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Genetic diversity Study of *Dioscorea*s Using Morphological Traits and Isozyme Markers Analyses

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Abstract

Yam is a popular tuber crop in Nigeria, contributing to food security and income of farmers. Research on yam improvement has been very slow due to insufficient information on genetic diversity of the crop. The objectives of this study are to identify, characterize and classify some *Dioscorea* species of yam in farmers' field in Nigeria using morphological and isozyme indicators. Twenty-eight genotypes of yam (*Dioscorea*) species comprising IITA elites, local landraces and adaptable farmers' varieties were planted at a space of 1m x 1m in a randomized complete block design in two replications. Two major morphological clusters were observed. Cluster I comprised relative species (*D. dumetorum, D. abyssica and D. schimperana*) with *D. alata* (water yam) and cluster II was dominated by *D. rotundata* and *D. cayenesis.* High correlation between morphological and molecular data analyses were observed in this study. Three isozyme markers were observed as good discriminant for yam: SKDH, 6-PGD and AAT. Isozyme markers analysis indicated that *D. alata* species were distantly related to *D. rotundata* and *D. cayenesis,* while *D. rotundata* species were closely related to *D. cayenesis.* The study provided important information for breeders which could be exploited for the development and improvement of the yam crop.

Keywords: Yam, Isozyme markers, genotypes, classification, morphology and polymorphism

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Introduction

Yam belongs to the genus *Dioscorea*, which is the main genus of the family *Dioscoreaceae*, which is widespread throughout the tropics and subtropics with over 600 species (Norman *et al.,* 2011). Only a few produce edible tubers of which *D. alata* (water yam), *D. rotundata* (white yam) and *D. cayenensis* (Yellow yam) are the most important. The major yam species are tropical plants and do not grow well below 20^0 C. They require at least 1000 mm annual rainfall. Their growing period is between 7-9 months. Yams are perennial plants with a vine-like habit. According to species, they produce one large tuber or several smaller ones. They are normally grown as an annual crop, where whole seed tubers or pieces of tuber are planted on mounds or ridges or sometimes in trenches or holes filled with organic matter (Lebot, 2009).

Nigeria is the leading producer of yam, and accounted for 71% of the world production (IITA, 2009 and FAO, 2011). Some of the major problems of yam cultivars were lack of distinctiveness and uniformity in characters, thus resulting in the overlap or duplication of cultivars. Therefore, reliable methods for identification and classification may facilitate in the crop germplasm characterization and its improvement.

Genetic diversity is an important requirement for a successful breeding programme. Morphological characterization is one of the steps in genetic diversity study and it has been used as a powerful tool in classification of cultivars and also to study their taxonomic status (Norman *et al.,* 2011 and Tamiru, *et al.,*

2011). Akoroda (1982) used two numerical taxonomic methods, cluster and minimum spanning tree to classify 20 Nigerian cultivars of yellow yam *D. cayenensis*. He observed that, classification from both methods were complementary and facilitated a better understanding of the features of the cultivars. .Mignouna *et al.* (2002) reported that genetic erosion can be controlled by collection and characterization of existing germplasms for diversity studies and crop improvement. Literature reviews reported morphological characterization, identification and classification of other *Discorea* species such as (Mulualem and WeldeMichael, 2013) and (Siadjeu *et al.,* 2015).

Due to environmental dynamism that influenced morphological characters, it is very important to complement morphological with molecular characterization. The genome of the crop can be studied directly, using biochemical or molecular markers. Isozymes, being biochemical expressions of the genetic make-up of the plant, can be used to greatly improve the accuracy of cultivar identification. Bressan *et al.* (2014) used isozymes analysis in delineating yam cultivars into different species. The results revealed clear genetic diversity of the yam species between and within communities under study.

Onyilagha (1988) used polyacrylamide gel elctrophoresis on 13 cultivars of *Dioscorea* (5 of *D.rotundata* and 8 of *D.cayenesis*). The two taxa have some common morphological features, leading to taxonomical confusion in the past. Four hundred and fifty three accessions of cultivated yam varieties belonging to the *D. cayenesis* - *rotundata* complex were collected in West Africa and characterized by their isozymic patterns by Hamon and Toure (1990). These accessions which belong to 20 variety groups, on the basis of their morphological characteristics, were studied using starch electrophoresis. The results showed a remarkable agreement between the morphological polymorphism and enzymatic variation.

