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MOLECULAR CHARACTERIZATION OF *DIPCADI FILAMENTOSUM* MEDIK USING SSR MARKER

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ABSTRACT
The genetic variations and phylogenetic relationships were accessed in 24 populations of Dipcadi filamentosum using Simple Sequence Repeats (SSR) markers. The specimens were collected from 11 sites in 8 states in Nigeria. These were transplanted into 5 liter sized buckets filled with sandy-loam soil at the Botanical Garden of the University of Ilorin in Ilorin, Nigeria. Genomic DNA was extracted from the leaves using CTAB method. The degree of relatedness and genetic diversity among the populations was assessed using SSR. Four sets of SSR primers were used on each population and diversity among the populations were studied. The dendrogram constructed from the dissimilarity and Unweighted Pair Group Method of Arithemathic Averages (UPGMA) showed that 23% of the analyzed populations were unrelated. The Yobe population from north – east forms an independent cluster at 74%. While at 77 – 100% similarity all other populations were shown to be related. The delimitation by these markers on the Dipcadi populations ranged from 0.7250 to 0.8875 respectively. Genetic diversity estimates were between 0.5427 and 0.7149 while the Heterozygosity were between 0.4638 and 0.7586. Genetic diversity among the population under study was evident by the polymorphism obtained in the study. Thus, by means of the SSR markers the genetic diversity in the populations was determined.
KEY WORDS: *Dipcadi filamentosum, phylogeny, polymorphism, population, genetic diversity*

INTRODUCTION

Dipcadi filamentosum belongs to the Family Hyacinthaceae, sub family: Ornithogaloideae. The Hyacinthaceae comprises of about 46 genera which includes about 700 to 900 species of bulbous plant which are mainly distributed through Europe, Africa and South-West Asia, with a small genus in South America (APG 11, 2003). *Dipcadi* is represented by 55 species which are distributed in the Southern Mediterranean region in Africa, Arabia, Socotra, Madagascar and India (Mabberley, 1990).The greatest diversity is found in South Africa where about thirteen (13) different species exist at present (Stedje & Nordal, 1987) while India houses the second highest diversity of about nine different species (Deb & Dasgupta, 1978).

D. filamentosum is a wild species found in many regions in Nigeria especially in areas where shady trees are absent (Temikotan *et al*., 2013). It is a perennial herb

that is endangered and rarely cultivated. The plant is mostly found in seasonal climates with a pronounced dry season and is rare in tropical lowlands and forested areas. It grows well in sandy – loam soil. It has one to many leaves that are long and narrow arising from the base. The leaves form a bulb which opens with lanceolate or linear lanceolate blades while flowers are bell-shaped or tubular (Manning *et al*., 2004). In Nigeria the plant is found in the wild and three species have been identified up to date (Adelanwa, 2008).

The plants in the family Hyacintheceae have received attention as a potential source of unique alkaloids, such as bufadienloides, homoisoflavonones and steroidal compounds that may have a range of medicinal properties (Pohl *et al*., 2000). The leaves of *D. filamentosum* have been reported to contain other phytochemical such as tannins, saponin and flavonoids (Abdulkareem *et al*., 2014). It is used in horticulture as ornamental plants.

The study is carried out in order to initiate a DNA marker based genetic assessment of the Nigerian populations of *D. filamentosum* genetic resources. The assessment of its genetic diversity is important in the promotion of the conservation, effective management and protection of the plant.

MATERIALS AND METHODS

Sampling method

Dipcadi filamentosum collections were done using the cluster sampling method. Each geographical location was taken as a cluster. In each of the cluster, random sampling of the plant populations was carried out to ensure fair representation of the population of the plant in those regions in Nigeria.

Twenty four populations of *D. filamentosum* collected from four geographical zones (North West, North east, North central and South west) in Nigeria were brought for cultivation at the Biological Garden of the University of Ilorin, Nigeria and planted in polythene bags with a 5L capacity under controlled environmental conditions. The plants were watered once a week and weeds were handpicked. Locations from where samples were collected are shown in table 1.

Genomic DNA Extraction

The fresh leaves of *D. filamentosum* measuring 0.2g from each of the 24 samples were used for DNA extraction. These were frozen in liquid nitrogen and ground into fine powder. Total DNA was isolated using the CTAB method according to Doyle and Doyle (1987). The quantity and quality of DNA were assessed using the spectrophotomter/Nanodrop and 0.8% agarose gel electrophoresis respectively. Optical density at 260 nm and 280 nm were measured and purity percentage was calculated to estimate the purity of the DNA sample. The DNA samples were stored at - 4^0C.

