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Morphological and Molecular Characterization of Cocoyam (*Colocasia esculentum* and *Xanthosoma sagittifolium*) (L) Schott germplasm from Nigeria using Simple Sequence Repeat-SSR marker

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ABSTRACT: *Colocasia esculentum (L) Schott and Xanthosoma sagittifolium (L) Schott, commonly called cocoyam is one of the orphan crops with great potentials, but neglected by researchers and growers despite their high demands by consumers in recent times. Consequently, some of the local varieties have become almost extinct; this calls for the need to revive interest in these crops. This study was undertaken to characterize six cocoyam accessions collected from Cross River and Akwa Ibom States, Nigeria, using 13 qualitative and 6 quantitative Agro-morphological descriptors as well as simple sequence repeat (SSR) primers. The crops were laid out in a field using the Randomized Complete Block Design (RCBD) in 4 replications. Analysis of variance (ANOVA) test showed that the 6 accessions were not significantly different (P>0.05) in crop span, crop height, number of leaves, leaf width and leaf length. This indicates that these features cannot be used to distinguish between Colocasia and Xanthosoma spp. The qualitative traits were more useful in distinguishing between the two cocoyam species. Three of the primers used for molecular studies successfully amplified a few of the cocoyam accessions, indicating their suitability for genetic diversity studies in Colocasia esculenta and Xanthosoma spp.*

KEYWORDS: Colocasia esculenta and Xanthosoma sagittifolium, Germplasm, Characterization, Simple Sequence Repeat-SSR

INTRODUCTION

Cocoyams are herbaceous plants belonging to the Monocotyledonous family Araceae and grown primarily for their edible corms. Edible cocoyams cultivated as food crops belong to the genera *Colocasia* Schott and *Xanthosoma* Schott and are generally made of a large spherical corm (swollen underground storage stem), from which a few large leaves emerges. The petioles of the leaves stand 45^{o} or erect and can reach the lengths of 1 m. The leaf blades are large and heart-shaped and can reach 50 cm in length. The corm produces lateral buds which give rise to cormels and suckers or stolons. They are often grown as annuals and harvested after one season. (Rao, Matthew, Eyzaguirre and Hunter (eds) 2010).

Economically, cocoyams are classified as root or tuber crops and among the oldest domesticated crops of the ancient world (Perez Ponce, 2007; Lebot, 2009; Owusu-Darko *etal*., 2014). However, little is known about their levels of production, cultivation, types as well as taxonomy (Talwana*etal*., 2009, Baruwa and Oke, 2012). The genera *Colocasia* also referred to as *taro* or old *cocoyam, arrowroot, eddoe, macabo or dasheen,* originated from Southeast or Central Asia. Whereas *Xanthosoma* also referred to as *tannia or new cocoyam, Chinese taro* originated from Central and South America (Deni Brown 2000, Onwueme, 1978; Singh, 2004; APG, 2009; Mwenye, 2009).

There are two major varieties of *Colocasia* namely *Colocasia esculenta*, with a large central corm with suckers or stolons and *Colocasia antiquorum* characterized by a small central corm and a large number of smaller cormels (Gomez-Beloz and Rivero 2006; Seetohul *etal*., 2008). In the other hand, most edible species of *Xanthosoma* are either *Xanthosoma sagittifolium* or *Xanthosoma maffafa* (Okoye et al., 2006). Their corms are often difficult to distinguish from those of *C. esculenta* after their leaves have been removed (Matthews, 2010).

Furthermore, between 1970 and 1980 *taro* was among the third most consumed staple food crops in West Africa and Nigeria in particular. During this time it was a major article of trade, celebrated yearly with a festival called "Ede Oye" in the Southern parts of Nigeria *(Ubalua et al., 2016*). Presently, the status of the crop is that of an orphan crop, largely neglected by the scientific community and growers. The situation is worsened by the increasing human population with increasing neglect on it production left only to the resourced poor farmers. This beg for urgent need to reverse the trend through intensified efforts on the breeding of improved varieties of this crop in order to make it more attractive to consumers and growers in Nigeria. The starting point is proper documentation of available diversity of this crop. Thus we characterize the cocoyam germplasm from different agro-ecological zones of Nigeria, using Agro-morphological and Simple Sequence Repeat-SSR as a molecular marker.