Terauchi *et al*. (1992) traced the origin and the phylogeny of 14 cultivars of Guinea yams by using the restriction fragment length polymorphism analysis of chloroplast DNA and numerous ribosomal DNA, and it was revealed that two cultivated species (*D. rotundata* and *D. cayenensis*) exhibited the same chloroplast genomic type. The lack of sufficient data on yam genotypes and their characteristics restrict their improvement and conservation, leading to the genetic erosion of yam (Dansi *et al.,* 1997). Therefore, the aim of this study was to identify, characterize, and classify some *Dioscorea* species of yam grown in the farmers' field in Nigeria using using morphological traits and isozyme markers analyses.

Materials and Methods

The experiment was carried out on the experimental field of International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria. 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 mean relative humidity of about 80%. Twenty seven cultivars of *Dioscorea* spp., comprising of IITA elites, local landraces and adaptable farmer cultivars were planted at a space of 1m x 1m in a randomized complete block design with two replications. Seven plants per genotype per replicate were planted and five plants per genotype per replication were tagged for record taking in a total of six plots of size 224 m^2 per plot. The plots were ploughed and ridged at a distance of 1m apart, and the total experimental farm size was 0.134 ha. The genetic materials used in this study, status and country of origin are indicated in Table 1.

Morphological data collection: Data collection, in respect of pigmentation of stem, twinning habit, direction of twinning, started at the emergence of the shoot. Other parameters observed weekly were plant type, branching, density of spines, site of spines, waxiness of both upper and lower leaf surface, hairiness of the stem and leaf, secondary sprouting, onset of leafing, leaf type, vein and petiole pigmentation and stipule waxiness. At 4 months after planting, the length and the width of matured leaf were taken, the leaf area and the petiole length and colour, while the tuber shapes were taken after harvest. Data collected were based on International Plant Genetic Resources Institute (IPGRI, 1990) evaluation system.

Biochemical data collection: 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***:** A = 7.50 mL, C = 15.0 mL, distilled H_2O = 37.5 mL, E = 0.41 mL, and TEMED = 18.75 mL.
*The stacking gel***:** B = 2.5 mL, D = 5.0 mL, distilled H_2O = 12.5 mL, E = 60.0 µL, and TEMED = 24. 0 µL

Table 1: Status and source of yam cultivars

Cultivar code No.	Cultivars	Status	Species	Source
1	TDr 87/00504	Improved	D. rotundata	IITA
2	TDr 90/01035	Improved	D. rotundata	IITA
3	TDr 87/00272	Improved	D. rotundata	IITA
4	TDa 85/00247	Improved	D. alata	IITA
5	TDa 85/00257	Improved	D. alata	IITA
6	TDa 85/00247	Improved	D. alata	IITA
7	TDa 85/00236	Improved	D. alata	IITA
8	TDr 90/ O1790	Improved	D. rotundata	IITA
9	TDc 760	Improved	D. cayenensis	IITA
10	D.schimperana	Land race	Wild relative	Nigeria
11	TDr 87/0084	Improved	D. rotundata	IITA
12	TDr 90/00015	Improved	D. rotundata	IITA
13	TDa 86/00614	Improved	D. alata	IITA
14	TDr 90/01190	Improved	D. rotundata	IITA
15	TDr 90/01786	Improved	D. rotundata	IITA
16	D. abyssica	Land race	Wild relative	Nigeria
17	TDr 90/01/814	Improved	D. rotundata	IITA
18	TDa 86/00600	Improved	D. alata	IITA
19	TDr 87/00255	Improved	D. rotundata	IITA
20	TDa 85/00249	Improved	D. alata	IITA
21	TDr 87/00213	Improved	D. rotundata	IITA
22	TDa 85/00243	Improved	D. alata	IITA
23	TDa 85/00611	Improved	D. alata	IITA
24	TDr 87/00102	Improved	D. rotundata	IITA
25	TDa 87/01096	Improved	D. alata	IITA
26	TDr 87/00197	Improved	D. rotundata	IITA
27	*D.dumetorum*	Land race	Wild relative	Nigeria

