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Characterization of EST-derived microsatellites in cassava (*Manihot esculenta* Crantz.)

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ABSTRACT

Cassava (Manihot esculenta Crantz) is a staple food for millions of people both in the tropics and subtropics and is increasingly being used as an industrial crop for the production of starch and other products. Currently, the number of molecular markers available for cassava genetic studies is limited. This study was conducted for the characterization of Expressed Sequence Tags (EST)-derived microsatellites or Simple Sequence Repeats (SSRs) in cassava (Manihot esculenta Crantz). Ninety six EST- SSRs previously identified insilico, were validated and screened for polymorphism using a diverse set of cassava genotypes. Using the optimized PCR conditions, these SSRs were screened for polymorphism using two diversity panels: panel 1 which comprises of cassava landraces and elite clones cultivated in Africa while panel 2 which comprises of cultivated cassava from Africa, Asia and Latin America, as well as wild Manihot species and castor bean (Ricinus communis) belonging to the family Euphorbiaceae. About thirty one SSRs were polymorphic in panel 1 while forty two were polymorphic in panel 2. A greater number of polymporhism was observed for panel 2 compared to panel 1, as expected since panel 2 comprises a greater genetic diversity. A total of seventy alleles were detected in panel 1 and ninety six for panel 2. The number of alleles per locus ranged from one to four by using these new polymorphic SSRs, with distinct genetic relationships between different genotypes. The cluster analysis showed consistency on the available information of the accessions under study, thus showing that the markers can be used for cassava genotyping.

Key words: Genetic diversity, *Manihot esculenta* , panel, and SSR markers

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INTRODUCTION

Cassava (*Manihot esculenta*_Crantz) is a species native to tropical America [1], initially cultivated by the native Latin Americans and later introduced into the Africa and Asian continents. The worldwide cassava production, on an area of 17,870,626 hectares is approximately 195,574,112 tons [2]. Its main value is the roots whose dry matter contains more starch and this form the basic carbohydrate element of the diet and the leaves are eaten as a preferred green vegetable in many parts of its cultivation. Leaves of cassava are a cheap but rich source of Vitamin A, B and C, and other minerals [3, 4, 5, 6, 7]. In Africa, cassava is cultivated mostly by subsistence farmers, often on marginal lands and for them, the crop is vital for food security and income generation. It is considered the most important source of dietary energy in sub-Saharan Africa, where average consumption exceeds 300kg per person per year. The crop's ability to tolerate drought under conditions considered marginal for most other crops, including low soil fertility has ensured its increasing worldwide economic importance, thus making it the fourth most important tropical crop globally [4]. Cassava is easy to grow and can be planted at any time of the year, provided there is enough moisture for stem cuttings to sprout [8].

High levels of heterozygosity, long growth cycle, poor seed set, vegetative propagation and severe inbreeding depression on selfing hamper cassava improvement by conventional breeding. The long growth cycle, which is from 8 to 24 months, means that it is visited by lots of pests that may also transmit diseases. Therefore, the use of molecular markers for cassava breeding can contribute to the solution of some of these constraints

In order to understand the important role of traditional farmers in maintaining and even amplifying genetic diversity in cassava landraces of a place in any countries, various studies have been undertaken with isozyme markers [9, 10, 11, 12], randomly amplified polymorphic DNA (RAPD) markers [13, 14, 15], and microsatellites or simple sequence repeats (SSR) [16, 14, 17], the latter being an appropriate marker for the detection of genetic polymorphisms, widely used to characterize genetic diversity in traditional crops [16, 18, 19, 20].

Microsatellite markers (highly polymorphic, co-dominant in nature and showing a Mendelian mode of inheritance) are widely utilized because they are reproducible, and also amenable for high throughput marker genotyping. These characteristics have made microsatellites a very useful molecular tool in genome studies and also for genetic mapping [21, 22, 18] .

Also, Expressed sequence tag (EST) collections constitute an inexpensive and efficient tool for gene discovery [23] . Development of ESTs is expected to increase our efficiency at identifying the key regulatory genes, physiological processes and also environmental interactions associated with growth and development. The use of EST-database will result in developing a unigene set that allows for cross species comparisons [24]. A collection of 18,166 high quality EST sequences has been developed for cassava and a unigene set comprising 8,577 sequences has been identified. In addition, a set of putative SSR markers has been identified *insilico* from this unigene set [25].

