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Research Article

Molecular Characterization of *Plectranthus esculenthus* and *Plectranthus rotundifolius* using Simple Sequence Repeats

D. Yakubu[1], I. Bawa[1], U. M. Sesugh[2]

[1]Department of Biochemistry, Federal University of Agriculture, Makurdi, Benue State, Nigeria.
[2]Department of Plant Breeding, Federal University of Agriculture, Makurdi, Benue State, Nigeria.

*Corresponding author's e-mail: yakubuydanjuma@gmail.com

Abstract

The *Plectranthus* genus comprises several species generally referred to as boldo, which are highly used in traditional medicine due to their anti-dyspeptic, analgesic and digestion-stimulating properties. This work was aimed at the molecular characterization of two species of *Plectranthus* genus *(P. rotundifolius* and *P. esculenthus)* and the intraspecific diversity of *P. esculenthus* (Bebot, Riyom and Longat), by means of the SSR technique and morphological markers. This study may be the nobel of SSR technique to characterize *P. esculenthus* and *P. rotundifolius* in Nigeria. Morphological analysis featured eleven characters; Plant height, length of branches, intermodal distance, length of tubers and tuber girth and leaf area were measured using a meter rule graduated in centimeters. In the cases of number of sprouted stems, number of leaves, number of branches as well as number of tubers, physical counting were noted. For tuber weight, a weighing balance was used. Principal components analysis was carried out on the data obtained, where the individual components responsible for the variation of the studied taxa were analyzed. Morphological characters derived from leaf, stem, root, and tubers were analysed numerically using cluster method. A cluster analysis was carried out on the DNA product. Morphological variability was observed at the cluster distance with *P. rotundiifolius* and *P. esculenthus* (Longat) having the shortest distance between clusters at (0.1) and *P. esculenthus* (Riyom) at (0.6) while *P. esculenthus* (Bebot) was the farthest away with a with morphological variability distance of 1.5 (15%). Principal components analysis captured the morphological traits at the third principal component (53.1, 85.3 and 100%) showing that the morphological traits measured may not be the true representative of the morphology of the plants as more traits are needed to distinguish them. Genetic variability was observed between the *P. esculenthus* (longat) and *P. rotuntifolius species* (50%), followed by *P. esculenthus* "Riyom" (15%) and *P. esculenthus* "Bebot" (10%). The genetic cluster analysis showed that the two plants are not the same as *P. esculenthus* (Riyom) was found to be more closely related to *P. roduntifolius* which is in contrast with the morphological cluster which placed *P. esculenthus* (Riyom) as the more closely related species to *P. roduntifolius*. The results of this study suggest that these plants should be regarded as diffe rent based on SSR markers contrary to current treatment which was based on only morphological traits.

Keywords: Genetic similarities; Morphological characterization; Molecular characterization; Simple sequence repeats; *Plectranthus* sp..

Introduction

Genetic diversity which is defined as the total number of genetic characteristics serves as a way for populations to adapt to changing environments [1]. It is more likely that some individuals in a population will possess variations of one or more alternative forms of a gene which may arise by mutation and this population will continue for more generations because of the success of these individuals [2].

Genotypic and phenotypic diversity have been found in all species at the protein, DNA, and levels of organisms; in nature, this diversity is nonrandom, heavily structured, and correlated with environmental variation and stress [3]. The interdependence between genetic and species diversity is delicate, these changes in species diversity lead to changes in the environment, leading to adaptation of the remaining species [1]. Changes in genetic diversity such as loss of

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species, leads to loss of biological diversity [2]. Loss of genetic diversity in domestic animal populations has also been studied and attributed to the extension of markets and economic globalization [1,3]. The genetic diversity is important as is directly relatable to the adaptive ability of a species, and second, a population's fitness is closely related to its heterozygosity [4]. One of the ways of illustrating the importance of genetic diversity is by highlighting what happens when there is a lack of genetic diversity. Inbreeding is commonly associated with genetic diversity, which causes a decrease in the reproductive fitness of a population because of a decrease in its heterozygosity from repeated matings through closely related individuals [5]. Genetic diversity assessment has potential uses in evolution, breeding and conservation of genetic resources [6].