Table 2: Biochemical Analysis and solutions

Stock solutions:	
Solution	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

Sample preparation and enzyme extraction: Samples of young fresh leaves from the field were collected in the test tubes placed in an ice container and then carried to the laboratory. The samples were crushed in an extraction buffer solution: Trizma base = 1.211 g, potassium chloride = 0.725 g, ethylene diaminetetraacetic acid = 0.298 g, sucrose = 3.420 g, 2 M-E (mercaptoethanol) = 60.0 µL, distilled H_2O = 100 mL at pH 7.5, and 200 mg of polyviylpolypyrrolidone at a ratio of 1:5 (W/V) per sample. After crushing, the samples were centrifuged at 4°C for 10 min at 10.000 × g. Furthermore, approximately 20.0 µL of the supernatant was loaded in each slot. Two standard genotypes were loaded on each slab to provide the reference bands. The stock electrode buffer was Davis 10× Tris/glycine at pH 8.57 at room temperature and 10× concentration. Model SE700 (Hoefer Scientific Instrument) of an electrophoresis tank and model PS2500 of a power supply unit (Hoeffer Scientific Instrument) were used for electrophoretic analysis. The slab polyacrylamide gels used were prepared using the stock solutions. For electrophoresis, an initial voltage between 200 V and 300 V and a constant current of 60 mA for 3 hours at 4°C was used. The gels were then stained according to the following protocols:

Staining markers and constituents:

AAT: Aspartate amino transferase (E.C.2.6.1.1.): Modified Vallejos method (1983)
Tris-HCL pH 8 (0.1 M) = 10 mL, H_2O (distilled) = 90 mL, Aspartic acid = 0.30 g, α-ketoglutaric acid = 0.15 g, pyridoxal-5-phosphate = 0.01 g or a pinch, and Fast Blue BB salt = 0.15 g

6-PGD: 6-Phosphogluconate dehydrogenase (E.C.I.I.I.44): Modified Vallejos method (1983)
Tris-HCl pH 7.5 (0.1 M) = 50 mL, $MgCl_2$ (1 M) = 0.25 mL, 6-P-gluconic acid = 10 mg, NADP (20 mg/mL) = 0.38 mL, MTT (10 mg/mL) = 1.0 mL, and PMS (10 mg/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 (0.1 M) = 50 mL, $MgCl_2$ (1 M) = 0.5 mL, glucose-6-phospate = 20 mg, NADP (20 mg/mL) = 0.38 mL, MTT (10 mg/mL) = 1.0 mL, and PMS (10 mg/mL) = 0.2 mL

SKDH: Shikimate dehydrogenase (E.C.I.I.I. 25): Modified Vallejos method (1983)
Tris-HCL pH 7.5 (0.1 M) = 50 mL, shikimic acid = 50 mg, NADP (20 mg/mL) = 0.38 mL, MTT (10 mg/mL) = 1.0 mL, and PMS (10 mg/mL) = 0.2 mL

The gels were equilibrated with the staining buffer for approximately 10 min in the incubator at 37°C. This was replaced with the complete staining solution and incubated in the dark at 37°C for 30 min or until the bands developed.

Statistical analysis: The morphological data were subjected to the hierarchical cluster analysis in GENSTAT computer program, which drew dendrograms. For the isozyme analysis, a numerical code for scoring the enzyme

phenotypes was combined with the copies of banding.

Genetic analysis: Enzyme loci were evaluated for polymorphism and enzyme activity. The allelic frequencies for each genotype were recorded on the basis of the repeatability and interpretation of a dendrogram. To study the genetic relationships among the genotypes, the presence or absence of isozyme bands was transformed into a binary character matrix (1 = presence and 0 = absence of an isozyme band). Thus, isozyme variability was created and a binary matrix was derived. Pairwise distance matrixes among the genotypes were again derived using the numerical taxonomy and multivariate analysis system (NTSYS-PC), Version 2.1 (Rohlf, 2000), and Jaccard coefficient of similarity (Jaccard, 1908). A genetic diversity dendrogram for the genotypes was created using the unweighted pair-group method with an arithmetic mean cluster analysis (Sneath and Sokal, 1973; Swofford and Olsen, 1990).