The objectives of this study therefore were to validate 96 candidate EST-derived SSRs and to screen these for polymorphism using a diverse set of cassava genotypes, wild *Manihot* species and castor bean, another genus in the family *Euphorbiaceae.* The EST-SSRs developed in this study should increase the number of microsatellites available for cassava. These SSRs can then be utilized for genetic mapping and diversity studies in cassava as well as for comparative genetic studies between cassava and other taxa from the *Euphorbiaceae* such as castor bean and leafy spurge.

MATERIALS AND METHODS

Plant materials

Forty-seven cassava accessions were selected for this study from a collection of landraces and International Institute of Tropical Agriculture (IITA) improved accessions. These accessions have been grown in Ibadan and Ubiaja, Nigeria over several years.

Two panels of cassava genotypes were used; labeled as panel1 and panel 2. Panel 1 comprised of 24 African cassava genotypes (Table 1). Panel 2 comprised of cassava genotypes from around the world, castor bean and several wild *Manihot* species (Table 2).

DNA extraction

Genomic DNA was extracted from young leaf samples [26] . An amount of 100 mg leaf tissue were ground into powder in liquid nitrogen and transferred to 2mL Eppendorf tubes containing 800 µl of CTAB extraction buffer [30 mMEDTA pH 8.0, 0.1M Tris-HCL pH 8.0, 1.2 M NaCl, 3% CTAB, plus 0.7% β –mercaptoethanol] and then left at the roomtemperature until the samples were ready. 100µLof 20% SDS was then added to the samples, mixed thoroughly for 1 minute and then incubated at 65^{o}C for 15 minutes. After incubation, the samples were allowed to cool for 2 minutes, 300 µL of ice-cold 5M Potassium Acetate was added to each tube, mixed gently and then incubated on ice for 20 minutes. The samples were subsequently centrifuged at 12,000 rpm for 10 minutes.The upper aqueous phase was carefully removed into two clean 1.5ml microfuge tubes. Seven hundred microlitres of ice-cold iso-propanol was added to each tube and mixed by inverting gently 8 to 10 times. The samples were then incubated at -80̊C for 1 hour and then spun in the centrifuge at 12,000 rpm for 1 minute. The supernatant was then transferred to a fresh tube with 350µL of chilled isopropanol and stored at - 4^{o}C for 1 h, which was thereafter once more centrifuged at 8,000 rpm for 10 min. After drying, the pellet was re-suspended in 250 µL of high salt TE buffer (10 mMTris-HCl pH8.0, 1mM EDTA) and 4 µL RNAse (10 mg/mL) were added to each tube and incubated at 37^{o}C for 1hour.The concentration of each sample was determined using the DNA quantification kit and the purity was

determined using a spectrophotometer (Variant Cary 100 UV-VIS spectrophotometer). The quality of the DNA was checked on 1% agarose gel electrophoresis in 1 x TAE buffer by using 0.5µg/ml Ethidium Bromide staining technique for 5minutes and then distained in water, so as to visualize the DNA bands in the gel.

Table1: List of the 24 African cassava genotypes and their origins (Panel 1)

Lab number	Accession Name	Type/Origin
1	TME 28	Landrace, Nigeria
2	TMS 30572	Elite line, IITA, Nigeria
3	TME 419	Landrace, Togo
4	TME 117	Landrace, Nigeria
5	02/0577	Castor-cassava hybrid
6	TME 3	Landrace, Nigeria
7	TME 9	Landrace, Nigeria
8	00/0214	Inter-specific hybrid with *M tristis*
9	02/0593	Castor-cassava hybrid
10	TME 7	Landrace, Nigeria
11	TME 1	Landrace, Nigeria
12	TMS 4(2) 1425	Elite line, IITA, Nigeria
13	TMS 30555	Elite line, IITA, Nigeria
14	00/0210	Inter-specific hybrid with *M tristis*
15	TME 388	Landrace, Uganda
16	TME 1786	Landrace, Kenya
17	TME 530	Landrace, Malawi
18	TME 6	Landrace, Nigeria
19	TME 225	Landrace, Benin
20	TME 230	Landrace, Togo
21	TME 232	Landrace, Togo
22	TME 279	Landrace, Nigeria
23	TME 638	Landrace, Ghana
24	TME 568	Landrace, Angola

.