MATERIALS AND METHODS

Sample Collection

Six land races of cocoyam germplasm were collected from different locations including Akwa Ibom State and Cross River States in Nigeria (Table 1, Plate 1).

Table 1. Cocoyam accessions used, their identifications and collection sites

Code	Scientific name	Local Name	Location
AKI	(*Xanthosoma sagittifolium* L. Schott)	Ikpong Asimeka	Obio Etoi, Akwa Ibom
AK2	(*Xanthosoma sagittifolium* L. Schott)	Afia Ikpong	Ini LGA
AK3	*Colocacia Esculenta*	Ikpong Animbo	Cross River
AK4	(*Xanthosoma sagittifolium* L. Schott)	Ndat Ikpong	Akwa Ibom
AK5	*Colocacia esculenta*	Ikpong Panya	Ikom
AK6	*Colocaccia esculenta*	Afia Ndudut	Cross River

Experimental Design and site
The experiment was laid out in a Randomized Complete Block Design (RCBD) in the University of Calabar, Research and Experimentation farms. Each block was planted with the 6 land races Randomized within it, with a planting distance of 1m between blocks and 0.5m within blocks. The molecular characterization aspect of the work was done in the molecular biology laboratory of the same University of Calabar.

Morphological Characterization:
Descriptors of Cocoyams (*Colocasia esculentum and Xanthosoma sagittifolium (L.)Schott*) developed by International Plant Genetic Resources Institute (IPGRI)/International Institute of Tropical Agriculture (IITA) (1999) were used for data collection. Data were collected on the following parameters: Plant span, Plant height, Leaf length, Leaf width and number of leaves per plant.

Molecular Characterization
Genomic DNA was extracted from the 6 land races of cocoyam and characterized using Simple Sequence Repeats (SSR).

Extraction of Genomic DNA
Genomic DNA was extraction using C-TAB (Cetyl trimethyl ammonium bromide) method. Briefly, fresh leaf samples were collected from the Research farm into1.5ml Eppendorf tubes containing Zirconia beads and stored in a -80°C freezer (U410 New Brunswick high efficiency freezer) to freeze-dry overnight. The samples were then collected and crushed in a Retsch MM400 grinder at 2500rpm for 3mins. The beads were removed with the aid of a magnet and 500µl of *CTAB* extraction buffer was

added to the ground tissues and vortex briefly. The mixture was then incubated on an Eppendorf thermomixer F1.5 at 65°C for 1h.

After incubation the mixture was centrifuged for 10mins at 15,000rpm. The upper phase was carefully extracted and transferred to a fresh tube, then 500µl of chloroform:isoamyl (24:1) was added and mixed properly by inverting the tubes. The mixture was centrifuged at 15,000rpm for 10mins and the upper phase extracted and transferred to fresh tubes. This step was repeated and finally, 350µl of 2-propanol was added to the extract, mixed by inversion and the mixture was centrifuged at 15000rpm for 10mins. After centrifugation a clear white pellet appeared at the bottom of the tube, the aqueous phase was decanted carefully to avoid losing the pellet.

Approximately 500µl of 70% ethanol was added to the tubes and tapped gently with fingers to release the pellet, then centrifuged for 10mins at 15000rpm. The aqueous phase was carefully decanted and the pellet allowed to dry for 30mins in open air. Approximately30µl of TE buffer was added to the DNA and then stored in a Liebherr medline -20°C freezer until required.

DNA quantification

The DNA concentration was evaluated using a JenWay Genova Nano system. Approximately1.5µl of the DNA was tested on the nanodrop using 1.5µl of the TE buffer as a blank. The concentration was adjusted by diluting the DNA with TE buffer to bring to 250ng/µl concentration.