PCR Amplification

SSR (Simple Sequence Repeat) was carried out using four primers (Inqaba, South Africa). PCR was done in a volume of 25 µl (Table 2). Amplification was performed in the thermal cycler (master cycler, Eppendorf, Hamburg, Germany) set at a program of 94 ^{0}C for 4 min, 40 cycles of 94 ^{0}C for 5 sec, primer annealing 57^{0}C for 10 sec, extension at 72 ^{0}C for 20 sec, final extension at 72 ^{0}C for 5 min. The PCR products were then loaded into 10 % polyacrylamide 1 x TBE buffer gels which were stained with Ethidium bromide to show the microsatellite band. The PremixTaq Version 2.0 contained blue dye that allows monitoring of progress of migration during electrophoresis. The bands were viewed in the UV light documentation unit (LiuYi, China).

Data Analysis

Each band observed was treated as a SSR marker. All SSR loci were characterized as co- dominant markers and were scored as present (1) or absent (0). The number of polymorphic bands was determined. Percentage of polymorphism was obtained by calculating the proportion of polymorphic bands over the total number of bands. Coefficient of similarity tree was produced by clustering the similarity data with the unweighted pair group method using the statistical software package NTSYS- pc Version 2.1 package (Rolf, 1998). The similarity coefficient was used to construct a dendrogram by the Unweighted Pair Group Method with arithmetic averages (UPGMA).

RESULTS AND DISCUSSIONS

The bands were scored across the lanes according to their molecular weight. Only clear reproducible bands were scored. The size of the bands ranged from 20 to 300 bp.

The allelic frequency was highest in primer 3 (0.5141) and least in primer 2 (0.3768). Primer 1 produced an allelic frequency of 0.4224 while primer 4 had 0.4765. Number of genotypes ranged from 9.0000 in primer 1 to 5.0000 in 2, and 4.0000 to 5.8000 in primers 3 and 4 respectively (Table 3).

A total of 14 polymorphic bands were observed in SSR marker used. Five polymorphic bands were obtained in primer 1, 3 bands in primer 2, 3 and 3.4 in primer 4 (Table 3). The delimitation of these markers on the populations ranged from 0.7250 to 0.8875 respectively (Table 3). Genetic diversity estimates were between 0.5427 and 0.7149 while the Heterozygosity was 0.7586 in primer 1, 0.4638 for primer 2, 0.5493 in primer 3 and 0.5080 in primer 4 respectively (Table 3).

The principal component analyses (PCA) for correlation of the performance of the various loci on the alleles revealed that the first three principal components account for approximately 62.10 % of the total genetic variations observed in 24 genotypes. Component one accounts for 28.37 % of the genetic variation observed in the alleles

while principal components two and three account for 19.98 % and 13.75 % of the observed genetic variations respectively (Table 4).

The biplot of the PCA of *Dipcadi filamentosum* showed that quadrant I had populations from Katsina _1, Katsina_2 Kabba_2, Ekiti_2 and are delimited by primer 4. The Katsina populations were on the centroid. Six populations were observed in quadrant II and the primer responsible for their delimitation was primer 1. In quadrant III, three populations were delimited by primer 3. Populations on the centroid are Sobi and Kaiama big. Quadrant IV four populations and the primer delimiting the populations was primer 2. Kaiama medium was observed to be far from the centroid (Figure 1).

The neighbour joining clustering for genetic variability has clustered the populations into 2 major clusters. Cluster 1 consist of nine populations with Sobi _2 and Kaiama big as being closely related. Sobi_1 and Kaiama _medium are clustered together while Sobi_1 was shown to have mutated than Kaiama _medium. Sobi_3 formed a sub cluster of its own. Populations from the South West consistently clustered with those from the North Central and North Eastern regions while in some cases like the Oyo_1 and Maiduguri, Ekiti_1 and Tanke_1 both from the south west populations are the progenitors of their northern relatives (Figure 2). However, in some cases those from the same geographical zones were clustered together.

In the cluster analysis, ten populations from diverse regions were clustered together as closely related. Some of these are Ogun_1 and Maiduguri, Katsina_2 and Oyo_2, Ekiti_2 and Kabba_3 and Katsina_3 and Ogun_2. Tanke _3 form the base from which other populations arose. Ekiti_1 and Katsina_1 are clustered together. The Yobe population forms a cluster alone.