Table 2: List of cassava genotypes, wild relatives and castor bean (Panel 2)

Lab number	Accession Name	Type/Origin
1	Castor bean	-
2	*M. glaziovii*	Wild relative
3	Kigoma Red	Landrace, Tanzania
4	Mbundumali	Landrace, Malawi
5	Bugwelu	Landrace, Zambia
6	Cm 6740-7	elite clone, CIAT, Columbia
7	Cm 523-7	elite clone, CIAT, Columbia
8	Cm 3306-4	elite clone, CIAT, Columbia
9	Cm 1335-4	elite clone, CIAT, Columbia
10	Mcol 1468	Landrace, Columbia
11	MBRA 12	Landrace, Brazil
12	MBRA 383	Landrace, Brazil
13	MBRA 1045	Landrace, Brazil
14	MECU 72	Landrace, Ecuador
15	MPER 183	Landrace, Peru
16	MTAI 18	Landrace, Thailand
17	IAC 12	elite line, Brazil
18	IAC 14	elite line, Brazil
19	*Feculabranca*	Sao Pual
20	*Brancelesatanna*	Sao Pual
21	*M. epruinosa*	Wild relative
22	*M. tripartita*	Wild relative
23	*M. brachyandra*	Wild relative

Microsatellite amplication

The amplication reactions were conducted in a final volume of 10 µl containing: 1.0 µl of DNA (10ng/µl); 1.0 µl 10x PCR buffer containing 15 mM $MgCL_2$ (Promega), 0.8 µl of dNTPs (2.5 mM each), 0.2 µl of TaqDNA polymerase (Promega; 5 u/µl), 2.0 µl of the forward and reverse primer (1 µM each) and 5.0 µl of distilled water. PCR reactions were performed with three different annealing temperatures for all primer pairs:

First annealing temperature condition
Touchdown 55 to 50°C:
1. 94°C for 3 minutes;
2. 94°C for 30 sec;
3. 55°C for 30 sec -0.5°C per cycle;
4. 4. 72°C for 1 minute;
5. Go to 2. 10 times;
6. 94°C for 30 sec;
7. 50°C for 30 sec;
8. 72°C for 1 minute
9. Go to 6. 25 times;
10. 72°C for 5 minutes;
11. 12°C (hold);

Second annealing temperature condition
Touchdown 58 to 55°C:
The same procedure as above

Third annealing temperature condition
Touchdown 60 to 55°C:
Repeat the same procedure as above.

The PCR products were then run on a 3.5% superfine resolution (SFR) agarose gel in 1 x TBE buffer at 80V for 2h. Primer pairs that failed to amplify any product or that have a faint product under these conditions were tested under the same three annealing temperatures with three different $MgCl_2$ concentrations: 1, 2 and 4 mM. The gels were stained with ethidium bromide and photo-documented under UV light.

Statistical Analysis
Generated data was assembled into a matrix. The data matrix was then analyzed using Numerical Taxonomy System of Statistic v.2.0j (NTSYS) [27]. Similarity indices between pairs of the cassava, castor bean and wild *Manihot* species were calculated for the combination of data from 96 primer pairs by selecting Similarity for Quantitative Analysis (SIMQUAL) using the method of [28] Similarity Coefficient. The similarity index of Jaccard between plants i and j is as given below:

$Sij = a / (a+b+c)$

$Dij = Sij$

a - denotes the number of characters present in both i and j
b - denotes the number of characters present in i and not in j
c - denotes the number of characters present in j and not in i
D - is the distance coefficient

The similarity data matrix was then used for cluster analysis, which was based on the unweighted pair-group method with arithmetic mean (UPGMA). The clustering obtained was used to generate dendrograms.