Polymerase Chain Reaction *PCR*

The samples were screened using a total of 3 SSR markers (Table 2) used by previous workers on other cocoyam cultivars. They were synthesized by Inqaba biotech West Africa ltd.The primers were centrifuged briefly and then diluted with nuclease free water according to the manufacturer's instructions to obtain stock solutions. A working solution was then prepared from the stock solutions by diluting 20ul of each primer with 180µl of nuclease free water in a fresh 2ml Eppendorf tube. The PCR cocktail solution was prepared to make 50µl reaction solution for 12 samples as shown in Table 3

Table 2. Microsatellite (SSR) primers used for the molecular screening

S/N	PRIMER CODE	FORWARD SEQUENCE	REVERSE SEQUENCE
1	(TG)6(GA)4	GTCCAGTGTAGAGAAAAACCG	CACAACCAAACATACGGAAAC
2	(CA)8	GTAATCTATTCAACCCCCCTTC	TCAACCTTCTCCATCAGTCC
3	(TGA)6(TGGA)4	AGCCACGACACTCAACTATC	GCCCAGTATATCTTGCATCTCC

Table .3. Components of PCR Cocktail

Components	25µl reaction	50µlreaction	12 samples
10×s.Taq Buffer	2.5	5	60
$MgCl_2$	2.5	5	60
dNTPs	0.5	1	12
P_F	0.5	1	12
P_R	0.5	1	12
Taq Poly Enzyme	0.125	0.25	3
DNA	--	-	
Nuclear free distilled water	17.375	34.75	429.00
TOTAL	25µl	50µl	588

Agarose gel electrophoresis

The Agarose gel 1% was prepared by dissolving 1g of Agarose in 100ml of TrisEDTA (TE) buffer and heated in a microwave oven for 4 to 5mins. The mixture was allowed to cool briefly and then 6µl of ethidium bromide stain was carefully added (in a fume hood) to the mixture and mixed by gentle shaking. The mixture was poured into a gel casting tray mounted with combs and allowed to solidify. The gel was then transferred to a horizontal gel electrophoresis tank containing 1X TE buffer and the combs gently removed. Approximately 1.5µl of loading dye was added to the PCR products. The mixture was briefly vortexed and gently loaded onto the gel inside the tank. Six (6) µl of DNA ladder was also loaded on the first well and the set-up was run at 130V for 1hr.

Photography and documentation

After electrophoresis the gel was transferred to a High performance UV transilluminator (UVP, United Kingdom), and viewed with a safety goggle and the pictures taken with EOS 1200D camera (Canon, Taiwan).

RESULTS AND DISCUSSION

Results

Morphological Studies

The means and standard errors of the six morphological attributes studied in 6 accessions of cocoyam are presented in Table 4.

Table 4. Means of morphological attributes studied in six cocoyam land races.

LAND RACES	ATTRIBUTES			
PS (cm)	PH (cm)	NL	LW(cm)	LL (cm)
Asimeka(A)	59.144.	73.6	218.8	23.8
Animbo(B)	84.756.	03.17	26.5	26.4
Ndat-Ndat(C)	40.824.	83.23	11.0	17.4
Afia ikpong (D)	53.637.	23.53	16.12	4.7
Panya(E)	55.9	37.93.	13.20.	526.2
Ndudut (F)	63.5	45.44.	3519.12	2.7
Overall Mean	59.641.	03.51	18.7	23.5

Legends: PS =Plant span, PH=Plant height, NL= Number of leaves, LW= Leaf width LL=Leaf length
.

Plant span

The mean Plant span did not differ significantly (P>0.05) among the land races studied as shown in the analysis of variance result (Table 5). It ranged from 40.8cm in *Ndat-ndat t*o 84.7cm in *Animbo* with an overall mean of 59.6cm in the 6 accessions (Table 4).

