour of outer surface of storage root cortex: white (w), Yellow (Y), Pink (P) and Purple (PP).
13. Tuber size: Small (S), medium (M) and Big (B)

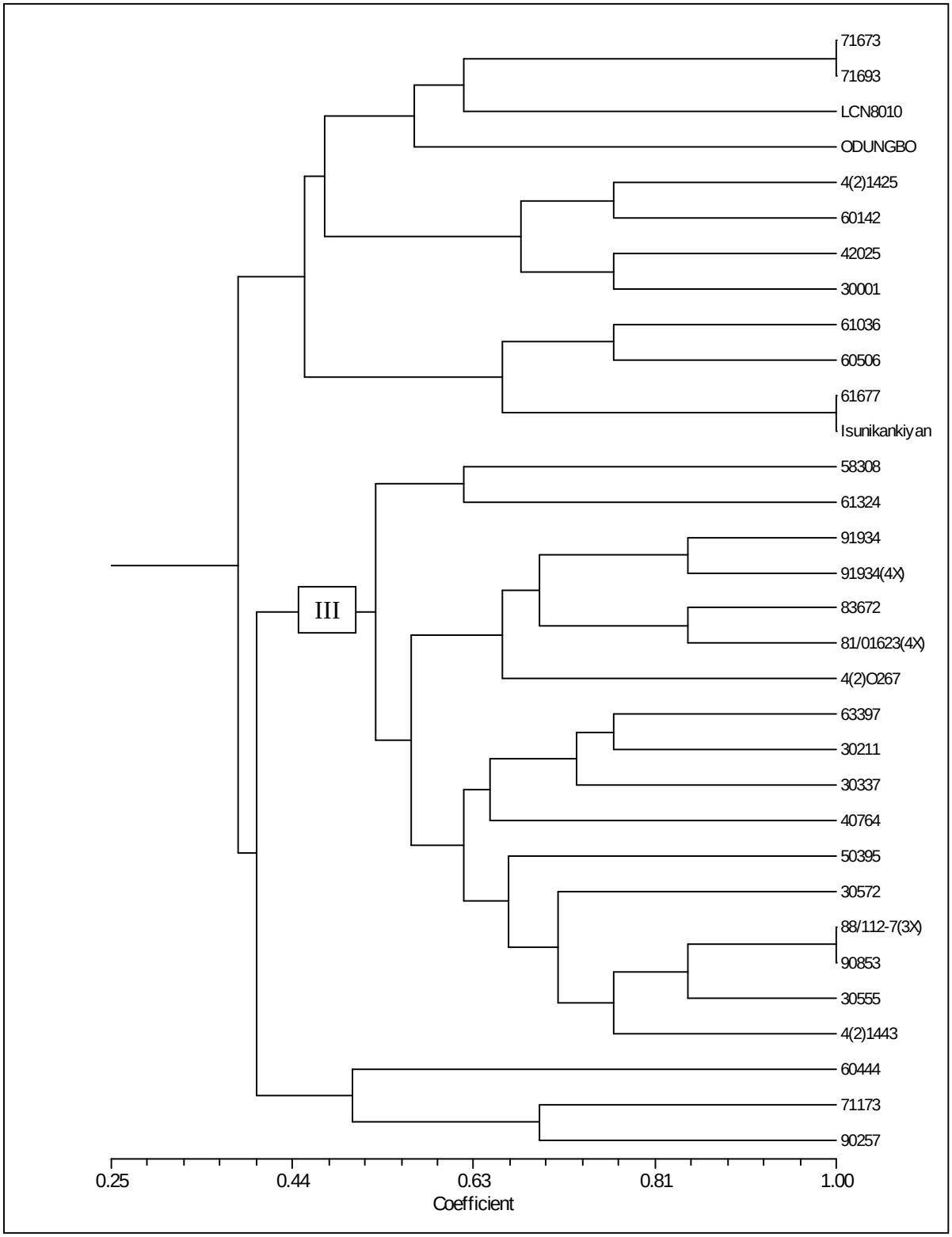

Fig. 1 : **Dendrogram showing genotypes clusters among 32 *Manihot* sp as revealed by morphological characters.**

The 31 isozyme loci were used to construct phylogenetic relationship dendogram among the 32 genotypes. These 31 loci are potentially useful isozyme markers that revealed the genetic diversity and relationships among the genotypes. At about 0.65 levels of coefficient of similarity, a genetic cluster was revealed, while at 0.70 level of coefficient of similarity, cluster I was observed comprising of 6 genotypes. Cluster II was revealed at 0.75 level of coefficient of similarity, while Cluster III and IV were observed at 0.83 level of coefficient of similarity (Fig. 2). Genotype TMS 88/112-7 (3X) and TMS 61677 had the highest number of identifiable loci, 16 and 15, of the markers examined, respectively, hence their genetic distinction among the genotypes examined. These genotypes possess up to 7 similar morphological characters (Fig. 2 and Table 3).