The selected microsatellite differentiated the *D. filamentosum* populations. A high level of genetic similarity was observed among the populations with about 77 % of the population from different regions being clustered as closely related. Similarly in the use of SSR markers by Doumbia *et al.* (2014) to characterize cowpea germplasm from Ghana and Mali, high level of similarity was observed in the 94 accessions studied. The 14% polymorphism obtained in this study is however at variance with the work of Abdulkareem *et al*. (2018) where a 100 % polymorphic level was obtained in their study of genetic diversity in *D. filamentosum* using RAPD markers.

The use of SSR molecular markers which are also known as microsatellites and are characterized as short sequence tandem repeats are 1 – 5 base pairs (bp) long are employed to capture the area of genetic variability among the genotypes. Molecular markers are said to be important tools in the studies of genetic diversity (Bered *et al*., 2004) due to their high resolution and reliability in the identification of plant populations. Genetic diversity in the populations of *D. filamentosum* was shown in the NJ clustering of diverse populations from different geographical zones as closely related, such as OYO_2 and Kaiama, Maiduguri and OYO_1, Kabba_2 and Katsina_2.

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According to Abel (2007) in Abdulkareem (2018) the variation in selection pressure results into inherent variations among different populations of the same species. According to Ghulam *et al*. (2007), molecular markers are more reliable for genetic studies than morphological characteristics because the environment does not affect them. Mohammadi and Prasanna (2003) asserted that genetic diversity among individuals and populations can be measured using both morphological, biochemical and molecular markers. This is evident in the current work as the genetic basis of *D. filamentosum* has become narrow probably due to the loss of genotype during the journeys that brought it from various regions and gene flow. Thus the use of SSR marker has identified the genetic diversity and variability that are hitherto hidden in the population as also opined by Doumbia *et al*. (2014) in their study of the assessment of genetic variability in cowpea.

The genetic variations among the studied *D.filamentosum* populations was 23 %. There was evidence of polytomy as some branches in the tree are not fully resolved. These unresolved branches are OYO_2, Kaiama small, Tanke_3, Tanke_2 and Ogun_1. In the dendrogram from the cluster analysis Yobe genotype formed the basest from which other species have separated i.e. other populations could be traced over the years to their ancestors from the populations collected during this study in Nigerian vegetation. The populations with above 95 % similarity could be termed the same species. Examples are: Sobi_2 and Kaiama_1; Unilorin and Kaiama medium; Kabba_1 and Oyo_1; Katsina_2 and Oyo_2; Katsina_1 and Ekiti _1.Four different gene regions that amass variation rapidly could not separate these accessions as being different from each other. However, all the genotypes are the same at about 75 % on the phylogenetic tree. This implies that the variations among the accessions are not much. The accumulation of variations due to environmental influences across the regions would later results in speciation when they would have synthesized enough amino acids for their survival and incorporated the same in their genome as shown in the neighbor joining cluster analysis. Hence, the accessions appear to be the same species or about 2 species if the reference line is drawn from about 75 % similarity. The high polymorphic results obtained in the work of Abdulkareem (2018) using ISSR markers is at variance with the current work.

The four SSR primers used have generated only 14 polymorphic bands. Therefore, the molecular DNA marker analyses which are not affected by the environment have been suggested for the determination of genetic similarity among genotypes (Gilbert *et al*., 1999).

DNA markers such as SSR used in this study have the advantage of being large in number, locus specifics and not affected by the environment (Tanksley *et al*., 1989; Waugh & Powel, 1992; Rafalski & Tigey, 1993).

The locus specific nature of this marker has shown that the populations of *D. filamentosum* have genetic variability. This is evident from the heterozygosity value

and gene diversity rate of 0.54 - 0.71 obtained in this study. It showed that the microsatellite marker used in this study are more faithful in assessing plant genetic diversity due to their reproducibility and abundance throughout the plant genome (Powell *et al*., 1996, Feng *et al*., 2009).

CONCLUSIONS

The use of SSR markers in the assessment of the genetic diversity of the populations of *D. filamentosum* in this study has been established. The presence of polytomy on the evolutionary tree showed that the plant is still evolving. The effect of gene flow among the populations brought about the clustering of populations from diverse regions as same or closely related. This study will provide a basis for the characterization of the plant using molecular tools.

Table 1. Population of *D. filamentosum* collected from various sites in Nigeria.