Table 5. Analysis of Variance for Leaf span in the 6 cocoyam accessions studied

Source of variation	df	ss	ms	vr	F pr
BLOCK	2	848.5	424.3	0.96	
Accession	5	3156.9	631.4	1.42	0.296
Error	10	4435.5	443.6		
Total	17	8440.9			

Plant Height

The mean Plant height did not differ significantly (P>0.05) among the land races studied as shown in the analysis of variance result (Table 6). It ranged from 24.8cm in *Ndat-nda*t to 56cm in *Animbo* with an overall mean of 41.0cm in the 6 accessions (Table 4).

Table 6. Analysis of Variance for Plant Height in the 6 cocoyam accessions studied

Source of variation	df	ss	ms	vr	F pr
BLOCK	2	844.0	422.0	2.26	
Accession	5	1628.6	325.7	1.74	0.221
Error	9	1680.4	186.7		
Total	16	3944.7			

Number of leaves

The mean number of leaves did not differ significantly (P>0.05) among the land races studied as shown in the analysis of variance result (Table 7). It ranged from 3.13 in *Panya* to 3.62 in *Asimeka* with an overall mean of 3.51 in the 6 accessions (Table 4).

Table 7. Analysis of Variance for Number of leaves in the 6 cocoyam accessions studied

Source of variation	df	ss	ms	vr	F pr
BLOCK	2	4.7305	2.3653	2.63	
Accession	5	3.1556	0.6311	0.70	0.637
Residual	9	8.1038	0.9004		
Total	16	15.6838			

Leaf width

The mean leaf width did not differ significantly (P>0.05) among the land races studied (Table 8) and ranged from 9.2cm in Ebio to 24.6cm in *Osobo* with an overall mean of 18cm in the 6 accessions (Table 4).

Table 8. Analysis of Variance for Leaf Width in the 6 cocoyam accessions studied

Source of variation	df	ss	ms	vr	F pr
BLOCK	2	115.63	57.81	2.27	
Accession	5	391.91	78.38	3.07	0.068
Error	9	229.60	25.51		
Total	16	719.91			

Leaf length

The mean leaf length did not differ significantly (P>0.05) among the six accessions studied as given in the Analysis of Variance result (Table 9). It ranged from 17.4cm in *Ndat-ndat* to 26.4cm in *Animbo* with an overall mean of 23.5cm in the 6 accessions (Table 4).

Table 9. Analysis of Variance for Leaf length in the 6 cocoyam accessions studied

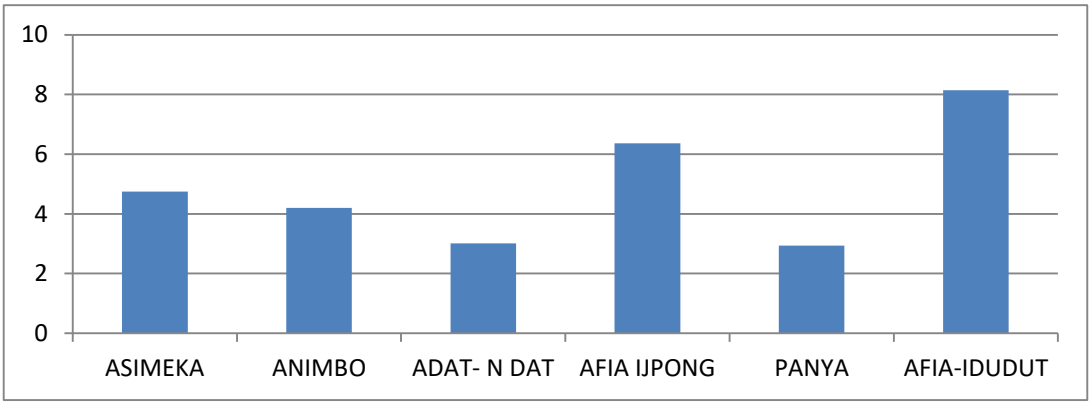
Source of variation	df	ss	ms	vr	F pr
BLOCK	2	112.73	56.36	3.13	
Accession	5	163.45	32.69	1.81	0.206
Error	9	162.15	18.02		
Total	16	430.36			

Molecular Studies

DNA Extraction (yield in µg/ml).