Living stone potato (*Plectranthus esculentus*) is a dicotyledonous perennial shrub belonging tothe family Lamiaceae. The ability of a cultivar to flower and set fruit determines the extent to which it can be improved upon by conventional breeding methods. Measuring genetic diversity is significant to examine variations present among the organisms on the basis of genetic markers at phenotypic, biochemical and genotypic level. It is a prerequisite for efficient breeding plans, collection expeditions or germplasm exchange in order to acquire specific characteristics [7].

Plectranthus rotundifolius or *Solenostemon rotundifolius*, commonly known as *native* or *country potato* in Africa and called "Chinese potato" in India is a perennial herbaceous plant of the mint family (Lamiaceae) native to tropical Africa. *P. rotundifolius* is closely related to the coleus plants widely cultivated as ornamentals and is often classified as a member of the genus *Solenostemon* rather than *Plectranthus*. SSRs based on simple PCR assays, with the advantages of high polymorphism, co-dominance specificity, extensive distribution and low cost, become the most common means of geneticmapping, germplasm identity, gene localization, molecular marker–assisted selection breeding and genetic diversity analysis [9], [10]. The use of short sequence is necessary to increase the probability that, although the sequences are random, they are able to find homologous sequences suitable for annealing [8].

The Research was aimed at studying the genetic diversity of *Plectranthus* spp *(P. esculenthus and P. roduntifolius)* using Simple sequence repeats (SSR) technique as the *Plectranthus* genus have similarities and because of this several terminologies have been used to refer to the same species of the *Plectranthus* genus which interferes with the collection of information about the ethnobotanic use of this genus, also there has been difficulty in finding morphophenological markers to discriminate these species [5].

Study of biodiversity could help to identify resistant varieties with higher survival rates, as a pre-requisite for a targeted breeding program. Genetic variability evaluation in is the first step towards mapping characteristics of interest for future genetic improvement research. The research tested the usefulness of SSR technique in discriminating between wild potato plants that are not easily distinguished by their morphological characters. It is necessary to use genetic markers as the backbone for distinguishing plants since morphological markers are affected by the environment [11]. The study was carried out on the *Plectranthus species (P. esculenthus and P. roduntifolius)* found in Jos Plateau state Nigeria.

Materials and methods

Potato plant materials

The plant materials used in the study were gotten and identified at the germplasm stock National Root Crops Research Institute Jos. They were Hausa potato and three cultivars of Livingstone potato namely Longkat, Bebot and Riyom. The Plant materials were kept in the Molecular Biology laboratory of the Department of Seed production of the Federal University of Agriculture Makurdi, Nigeria.

Morphological marker analysis

Morphological marker analysis was carried out using a similar technique described by [12]. The Morphological analysis featured eleven characters namely; Plant height, length of branches, intermodal distance, length of tubers and tuber girth and leaf area were measured using a meter rule graduated in centimeters. In the cases of number of sprouted stems, number of leaves, number of branches as well as number of tubers, physical counting were noted. For tuber weight, a weighing balance was used.

Principal components analysis was carried out on the data obtained, where the individual components responsible for the variation of the studied taxa were analyzed.

Experimental design and layout for morphological characterization

The experiment was conducted at the Teaching and Research Farm of the Federal University of Agriculture Makurdi. The experimental design adopted was the Randomized Complete Block Design (RCB). Five (5) replicates for all the *species* were maintained in a RCB experimental design for a period of 3 months. Nitrogen, Phosphate and Potassium (N.P.K) fertilizer using the formula 5-10-10 was applied at 40 Days After Planting (DAP) using the broadcasting method at 2 kg per 100 square meters. Weeding of the farm was initially done at 2 weeks intervals before the application of fertilizers and later done more frequently as much as required to keep the farm weed free.

Data analysis

The data obtained were entered into Microsoft Excel package and then imported into Minitab 17 software for further analysis.

Molecular marker analysis

Molecular markers analysis was carried out in the Molecular Biology laboratory of the Federal University of Agriculture Makurdi, Benue State.

DNA extraction from the leaves

Genomic DNA was extracted from plants using basic protocol described by [12].

Selection of primers

The primer sequence was selected from a work done by [13]. The primer sequence that produced the highest number of polymorphic bands was selected out of the 40 primers tested.10 primers were used.

Preparation of primer working solution

The primers were constituted by transferring 50 µl of the forward and revers primers respectively into a new labeled tube using a micropipette. The mixture was then vortex for 10 sec and stored in a refrigerator for further use.