Geographical location	Place of collection	State of collection	Code for each location	*Annual rainfall (mm)	*Mean temp (°C)	Soil type
South Western	Ado Ekiti	Ekiti	EKT	1,605.40	26.87	Sandy loam
	Ogun	Ogun	OGN	1,126.05	25.05	Sandy loam
	Oyo	Oyo	OYO	1,318.10	26.61	Loamy
North East	Maiduguri	Borno	MAI	745.70	28.86	Sand
	Damaturu	Yobe	YOB	696.60	29.70	Sand
North Central	Sobi	Kwara	SOB	1,046.30	26.35	Sandy
	Tanke	Kwara	TNK	1,046.30	26.35	Sharp sands
	Unilorin	Kwara	UNI	1,046.30	26.35	Sandy loam
	Kaiama (big)	Kwara	KAB	1,046.30	26.35	Loamy
	Kaiama (medium)	Kwara	KAM	1,046.30	26.35	Clay loamy
	Kaiama (small)	Kwara	KAS	1,046.30	26.35	Loamy
	Kabba	Kogi	KBA	NA	NA	Loamy clay
North West	Katsina	Katsina	KAT	527.5	27.28	Sandy loam

Table 2. The names of SSR primers, base sequence, no of polymorphic bands and size.

	Primer name	Primer pair	Sequence (5'- 3')	Number of polymorphic bands	Size ranges (BP)
1	1F	Forward	TTCGCAAATGGATCCGGACA	5	150 – 300
	1R	Reverse	ATTTCCCGCGCATCATCCTA		
2	2F	Forward	TTTCGCAAATGGATCCGGACA	3	30 - 50
	2R	Reverse	CATTTCCCGCGCATCATCC		
3	3F	Forward	TAAGCGGGCGTGTCAATACT	3	20 - 100
	3R	Reverse	TCGCGAGTCTGGTATCTTTGG		
4	4F	Forward	GCTAAGCGGGCGTGTCAATA	3	50 – 20
	4R	Reverse	TGAACACTCATAGCCTCTCGC		

Table 3. Genetic variability of the accessions based on the Markers used

Marker		**Allele Frequency**	**Genotype No**	**Allele No/ polymorphism**	**Availability**	**Gene Diversity**	**Heterozygosity**
Hadiz	1	0.4224	9.0000	5.0000	0.7250	0.7149	0.7586
Hadiz	2	0.3768	5.0000	3.0000	0.8625	0.6629	0.4638
Hadiz	3	0.5141	4.0000	3.0000	0.8875	0.5427	0.5493
Hadiz	4	0.4765	5.8000	3.4000	0.8375	0.6112	0.5080