Isozyme phenotypes: This study revealed 31 isozyme loci with the enzyme profiles of the genotypes studied (Table 4). There are 3, 8, 9 and 11 loci for SKDH, 6-PGD, AAT and G-6-PDH, respectively. While AAT had 6.3 – 96.9% polymorphism, other isozyme markers had 6.3 – 62.5%, 6.3 – 93.8% and 12.5 – 96.9% polymorphism for SKDH, 6-PGD and G-6-PDH, respectively (Table 4). Locus with low percentage polymorphism indicates that the locus appears in most of the genotypes examined like L-7 of AAT with 6.3% polymorphism.

Table 4. Isozyme markers that revealed polymorphism among 32 *Manihot* sp.

S/N	***Isozyme Marker***	***Loci***	***% Polymorphism***
1	AAT	L-1	96.9
		L-2	96.9
		L-3	75.0
		L-4	81.3
		L-5	12.5
		L-6	96.9
		L-7	6.3
		L-8	78.1
		L-9	87.5
2	SKDH	L-1	62.5
		L-2	6.3
		L-3	34.4
3	6-PGD	L-1	90.6
		L-2	90.6
		L-3	6.3
		L-4	90.6
		L-5	84.4
		L-6	68.8
		L-7	15.6
		L-8	93.8
4	G-6-PDH	L-1	96.9
		L-2	96.9
		L-3	90.6
		L-4	75.0
		L-5	37.5
		L-6	46.9
		L-7	12.5
		L-8	31.3
		L-9	59.4
		L-10	84.4
		L-11	96.9

Fig 2**.** Dendrogram showing genetic diversity among 32 *Manihot* sp as revealed by isozyme markers

Discussion

Morphological characteristic in cassava genotype identification: Morphological characters have been proved to be a useful tool in *M. escultenta* classification (Maduakor and Lal, 1989). Three major cluster groups were identified. In cluster I, the genotypes possessed silver green stem colour and purple petiole colour these traits assist in variety identification during planting. This cluster comprised

local landraces such as Odungbo, LCN 8010, and Isunikankiyan, which are known to be sweet cassava. This cluster I, may serve as gene pool for breeding sweetness quality in cassava. The root texture of these genotypes is very easy to remove, thus reduced drudgery in peeling cassava for gari. Genotypes in this cluster could be used as parents in cassava population improvement for these traits. However, cluster I and cluster III have some common features; the genotypes are vigorous with light green petiole colour and dark brown storage root surface colour. TMS 58308 is a common parent to most of the genotypes in these clusters. The storage root surface texture of genotypes in cluster III is rough which, distinguishes it from cluster I that had varied surface texture. The three genotypes in cluster II had silver stem colour which, indicates the major dominant trait for this classification, this may be used as markers in cassava hybridization. The wide variability of the botanical characteristic indicates a high degree of intraspecific hybridization (Rogers and Appan, 1973), thus, these genotypes are distinguishable by morphological characteristics such as vigour, pubescence, the leaf and root characters within the clusters. These results will however assist plant breeders in population improvement of cassava cultivars.

Biochemical characteristic in cassava genotype identification: The results of this investigation are particularly useful in Nigeria farming systems as cassava cultivar duplication is common among communities. Naming of cassava cultivars especially TMS 30572 is common and based on the mode of introduction into the community. Isozyme markers were successfully used to discriminate cultivars of M. esculenta (Resenda *et al*., 2004; Sumarani *et al.,* 2004; Chavarriaga-Aguirre *et al.,* 1999). It was observed that the genotypes exhibited enough polymorphism within the isozyme systems of AAT, 6-PGD, G-6-PDH and SKDH to identity conveniently and unambiquously most cassava genotypes examined. The first 3 isozymes system mentioned are especially useful for differentiating among genotypes because of the high level of genetic polymorphism observed and this will assist cassava breeders in germplasm screening and cross breeding activities. These results were also observed in other cassava accessions (Cardy and Kannenberg, 1982 and Weeden, 1983).

Relationships between biochemical and morphological characteristics in cassava genotype identification: The electrophoretic isozyme markers revealed more clusters than the morphological traits., clustering of genotypes based on morphological characters, as well as distinctiveness of genotypes was observed much earlier, which possibly indicates effect of environment on the morphological traits as compared to isozyme markers which, is are affected by environment (Lee and Ronalds, 1967).

The genetic clusters revealed that most of the genotypes in cluster I correspond to morphological cluster I with silver green stem and purple petiole colour common to all the genotypes indicating that these traits could be used in cassava classification.

Conclusion

The combination of isozyme markers and morphological characters can effectively be used for identification of duplicates in cassava germplasm collections among communities and farmers' fields, as well as monitoring vegetative propagation of these cultivars. In breeding programme, these results will serve as important information for cassava population development and improvement that can be exploited by breeder.

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