Polymerase chain reaction

Polymerase Chain Reaction was performed using a thermocycler (Applied Biosystem version) in a touch down fashion and covered the following steps: (i) Initiation: This was done at a temperature of 94°C for 5 min. (ii) Denaturation: This was carried out at a temperature of 94°C for 1 min. (iii) Annealing: This was carried out at between 30.0 and 4.0°C (depending on the annealing temperature of the primer). (iv) Polymerization: This was done at a temperature of 72°C for 2 min. (v) Steps 2 to 4 were repeated for 30 cycles. (vi) Final extension: This was carried out at a temperature of 72°C for 5 min.

Agarose gel electrophoresis

Agarose gel electrophoresis was done using the following steps: Preparation of gel: Five grams of agarose powder was weighed into an Erlenmeyer flask containing 500 mL of Tris base Acetic acid and EDTA (TAE). The content of the flask was then swirled and the top covered with a paper towel. The flask was microwaved till the agarose powder was completely dissolved and the content of the flask crystal clear. This was allowed to cool a little and then 1.5 µl of Ethidium bromide (EtBr) was added. The flask content was emptied into a casting tray with combs already inserted. After cooling, the combs where removed and the casting tray was then placed in a gel thank containing adequate 10X Tris base Acetic acid and EDTA (TAE) buffer.

Loading into wells

After PCR amplification, 1 µl of DNA loading dye was added into each of the tubes and then made to spin for 10 sec using a centrifuge. Ten µl of the content of the tubes was then carefully loaded into separate wells using a micropipette.

Gel visualization using U-V light

After the DNA had separated, the gel was transferred to a Bench top transilluminator and the gel image was captured using a digital camera for scoring and analysis.

Data analysis

The gel image formed was scored as 1 for the presence of amplification band 0 for the absence of amplification for each accession per primer. Dendogram was generated using Minitab 17 software

Results and discussion

Morphological studies

Variability and mean performance of species of wild Potato for morphological traits

Morphological analysis featured eleven characters namely; number of sprouted shoots per tuber, Plant height (cm), number of branches, length of branch (cm), number of leaves, size of leaves (cm), Internodal distance (cm), Number of stem/stand, Tuber weight (g), Tuber length (cm) and Tuber diameter (cm). Analysis of variance revealed significant variability ($p<0.05$) among the species of wild potato for all the traits measured (Table 1). Number of shoots per tuber varied significantly ($p<0.05$) among the species of wild potato, with *P. esculentus "*Longat" cultivar (PEscuLG) having the highest number, followed by "Bebot" cultivar (PEscuBt), *P. roduntufolus* and *P. esculentus "*Riyom" having the least number.

Magnitude and nature of variability among species of wild potato

The first principal component captured 53.10% of variability among the species for measured traits, while the second and third principal components captured 32.19 and 14.70% respectively (Table 2). Thus, cumulatively the first three principal components captured 100% of the variability for measured traits among the species. The analysis also showed that number of shoots per tuber, number of leaves, internode distance, tuber length and tuber diameter with high positive loadings contributed significantly ($p<0.05$) to the variability among the species as captured in the first principal component. Other traits with high positive loadings that contributed significantly to the variability are plant height, number of branches, length of branches and number of tubers per stand in the second principal component and size of leaves in the third principal component. Traits such as internode distance, number of shoots per tuber, tuber length and tuber weight, over-lapped in their positive contribution to variability among the three principal components.

Table 1. Selected SSR primers

Primer Name	Primer sequence
OPX 1	F (AGCTGCTCAGCATCAAGAGA) R (ACCACCTCAGGCACTTCATC)
OPX 2	F (GCGTCAGCGATTTCAGTACTA) R (TTCAGTCAACTCCTGTTGCG)
OPX 3	F (AGAAATTGGCAGAGCATTTAGCTG) R (GGATTAGACAAACCTTCTTTTCCACA)
OPX 4	F (GAAGCGACTTCCAAAATCAGA) R (AAAGGGAGGAATAGAAACCAAAA)
OPX 5	F (TGGGAAGAATCCTGAAATGG) R (TGCTCTACCAATTAACGGCA)
OPX 6	F (GCTGTGTTTTCAATTTCTTCAGCA) R (GTCTCGTTGTTCAGCTTCATCAAA)
OPX 7	F (CTTATGTTTTGATCTATTACACATGGCA) R (ACTCGAGACCTCTGATTTATGGGA)
OPX 8	F (ACCATCCACCATGTCAATGC) R (CTCATGGATGGTGTCATTGG)
OPX 9	F (CGCACGTATAGAATTAGAATTAAGAAA) R (CCCCTCGCATTAGAATTTTG)
OPX 10	F (TGGAATCCGAATTACGCTCT X) R (AGGTTTTACCACTCGGGCTT)