The following yields were obtained from the 6 accessions studied:

Sample DNA	yield (µg/ml)
Asimeka	4.753
Animbo	4.200
Ndat ndat	3.01.8
Afia Ikpong	6.366
Panya	2.948
Afia Idudut	8.14.9



Legends: DNA Extraction yields from samples (yield in µg/ml).

PCR RESULTS

Three primers used for molecular studies successfully amplified most of the cocoyam accessions, although stutter bands were present. Nevertheless, they can still be used in future diversity studies of *Colocasia & Xanthosoma spp* involving a lot more accessions.

The level of genetic diversity could not be ascertained from available molecular data in view of some research bottleneck.

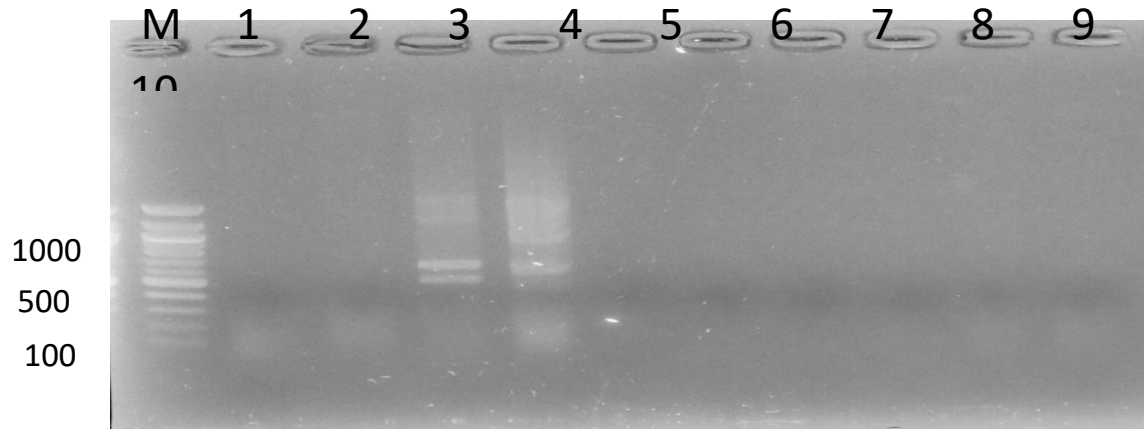


Plate 3. Agarose Gel electrophoresis picture from primer (CAC)5

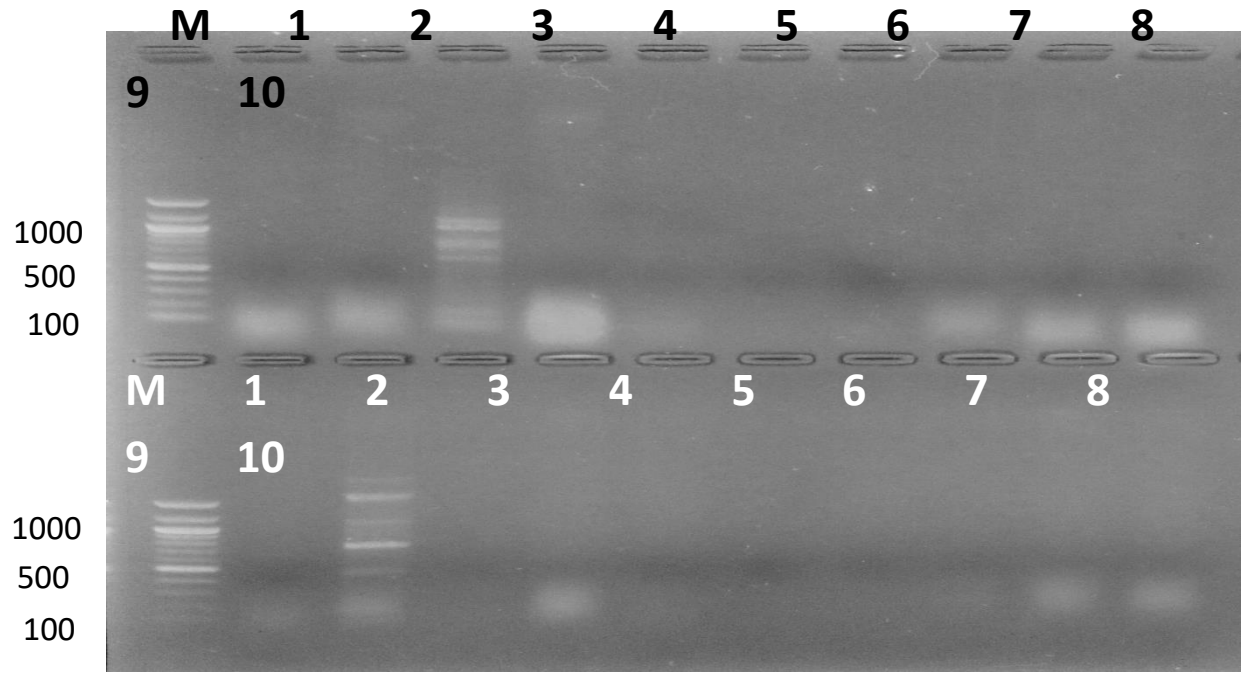


Plate 4. Gel picture from primer (CT)15 top; primer (CT)18 bottom

DISCUSSION

Distinct characters among the different accessions were investigated using morphological descriptors according to Nya and Eka, (2015). The six different accessions of *Cocoyams* were analyzed for morphological variation based on the phenotypic expressions of the plant samples collected. The samples collected from different geographical areas within Nigeria were completely different by their leaves shapes, size and colour. The plant sample appeared healthy with big round vines, leaves which were succulent in nature, while other samples had smaller long leaves which were dark green in colour and some showed light green with much smaller heart shape leaves in nature. These distinct features observed in the plants could be attributable to varying environmental factors and soil conditions, excessive sunlight and water availability in the locations from where they were raise and grown. Analysis of these six cocoyam land races obtained from farmers in different geographical areas within Nigeria and grown at the University of Calabar Research and Experimentation farms showed insignificant differences ($P>0.05$) in plant span, plant height, number of leaves, leaf width and leaf length. This indicates low diversity among the 6 land races studied. It also showed that these features cannot be used to discriminate between *Colocasia* and *Xanthosoma* spp.