Results

Yam morphological characters were fewer compared to cassava and sweet potato. However, four major morphological characters were significantly observed in this study (Table 3).

Table 3. Major distinguishing morphological features among 27 yam cultivars

genotype	Genotype code No.	Distinguishing Characters 1	2	3	4
TDr 87/00504	1	MB	FS	LG	G
TDr 90/01035	2	MB	FS	LG	G
TDr 87/00272	3	MB	FS	LG	G
TDa 85/00247	4	NB	AB	GP	G
TDa 85/00257	5	NB	AB	GP	G
TDa 85/00247	6	FB	AB	GP	G
TDa 85/00236	7	NB	AB	GP	PG
TDr 90/ 01790	8	MB	FS	LG	G
TDc 760	9	FB	MS	GP	G
D.schimperana	10	NB	AB	GP	G
TDr 87/0084	11	MB	FS	GP	G
TDr 90/00015	12	FB	FS	GP	G
TDa 86/00614	13	FB	FS	GP	G
TDr90/01190	14	MB	MS	LG	G
TDr 90/01786	15	MB	FS	GP	G
D. abyssica	16	NB	AB	P	PG
TDr 90/01/814	17	MB	FS	G	G
TDa 86/00600	18	FB	AB	LG	G
TDr 87/00255	19	MB	FS	LG	G
TDa 85/00249	20	NB	AB	P	PG
TDr 87/00213	21	MB	FS	LG	G
TDa 85/00243	22	NB	AB	P	PG
TDa 85/00611	23	NB	AB	LG	G
TDr 87/00102	24	MB	MS	LG	G
TDa 87/01096	25	FB	AB	GP	PG
TDr 87/00197	26	FB	FS	LG	G
D.dumetorum	27	NB	MS	G	G

Character codes

1. Branching habit: Few branches (FB), many branches (MB) and No branch (NB)
2. Spine density: (AB), Few spines (FS) and many spines Absent (MS)

3. Colour leaves at emergence: light green (LG), Green (G), Green purple (GP) and purple (P)
4. Petiole colout: Green (G), purple green (PG) and purple (P)