RESULTS

Validation of EST-SSR markers
A total of 86 candidate EST-SSR primers identified with clear bands were used for PCR reactions. The PCR reactions were carried out for each genotype in diversity Panel 1 and Panel 2. For each reaction, 5 µl of the PCR reaction was run on a 3.5% SFR gel. Polymorphic and monomorphic SSR loci were detected in both panel 1 and panel 2 (Fig.1). The number of polymorphic (P) and monomorphic (M) loci for each genotype in the diversity panel 1 and panel 2 for all EST-SSRs is summarized in Table 3 and 4 respectively. In some cases, it could not be unambiguously determined whether a particular SSR locus was monomorphic or polymorphic. Loci that were likely monomorphic and polymorphic were indicated as M? and P? respectively. The allele size ranges were estimated by

comparing against a DNA marker that produced bands ranging between 50 to 500 bp. However, as shown in Fig.1a and Fig.1b, lanes with no bands were observed, indicating either absence of alleles (null alleles) or alleles below the detection limit. Due to the limited resolution of the SFR gels to about 5 bp, the number of alleles determined in this assay, represent a minimum estimate. The actual number of alleles per locus as well as the number of alleles per genotypes may be higher.

For some EST-SSR loci, the bands observed on the SFR gels were larger than the expected size, based on the predicted location of the primers on the EST sequence. Since the primers for PCR were based on cDNA sequences while the PCR reaction for amplification of the EST-SSR was carried out on genomic DNA, these larger bands most likely indicate the presence of intron sequences.

Cluster analysis and genetic relationships in diversity panels of Africa cassava accessions

Panel 1:

The Numerical Taxonomy System of Statistic (NTSYS) Analysis showed genetic similarity among cassava accessions, with the coefficient of genetic similarity ranging from 0.43 to 0.86. At 0.70 similarity coefficient, the 24 cassava accessions clustered into ten main groups (Table 5). The dendrogram further revealed strong genetic relationship between the Nigeria landraces TME 279, TME 232, TME 230, TME28, TMS 30572, TME 117, TME 3, TME 9, TME 7, TME 1, TMS 4(2)1425, TMS 30555, TME 6, TME 230, TME 232 and TME 279. Strong similarities were also detected between TME 1786 from Kenya and TME 530 from Malawi, and between TME 225, TME 638 and TME 568 (Fig.2)