For sometimes now morphological information has been the basis for characterization of rich plants diversity (Endress, 2000). Nevertheless, there remains considerable reservation about the precise role of morphology in characterisation as they are inadequate for definite identification of plants up to species level, due partly to vagaries in environmental and soils condition (Swarna*et al.*, 2015). Furthermore, these morphological descriptors are often restricted as the characters may not be obvious at all stages of the plant development and growth. Thus, this calls for the use of additional descriptors involving in-depth analysis at the molecular level as essential to identifying and authenticating the rich diversity of Cocoyams.
The 3 primers used successfully amplified one or two of the land races, although stutter bands were present. These 3 primers can therefore be useful in future diversity studies of *Colocasia* and *Xanthosoma* spp of Cocoyams involving more land races. The level of genetic diversity could not be ascertained from available molecular data in this study.

Furthermore, the genetic composition of the plant species have been shown to be insignificantly different, this showed that the perceived observable differences *phenotype* are accountable due to environments rather than genotype *genetic component*. As a result their identification from among their land races has become essential in germplasm study and for the formulation of appropriate improvement plan (Swarna *et. al.*, 2015). However, rising from our focus and the objectives of our study, molecular characterization of Cocoyams - *Colocasia* and *Xanthosoma* species may be considered as fundamental important for generating new improved varieties for tackling global food insecurity.

Conflict of interest: The authors declare that they have no conflict of interest.

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