Key:
OPX 1= SSR PRIMER 1, OPX2=SSR PRIMER 2, OPX3=SSR PRIMER 3, OPX 4= SSR PRIMER 4, OPX 5= SSR PRIMER 6, OPX 7= SSR PRIMER 7, OPX 8= SSR PRIMER 8, OPX 9= SSR PRIMER 9, OPX 10= SSR PRIMER 10

Table 2. Variability of measured traits among four species of wild potato as revealed by Analysis of Variance

Specie	NP(cm)	PH(cm)	NB(cm)	LB(cm)	NL(cm)	SL(cm)	ID(cm)	NS(g)	TW(g)	TL(g)	TD(g)
PEsculG	4.00	54.08	15.26	42.80	16.80	42.00	3.60	84.80	740.00	12.60	7.66
PEsculBt	3.00	57.58	3.74	39.60	20.00	24.70	3.20	4.60	12.70	12.94	14.20
Produn	2.00	67.04	23.52	30.82	8.36	38.58	3.06	91.20	700.40	9.36	7.86
PEsculRM	1.00	39.96	11.26	28.70	11.30	56.04	2.80	22.00	492.50	10.38	7.64
LSD	0.00	1.81	0.69	0.80	1.19	0.16	0.22	11.65	239.55	2.63	1.83
P-value	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.0317	<.0001
CV	0	2.39	3.71	1.63	6.13	6.28	5.070	16.68	35.73	16.83	14.17

NP= number of sprouted shoots per tuber, PH=Plant height (cm), NB= number of branch; LB= length of branch (cm), NL = number of leaves, SL= size of leaves (cm), ID = Internodal distance (cm), NS= Number of stem/stand, TW=Tuber weight (g), TL= Tuber length (cm), TD= Tuber diameter (cm).CV= Coefficient of variance.

Morphological characters of plants have been used extensively both for producing classification and for diagnostic purposes and they are still indispensable to the taxonomists today [11]. The selective action of some environmental factors could foster the formation of genetic races or variety or ecotypes which then maintains a narrow phenotypic range. Variation may be in a plant species but taxonomically it may be difficult to interpret evolutionarily if it forms a cline, which is continuous from one extreme to the other as amply shown in the characters or traits showing significant variation in range of values. According to [11], the preponderance of intermediate characters coupled with similarity in shared characters is indicative of the weak specific boundary in the circumscription of *Solenostemon* and *Plectranthus* and points out that the species are the same

Table 3. Magnitude and Nature of Variability as revealed by Principal Component Analysis

Trait	Prin1	Prin2	Prin3
NP	0.285320	0.377573	0.110079
PH	0.040655	0.315794	-0.627706
NB	-0.315425	0.324468	-0.168621
LB	0.339361	0.280361	0.173894
NL	0.404623	-0.009257	0.163692
SL	-0.310752	-0.099056	0.498065
ID	0.227112	0.427048	0.180789
NT	-0.192329	0.469668	-0.041101
TW	-0.289961	0.347314	0.224737
TL	0.388780	0.052282	0.257688
TD	0.343799	-0.193943	-0.330224
Eigenvalue	5.841469	3.541392	1.617139
Proportion	0.531000	0.321900	0.147000
Cumulative %	53.10	85.30	100.00

NP= number of sprouted shoots per tuber, PH=Plant height (cm), NB= number of branch; LB= length of branch (cm), NL = number of leaves, SL= size of leaves (cm), ID = Internodal distance (cm), NS= Number of stem/stand, TW=Tuber weight (g), TL= Tuber length (cm), TD= Tuber diameter (cm).CV= Coefficient of variance