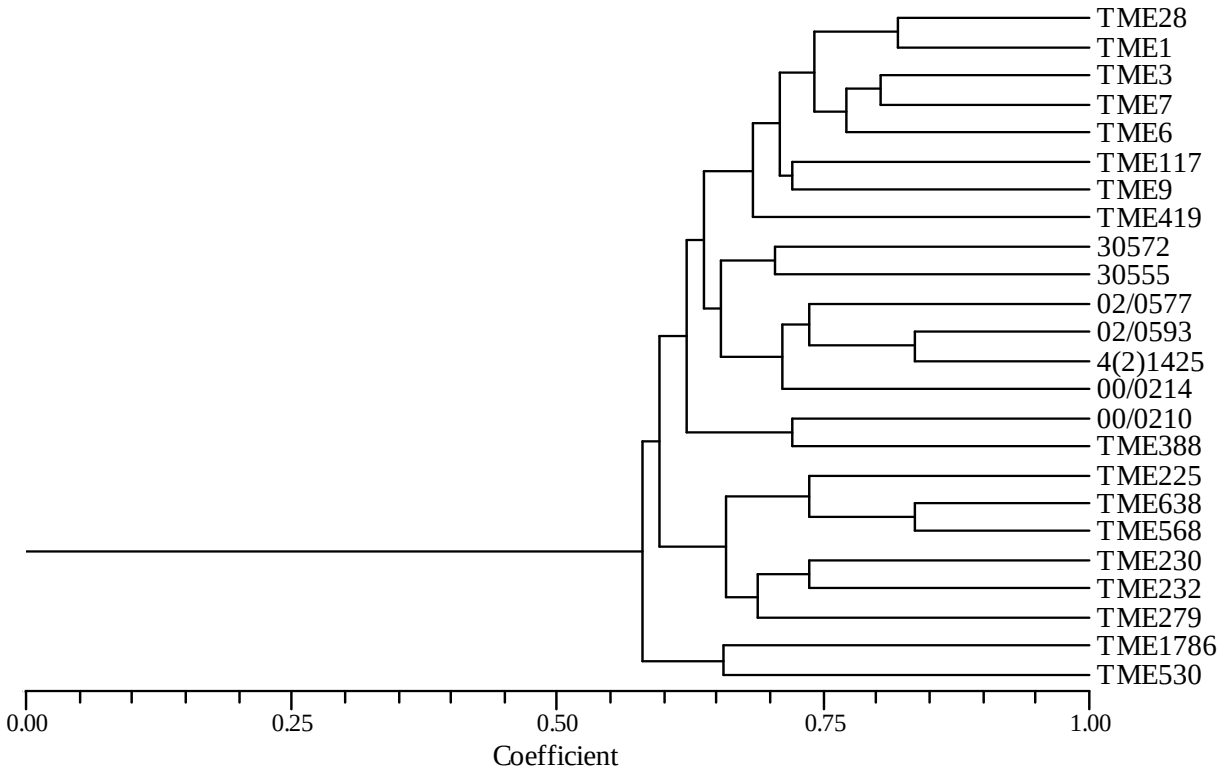

Fig 2.Dendrogram of panel 1 obtained with NTSYS similarity coefficient, representing genetic relationships among 24 Africa cassava (*Manihotesculenta)* accessions

Table 3.Summary of EST-SSR validation base on 86 EST-SSR loci in Panel 1

Polymorphism	Number
M+M(long)	46+2=48
M?	3
P(2)	19
P(3)	8
P?(4)	1
P?(2)	3
NA	3
NS	1

M- Monomorphic, P- Polymorphic, value in parentheses-number of alleles, M? and P? – loci showing resemblance of either M or P, long- allele size longer than expected suggesting the presence of introns, NA – No amplification, NS – Not scorable

Table 4.Summary of EST-SSR validation based on 86 EST-SSR loci in Panel 2

Polymorphism	Number
M+M(long)	14+7=21
M?+M?(long)	14+2=16
P(2)+P(2)(long)	30+2=32
P(3)	8
P?(2)	4
P?(4)	None
NA	3
NS	2

M- Monomorphic, P- Polymorphic, value in parentheses-number of alleles, M? and P? – loci showing resemblance of either M or P, long- allele size longer than expected suggesting the presence of introns, NA – No amplification, NS – Not scorable

Panel 2:

A total of 75 polymorphic bands were scored for cassava, castor bean and wild *Manihot* species. Dendrogram was then generated by complete cluster analysis based on similarity indices ranging from 0.13 to 0.94, which showed the genetic similarity among the panel 2 genotypes (Fig 3). Cassava, castor bean and *M. glaziovii* were clustered into two major groups at 0.15 similarity coefficients. Further distribution was done into eight main cluster groups at 0.75 similarity coefficient (Table 6).

Table 5: Dendrogram cluster groupings of the Panel 1

Cluster groups	Accessions
Group I	TME 530
Group II	TME 1786
Group III	TME 279
Group IV	TME 232 and TME 230
GroupV (two subgroups ‘a’ and ‘b’)	Subgroup ‘a’ – TME 638 and TME 568 Subgroup ‘b’ – TME 225
Group VI	TME 388 and 00/0210
Group VII (three subgroups ‘a’, ‘b’ and ‘c’)	Subgroup ‘a’ – 00/0214 Subgroup ‘b’- 4(2) 1425 and 02/0593 Subgroup ‘c’ 02/0577
Group VIII	30555 and 30572
Group IX	TME 419
Group X (four subgroups ‘a’, ‘b’, ‘c’, and ‘d’)	Subgroup ‘a’ – TME 9 and TME 117 Subgroup ‘b’ – TME 6 Subgroup ‘c’ –TME 7 and TME 3 Subgroup ‘d’ – TME1 and TME 28

Table 6: Dendrogram cluster groupings of the Panel 2

Cluster groups	Accessions
Group I	*M. epruinosa*
Group II	*M. brachyandra*
Group III ('a' and 'b')	
	Subgroup 'a' – *M. triparhta*and *Brancelesatanna*
	Subgroup 'b' – MBRA 1045 and MPER 183
GroupIV ('a','b','c' and 'd')	
	Subgroup 'a' – Bugwelu and Mcoli 468
	Subgroup 'b' – Cm 523-7
	Subgroup 'c' – Cm 6740 and MPER 383
	Subgroup 'd' – Mbundumali and Cm 3306 IAC 14 and *Feculabranca*
Group V	
Group VI ('a', 'b' and 'c')	Subgroup 'a' – MTAI 18 and IAC 12
	Subgroup 'b'- MECU 72
	Subgroup 'c' – MBER 12 and Cm 1335-4
Group VII	Kigoma Red
Group VIII	Castor bean and *M. glaziovii*