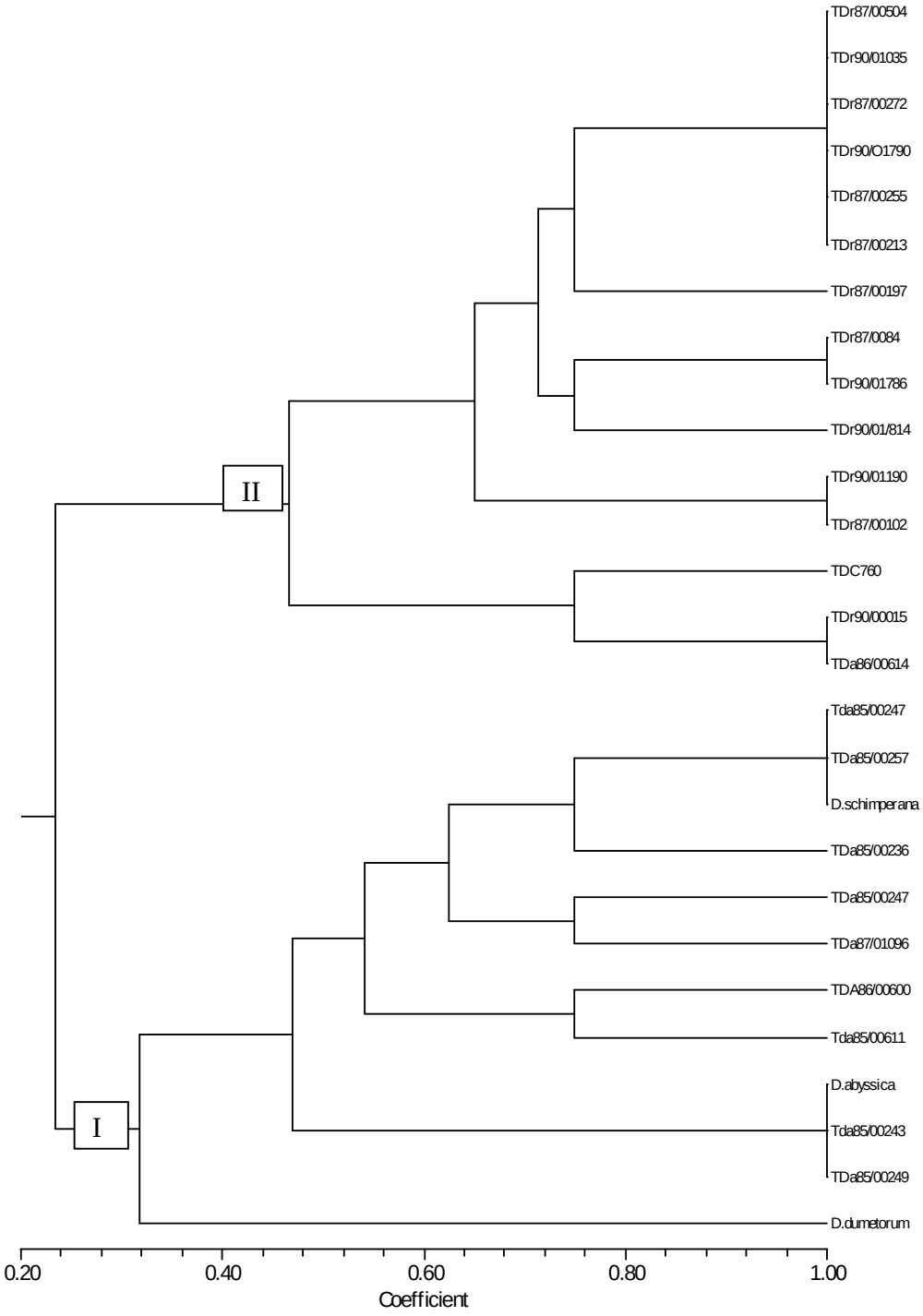

Fig. 1: **Dendrogram showing cultivar clusters among *Discorea* species as revealed by morphological characters.**

Two major clusters were observed between the coefficients of similarity of 0.32 and 0.50 (Fig. 1). From the coefficient of similarity of 0.60 and above, the number of clusters increased and the number of cultivars per cluster decreased; the cultivars exhibited individualistic morphological characteristics. *D. dumetorum* species had a coefficient of similarity of 0.30 with other species, indicating a high genetic distance. Cluster I consisted of 12 cultivars of three relative species, *D. dumetorum*, *D. abyssica*, and *D. schimperana*, with *D. alata* (water yam), thus indicating genetic similarities. Cluster II consisted of *D. rotundata* and *D. cayenesis*. This clustering indicated a high genetic diversity between *D. alata* and *D. rotundata* and a high genetic similarity between *D. rotundata* and *D. cayenesis*. Existences of sub-clusters were observed and there were no significant differences among the cultivars within the sub-clusters as compared with mega clusters mentioned earlier.

Isozyme phenotypes: Three isozyme markers were observed as good discriminants for yam (Table 4).There are 3, 5 and 10 loci for SKDH, 6-PGD and AAT, respectively. The G-6-PDH could not discriminate the yam species due to weak polymorphism. Isozyme marker AAT was the best discriminant with 50.0% – 96.4% polymorphism. Locus L-4 of AAT with low percentage polymorphism appears in all the genotypes examined, similarly for L-1 and L-2 for SKDH and 6-PGD, respectively.