Table 4. PRINCIPAL COMPONENT ANALYSIS (Correlation) using SSR markers

PRINCIPAL COMPONENT	EIGEN VALUE	% VARIATION
1	0.4628	26.373
2	0.3251	19.983
3	0.2236	13.747
4	0.1781	10.950

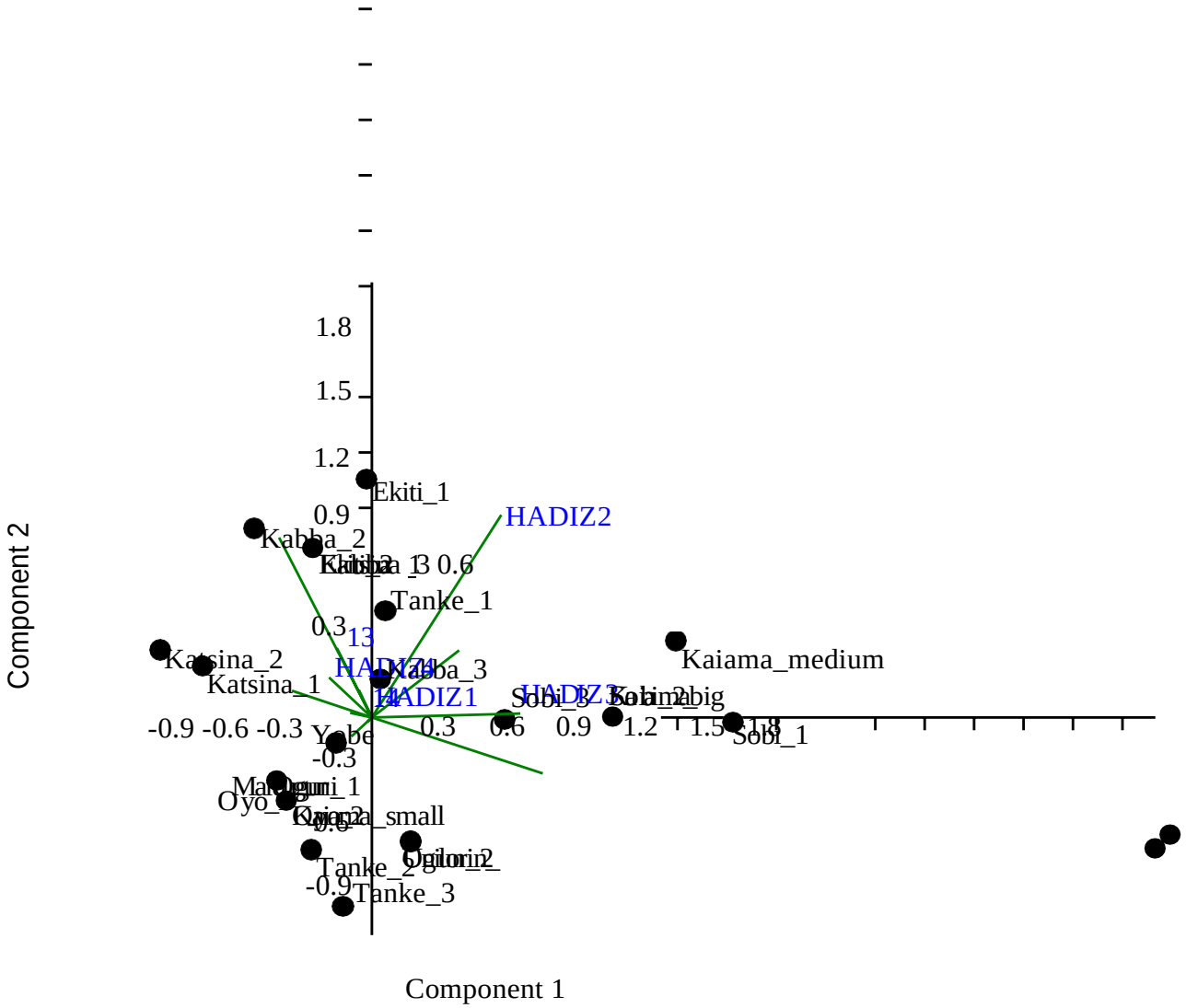

Figure 1. Biplot of Principal component analysis (PCA) of *D. filamentosum* using SSR markers.

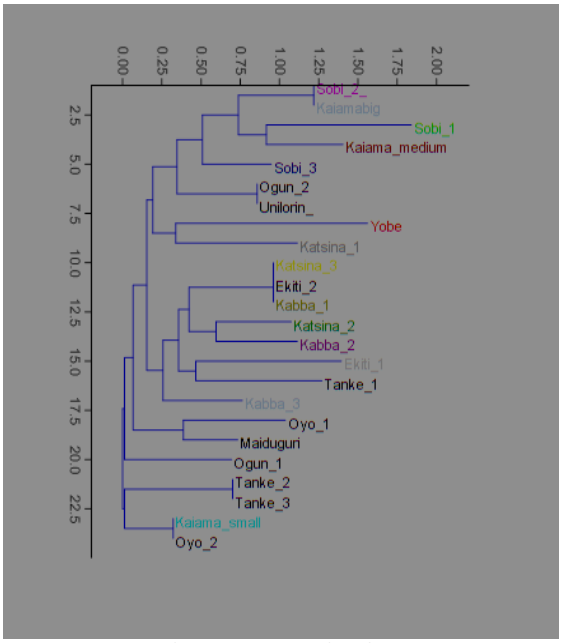

Figure 2. Neighbor joining clustering of 24 populations of *D. filamentosum* using SSR markers.

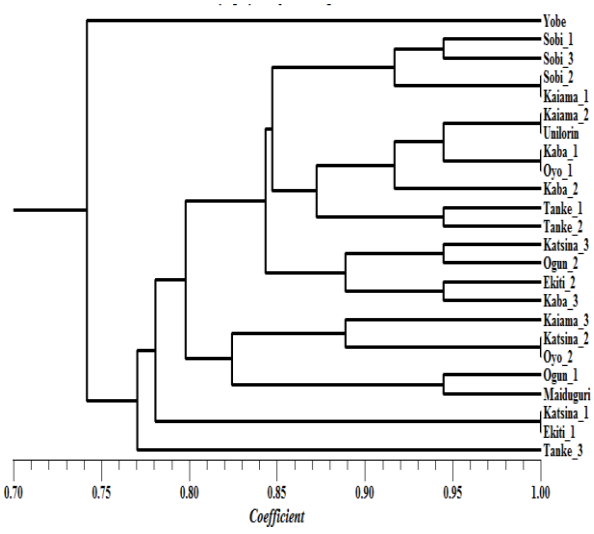

Figure 3. Dendrogram showing the clustering of the 24 populations of *D. filamentosum* using SSR markers.

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