DISCUSSION AND CONCLUSION

Various types of DNA markers have been developed [29, 30, 31, 32] to elucidate or show levels of polymorphism in array of crop such as cowpea (*Vignaunguiculata L.* [33], melon (*Cucumis melo L.*) [34], rice (*Oryza sativa L.*) [35], sunflower (*Helianthus annuus L.*) [36], almond (*Prunus pandorea and P. amygdalus*.) [37], sorghum (*Sorghum bicolor*) [38], grapevine *(Vitisvinifera*) [39] and wheat (*Triticum aestivum L.*) [40] Thus, high degree of microsatellite polymorphism that was observed in cassava in this particular study is comparable to the results of other crop species [41, 42]. This study equally showed that a large fraction of candidate EST-derived microsatellites markers were polymorphic in cultivated cassava and wild *Manihot* species. The 96 putative EST-derived microsatellites which were screened for polymorphism using a diverse set of cassava genotypes, also showed clear poly-morphisms. In panel 1, which comprised of African cassava genotypes, about 31% of the candidates EST-SSR markers were polymorphic while in panel 2, which comprised of African, Asian, and Latin American cassava genotypes as well as wild *Manihot* species and castor bean, about 51% of the EST-SSRs were polymorphic,as expected for this more diverse panel. Here, it should be noted that these numbers represented minimum estimates since the resolution on the SFR gel system was about 5bp and thus alleles with size differences smaller than 5bp might have been overlooked. Microsatellites markers have been used to study genetic diversity in a largenumber of plant species, including wheat, sunflower and many other crops. In this study, they also showed clear distinctions in the cassava accessions.

In addition to measuring genetic diversity, one of the objectives in our study was to verify how cassava accessions in respective panels originating from different regions in Africa, Asian, and Latin American countries were mutually related. Results showed a similarity index of 0.75 in both the panels. However, in panel 2-comprising of cassava accessions from Latin American, Asian, African and also castor bean and wild *Manihot*_species- showed large genetic relationships between them. Castor bean and *M. glaziovii* formed a distinct or similar group but genetically different from the rest of the accessions.*M. epruinosa, M. brachyandra* were alone and genetically distant from the rest of the accessions. Whereas, the remaining cassava accessions were all separated in their different groups. This result suggests that that the markers which were generated from cassava can also be used for other *Euphorbiaceae* species and genera. Panel 1- comprising of only African cassava accessions-was clustered in ten main groups.