Table 4: Isozyme markers that revealed polymorphism among 27 yam genotypes

S/N	Isozyme Marker	Loci	% Polymorphism
1	SKDH	L-1	67.9
		L-2	96.4
		L-3	96.4
2	6-PGD	L-1	71.4
		L-2	67.9
		L-3	96.4
		L-4	92.9
		L-5	96.4
3	AAT	L-1	82.1
		L-2	89.3
		L-3	89.3
		L-4	50.0
		L-5	71.4
		L-6	82.1
		L-7	96.4
		L-8	82.1
		L-9	85.7
		L-10	96.4

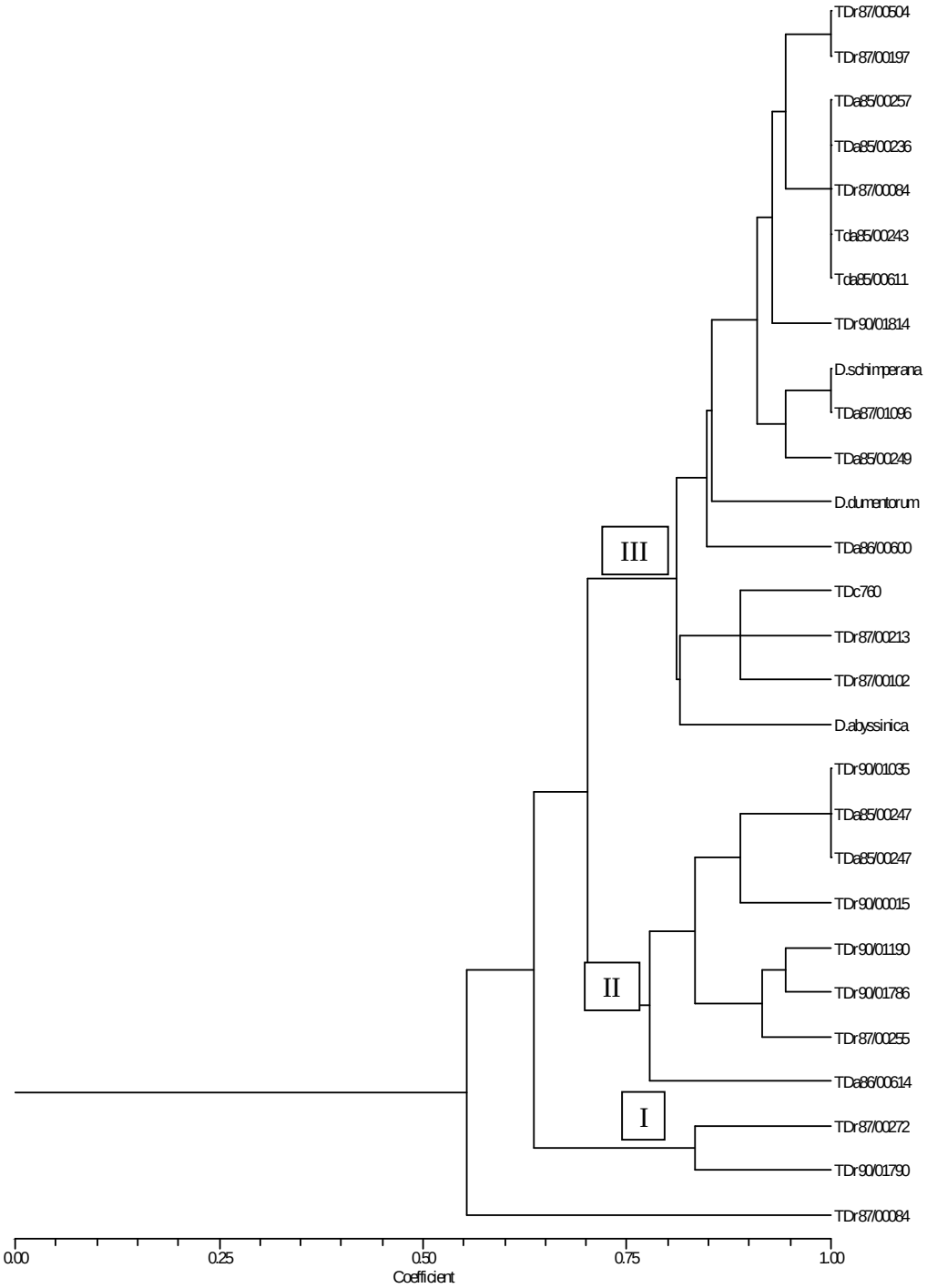

Fig.2.Dendrogram showing genetic diversity among 27 yam genotypes as revealed by isozyme markers