The first three principal components contributing to a cumulative variance of 100% is a significant result in this study. This implies that the 11 measured traits are not adequate morphological representation of the quantitative and qualitative variability among the species evaluated. This depicts that variation within the germplasm based on these traits is not huge; hence this shows that the plant breeder can make possible improvements on these plant species through intra and inter-species breeding where the plants with the best desired traits can be used

for targeted breeding. This is reflected in the low morphological distance at a similarity level of 1.5 (15%) between the extreme cultivars in the cluster analysis. [11] reported a morphological distance at a similarity level of 34% between the extreme cultivars in their characterization. The clustering of *P. roduntufolus, P. esculentus* Longat and Riyom cultivars in the same group, suggests an overlap in the range of values of measured traits between these species and shades some light on interspecies similarities in gradation of traits as shown in their mean performance. [5] Also reported that quantitative characters showed overlapping range of values for both between *Solenostemon rotundifolius* (*P. rotundifolius*) and *P.esculentus.*

Cluster analysis of species of wild potato based on morphological traits

The cluster analysis using the average linkage procedure based on the principal component analysis clustered the species of wild potato into two groups at an average genetic distance of 1.5 between clusters (Figure 1). Group one is subdivided into two sup groups. *P. esculentus* Riyom cultivar clustered into subgroup one unique to this cultivar, while group *P. roduntifolus*and *P. esculentus* Longat cultivar clustered together in subgroup two. *P. esculentus* Bebot cultivar clustered into group two separated from all the other species, this shows its uniquely different characteristics from the others.

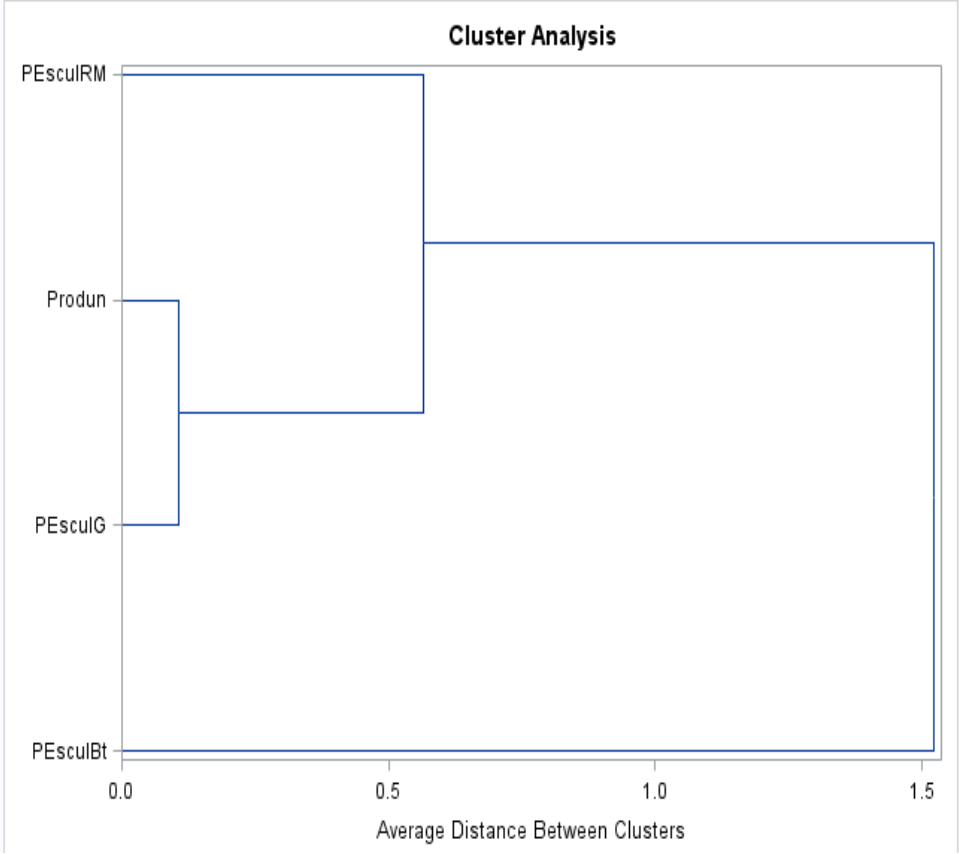

Figure 1. Dendogram showing variability among species of *Plectranthus* based on morphological traits

PEsculRM = Plectranthus esculenthus (riyom), Produn = Plectrenathus roduntifolius, PEsculG = Plectranthus esculenthus (longat), PEsculBt = Plectranthus esculenthus (bebot)

Variability is amply shown by the dendrogram of relationships among the taxa studied. In this study, the range of morphological variation is shown in this study in the leaves, stem and tuber characteristics as shown by the significant variability revealed from the analysis of variance. This significant variability among the species wild potato for measured traits in this study agrees with the report of [5], who reported significant morphological variability in wild potato. The study [14] also clearly illustrated the existence of a wide range of variation among the germplasm accessions of *P. edulis* collected from different regions of Ethiopia. The variability expressed in the range of phenotypes for measured traits in wild potato is a key element for evolution that will favour the survival of the species over generations and also provide platform for genetic improvement of desired traits. So too, the range of morphological variation observed in this study

makes possibility for genetic improvement favorable.