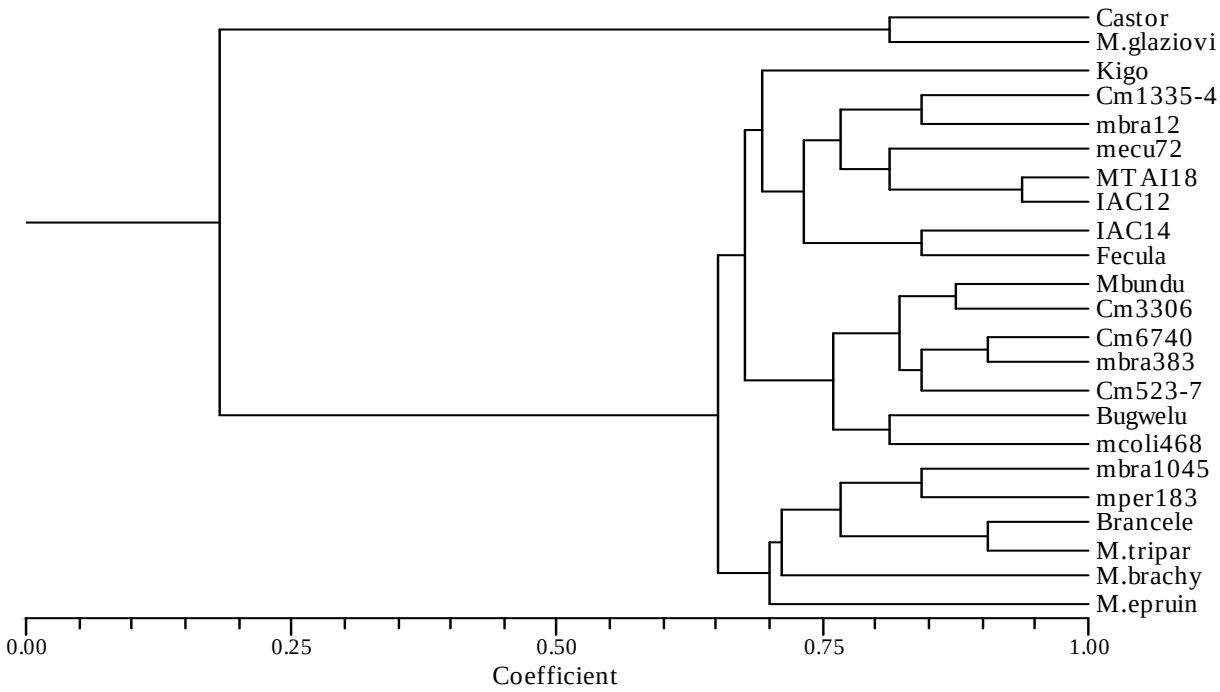

Fig 3.Dendrogram of panel 2 obtained with complete cluster analysis similarity coefficient, representing genetic relationships among cultivated cassava (*Manihot esculenta)*, wild *Manihot* and castor bean.

These African cassava genotypes could be easily used for characterization of cassava. The development of DNA marker technology has provided an efficient tool for germplasm conservation and management. In cassava, microsatellite markers have been used to characterize different accessions to determine their genetic structure and also how diverse they are.Microsatellite markers reveal differences among cassava accessions at the DNA level and so it provides a more direct, reliable and efficient tool for characterization of cassava genotypes.