Eighteen isozyme loci were used to construct the phylogenetic relationship dendogram among the 27 cultivars. These 18 loci of isozyme markers are potentially useful, which revealed the genetic diversity and relationships among the genotypes (Fig. 2). Three distinct clusters were obtained using the isozyme marker analysis. Clusters I and II

mainly consisted of *D. rotundata* genotypes similar to the cluster II of the morphological analysis (Fig. 1). Cluster III of the isozyme markers consisted mainly of *Dioscorea* relatives and *D. alata*, indicating that these species were closely related, as shown in morphological data cluster I (Fig. 1). The results showed a high correlation between the morphological and isozyme analyses in the genetic diversity study of yam (Fig. 2). Interpretation of these results was based on the conditions of this experiment and may not be generalized.

Discussion

Genetic diversity in yam germplasm is crucial for breeding and conservation programs. Genetic improvement in yam during selection depends on the availability of genotypes having a favorable allele for good agronomic traits, which depends on the available genetic diversity. The morphological data analysis revealed a closer genetic relationship between the relative species of *Dioscorea* used in this study and *D. alata*. Therefore, by creating broad based genetic variability of yam, the related species (*D. dumetorum*, *D. abyssica*, *and D. schimperana*) and *D. alata* could be genetically improved through hybridization, tissue culture technique, or molecular methods. Yam currently has narrowed genetic base because of incompatibility with other *Dioscorea* species. This study may provide insights into the feasibility in broadening the genetic base for the improvement of yam. In addition, the morphological data analysis revealed that the aforementioned yam species were distantly related to *D. rotundata* and *D. cayenesis*. However, *D. rotundata* and *D. cayenesis* were closely related (Fig. 1), and the hybrid species of *D. cayenesis–D. rotundata* and *D. rotunda–D. cayenesis* complexes have been identified (Zoundjihekpon *et. al.,* 1994; Dansi *et al.,* 1999; and Dansi *et al.,* 2000). The characteristics useful in distinguishing the genotypes within the clusters are branching, spine density, leaf type, and petiole color; these results were consistent with the results revealed by Akoroda (1983) and Velayudhan *et al.* (1989).

In this study, high correlation between the morphological and molecular data analyses; were observed. This corroborates similar reports of some tuber crops (Maquia *et al.,* 2013 and Efisue, 2013), but contrary to the earlier reports by Tairo *et al*. (2008). They observed insignificant correlation between the isozyme and morphological characters in sweet potato cultivars examined. Three of the four isozyme markers, AAT, 6-PGD, and SKDH, were highly effective in discriminating yam genotypes. However, AAT showed a higher level of genetic polymorphism than other markers in this study and previous studies. AAT is the most effective discriminant for some tuber crops with a higher level of polymorphisms than other isozymes markers (Efisue, 2013 and Bressan *et al.,* 2014). The isozyme marker analysis indicated that *D. alata* species were distantly related to *D. rotundata* and *D. cayenesis*. However, *D. rotundata* species were closely related to *D. cayenesis* (Fig. 2). Furthermore, these observations were associated with the morphological data analysis (Fig. 1). Other DNA-based molecular markers, such as simple sequence repeat (Muthamia *et al.,* 2013 and Norman *et al.,* 2012), random amplified polymorphic DNA, and amplified fragment length polymorphism (Asemota *et al.,* 1996; Dansi *et al.,* 2000; and Mignouna *et al.,* 2003), were also used in the genetic diversity studies of yam.

Conclusion

Yam improvement requires good quality agronomic traits that combine high yield and farmer' preferred traits and consumer values. These could be achieved through genetic diversity study of yam germplasm, which serves as gene pool for useful agronomic traits. This study clearly indicated the importance of the combination of morphological and molecular analyses in genetic diversity study of yam. Three of the four isozyme markers examined the AAT,6-PGD and SKDH were able to discriminate effectively yam cultivars, in particular, AAT is most discriminant due to its high level of polymorphisms. The study observed important information on diversity among cultivars, which breeders could exploit for the development and improvement of the yam crop.

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