Molecular characterization of Plectranthus species

The gel images produced were shown in Figure 2. all the ten SSR primers (OPX1-OPX10) tested amplified the DNA, and this clearly differentiates the plant samples that were used.

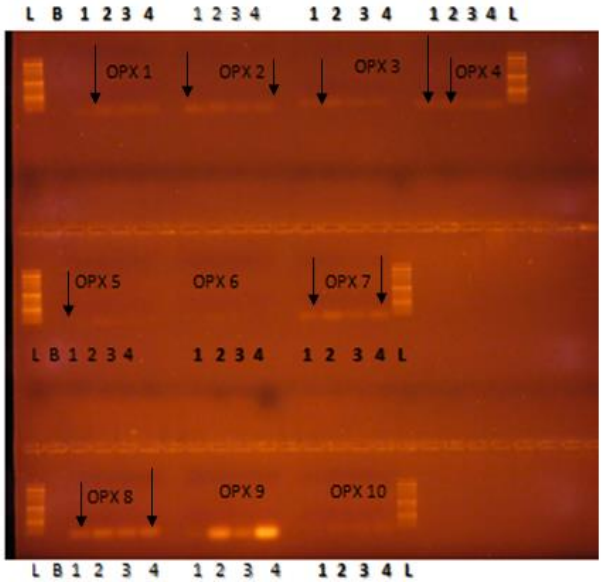

Figure 2. Gel image generated from DNA of *Plectranthus species* with SSR primers

P =SSR PRIMERS (OPX 1- OPX10), L= DNA ladder, B= Sample blank, 1= *P.roduntifolius*, 2= *P. esculenthus* (longat), 3= *P.esculenthus*(riyom), 4= *P. esculenthus* (bebot)

Polymorphism information of SSR primers

The number of polymorphic and monomorphic band produced by each primer is shown in table 4. The SSR primers produced 35 monomorphic and 5 polymorphic bands, making a total of 40 bands produced on the Agarose gel.

Dendogram showing genetic distances between Plectranthus species using SSR markers

A dendogram was created using the polymorphism shown by the SSR primers used. The dendrogram of relationships has provided a graphical summary of the levels and degree of closeness between the species and within the varieties of *P. esculentus* (Figure 3). Genetic variability was observed between the *P. esculenthus* (longat) and *Protuntifolius species* (50%), followed by *P. esculenthus* "riyom" (15%) and *P. esculenthus* "bebeot" (10%).

Morphological characters of plants have been used extensively both for producing classification and for diagnostic purposes and they are still indispensable to the taxonomists today [13]. The selective action of some environmental factors could foster the formation of genetic races or variety or ecotypes which then maintains a narrow phenotypic range.

Table 4. Polymorphism information of SSR primers

Primer	No. of Amplified Fragments		
	Monomorphic bands	Polymorphic bands	Total
OPX 1	4	0	4
OPX 2	4	0	4
OPX 3	4	0	4
OPX 4	4	0	4
OPX 5	3	1	4
OPX 6	2	2	4
OPX 7	4	0	4
OPX 8	4	0	4
OPX 9	3	1	4
OPX 10	3	1	4
Total	35	5	40

OPX 1= SSR PRIMER 1, OPX2=SSR PRIMER 2, OPX3=SSR PRIMER 3, OPX 4= SSR PRIMER 4, OPX 5= SSR PRIMER 6, OPX 7= SSR PRIMER 7, OPX 8= SSR PRIMER 8, OPX 9= SSR PRIMER 9, OPX 10= SSR PRIMER 10