Fig 1.Allelic variation of selected EST-SSR loci in Panel 1 and Panel 2

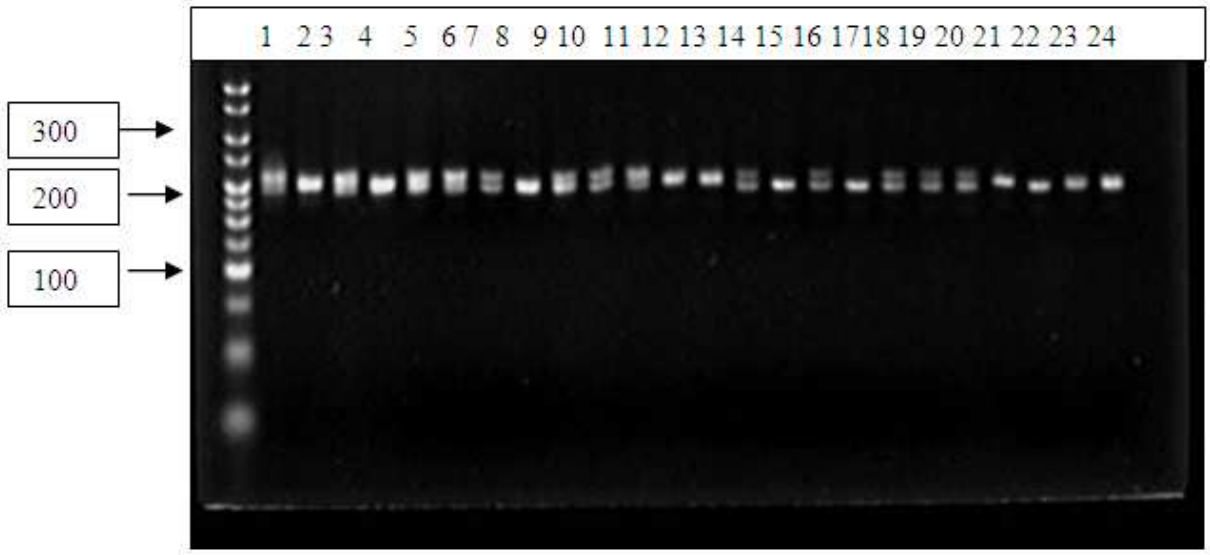

Fig. 1a: Example of allelic variation of panel 1 by primer pair # 153

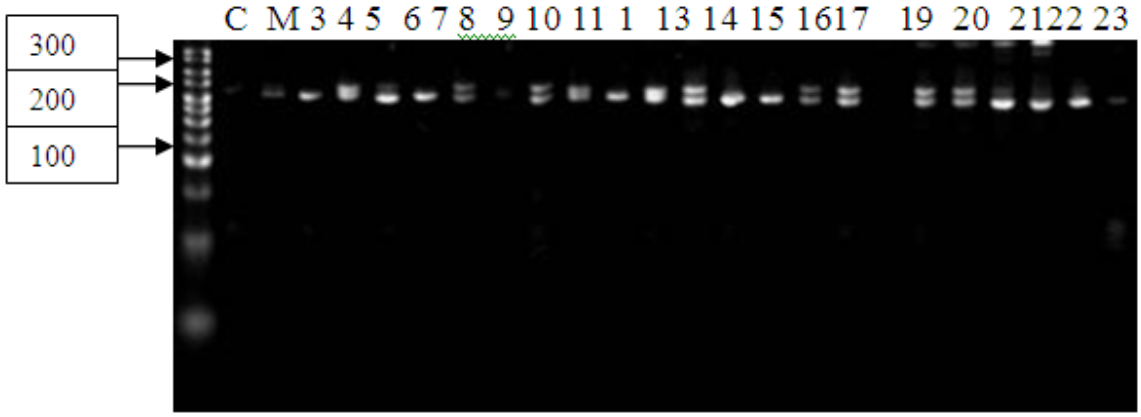

Fig. 1b: Example of allelic variation in panel 2 by primer # 157

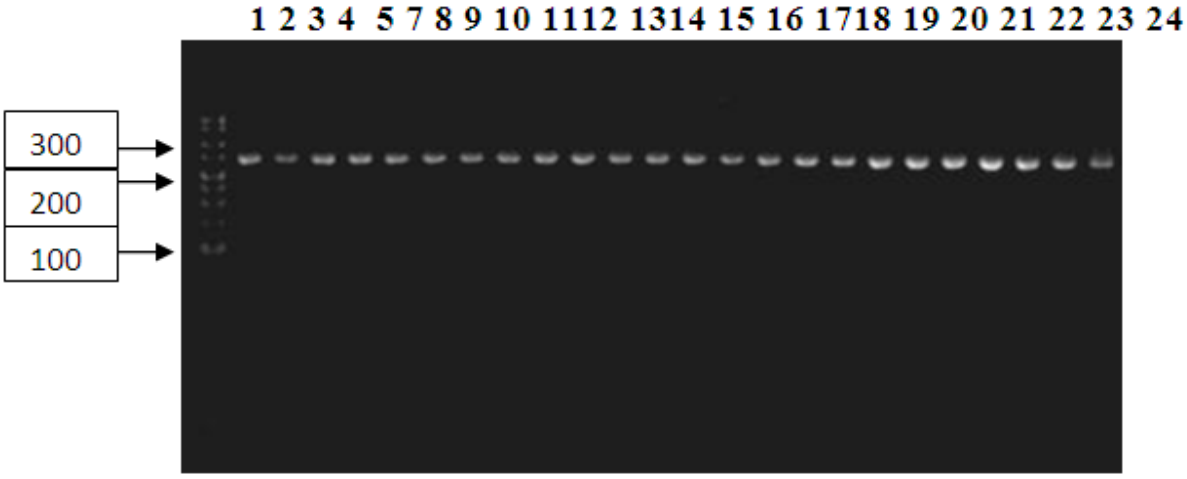

Fig. 1c: Example of a monomorphic DNA locus by primer # 145

The microsatellite markers used for evaluation in this genetic study could be readily used for genotype identification of cassava.

In this study we intended to contribute to a better knowledge on the genetic diversity of cassava and selected *Euphorbiaceae* species and similarity within and among different regions and promote germplasm conservation of an important genetic and autochthon resource.

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