Variation may be in a plant species but taxonomically it may be difficult to interpret evolutionarily if it forms a cline, which is continuous from one extreme to the other as amply shown in the characters or traits showing significant variation in range of values. According to [13], the preponderance of intermediate characters coupled with similarity in shared characters is indicative of the weak specific boundary in the circumscription of *Solenostemon* and *Plectranthus* and points out that the species are the same. This variability is amply shown by the dendrogram of relationships among the taxa studied. The dendogram generated from the amplified primer numbers shows that *P. roduntifolius* and *P. esculenthus* (riyom) are more closely related than *P. esculenthus* (Bebot and longat) which show more similarity, this is in contrast to the work done by [13] who proposed treating *P. esculenthus* (Bebot) as a different species based on their molecular traits. Considering the *Plectranthus spp* are cross pollinated plants, a low interspecific variability was expected. By dendogram inference and from observing a clear separation among the studied genotypes, this

finding does not support the use of these plants in synonymy. The genetic cluster analysis showed that the two plants are not the same as the *P*. esculenthus (riyom) was found to be more closely related to P. roduntifolius which is in contrast with the morphological cluster which placed P. esculenthus (riyom) as the more closely related speciesto P. roduntifolius. The results of this study suggest that these plants should be regarded as different contrary to current treatment.

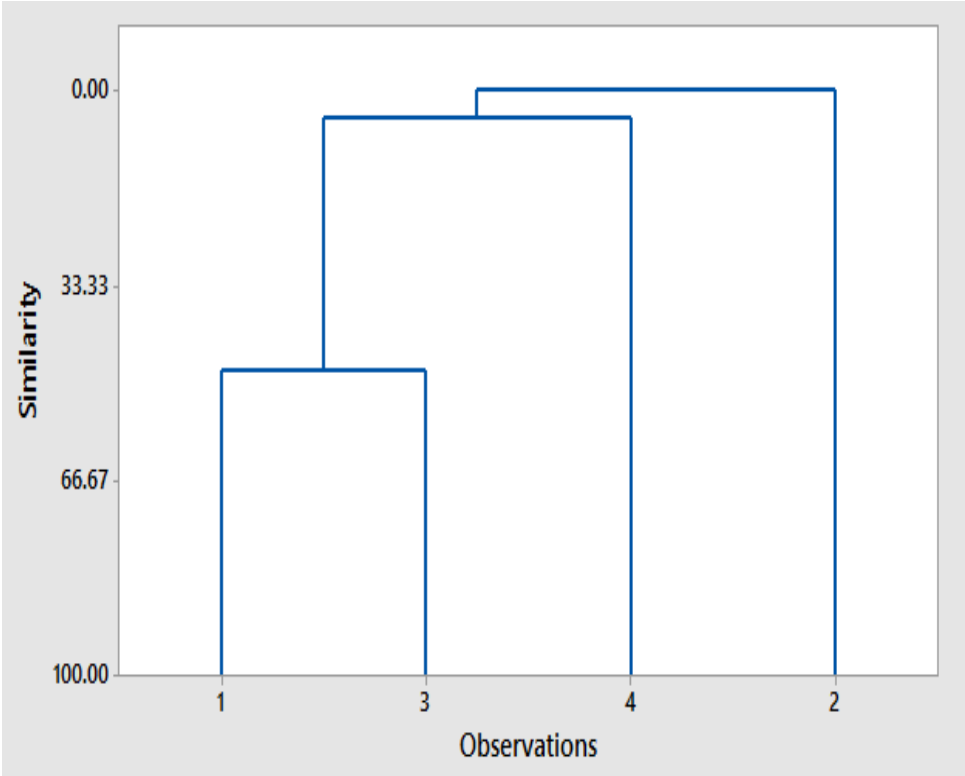

Figure 3. Dendogram from genetic characters analysed
1= *P. roduntifolius,* 2= *P. esculenthus (Longat),* 3=*P. esculenthus Rioym),* 4=*P. esculenthus (Bebot)*

Conclusions

This research confirmed the applicability of the use of both morphological and genetic markers to discriminate synonymy cases among *Plectranthus species* in Nigeria in view of the difficulty in characterization using morphological markers alone. *Plectranthus* has several species that are used in popular medicine. The genetic and morphological variability evaluation in this research is the first step towards mapping characteristics of interest for future genetic improvement research. The present study may be noble report on genetic similarity of Hausa Potato (*P. roduntifolius)* and livingstone Potato (*P. esculenthus)* using SSR marker. The genetic similarity and morphology data generated by four SSR primers revealed 50% genetic similarity exists in *P. esculenthus* and *P. roduntifolius*.. The penta-nucleotide primer showed high levels of genetic similarity. This implied that SSR primers can be used in checking similarities of the plant species.

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Competing interests

Authors have declared that no competing interests exist.

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