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DIVERSITY LEVEL OF GENOMIC MICROSATELLITE AMONG CULTIVATED GENOTYPES OF *Digitaria* SPECIES IN NIGERIA

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ABSTRACT

Acha (*Digitaria exilis* Kipps. and *D. iburua* Stapf.) are valuable indigenous food crops in West Africa. Despite several economic potentials of this crop, little or no attention is paid to its germplasm evaluation and improvement. In this study, we assessed genetic diversity and relationship among genotypes of cultivated acha in Nigeria, using Inter-Simple Sequence Repeat (ISSR) markers for the first time. Genomic DNA were extracted from the genotypes and we performed fragment amplification by Polymerase Chain Reaction (PCR). A total of 95 loci consisting of 790 bands were amplified by 13 ISSR primers, out of which 53.37% were polymorphic. Loci amplification per primer ranged from 5-10, with an average of 7.30 loci per primer. Eight of the primers had above 50% polymorphism. Cluster analysis separated the genotypes into two major groups; a group consisting of two *D. exilis* genotypes and the other comprising a mixture of genotypes. The *D. exilis* in the latter group were distant members and was only similar at 0.72 similarity index. The polymorphism we obtained in the present study showed that the ISSR markers are effective for assessment of genetic diversity of the genotypes. Clustering of *D. exilis* and *D. iburua* together suggests a common progenitor but could have been separated by geographical isolation mechanism.

Key Words: *Digitaria exilis*, *D. iburua*, genetic diversity, ISSR markers, orphan crops

RÉSUMÉ

Les fonios (*Digitaria exilis* Kipps. et *D. iburua* Stapf) sont des cultures alimentaires très importantes en Afrique de l'Ouest. Malgré les différentes potentialités économiques de cette culture, très peu ou aucune attention n'est portée sur l'évaluation et l'amélioration de son germplasm. Dans cette étude, nous avons évalué la diversité génétique et la relation entre les génotypes cultivés du fonio au Nigéria, en utilisant les markers Inter-Simple Sequence Repeat (ISSR) pour la première fois. Les ADN génomiques étaient extraits des génotypes et nous avons utilisé la technologie de Réaction en Chaîne de la Polymérase (PCR). Un total de 95 loci contenant 790 bands étaient amplifiées par 13 amorces d'ISSR, sur lesquelles 53,37% étaient polymorphiques. L'amplification des loci par amorces variait de 5 à 10, avec une moyenne de 7.30 loci per amorce. Huit des amorces ont plus de 50% de polymorphisme. L'analyse typologique a séparé les génotypes en deux grands groupes ; un groupe comportant deux génotypes de *D. exilis* et les autres comprennent un mélange de génotypes. Les *D. exilis* dans le dernier groupe étaient membres distants et étaient le seul similaire à 0,72 d'indice de similarité. Le polymorphisme que nous avons obtenu dans cette étude a montré que les markers ISSR sont efficaces pour l'évaluation de la diversité génétique des génotypes. Le regroupement de *D. exilis* et *D. iburua* ensemble suggère un parent commun mais pourrait être séparé par le mécanisme d'isolement géographique.

Mots Clés: *Digitaria exilis*, *D. iburua*, diversité génétique, cultures orphelines, markers ISSR

INTRODUCTION

Digitaria exilis (Kipps) Stapf. and *D. iburua* Stapf. (acha) also called fonio millets represent an integral component of millet biodiversity in the savannah zone of West-Africa. Acha belong to family Poaceae and genus *Digitaria*. The genus comprises of about 230 grasses, with a wide geographic distribution in the tropics and subtropics (Clayton and Renvoize, 1986). Acha are the most economically important crops in this genus. Although, a number of wild species occur as forage grasses, *D. iburua* (black acha) and *D. exilis* (white acha) are used for food in West and other parts of Africa (Adoukonou-Sagbadja *et al.*, 2006).

Acha is native to West-Africa where it is cultivated in the savannah regions, but the species *D. iburua* is currently limited in areas of cultivation. It is only found in Northern Nigeria, Togo and Benin, which represent a relic of formerly wider cultivation (Porteres, 1946; Haq and Ogbe, 1995). Unlike *D. iburua*, *D. exilis* has wider areas of cultivation and had been reportedly cultivated in Dominican Republic (Morales-Payan *et al.*, 2002). Acha meals are well appreciated for its taste and easy digestibility in many tribal areas of Guinea, Mali, Togo and Nigeria. The protein composition is rich in methionine and cystine, the two vital amino-acids usually deficient in major cereals (Vietmeyer *et al.*, 1996).

Despite the importance of this crop to traditional agriculture in West Africa, research efforts to improve the crop is still grossly inadequate. Consequently, the crop remain primitive facing diverse agronomical problems which, contribute notably to grain yield lost (Maji *et al.*, 2003). Traditionally, local farmers distinguish three groups based on the growth cycle: precocious, intermediate and late maturing types. The first insight into the characterisation of acha genetic diversity was based on morph-botanical characteristics (Porteres, 1976). However, the study did not include *D. iburua* due to its restricted cultivation areas. There are other reports on

acha characterisation using agronomic traits (Adoukonou-Sagbadja *et al.*, 2006; Dachi and Gana, 2008).

Diversity analysis based on morphological traits may be inflated and unrealistic, due to environmental influence. Since acha exhibit some level of isomorphism and is difficult to distinguish morphologically during the vegetative growth stage, molecular characterisation of the available germplasm is imperative. This will facilitate the use of genetic resources and estimation of genetic relatedness among the genotypes faster than the conventional methods (Semagn *et al.*, 2006). Thus, efficiency of genetic gain by selection can be improved if the patterns of genetic diversity within a population of breeding lines are known. This could be of significant importance in varietal identification, proper purity maintenance and implementation of germplasm conservation. Attempts using RAPD markers to assess the molecular variability of acha have been reported, but it involved few accessions from geographically restricted areas of Togo and Nigeria (Hilu *et al.*, 1997).

There is still a wide knowledge gap of genetic diversity and molecular characterisation of the West Africa acha genotypes. Furthermore, genotypes from Nigeria were not included in the previous molecular characterisation studies. The objective of this study was to assess the genetic diversity in the germplasm of two cultivated species of acha (*D. exilis* and *D. iburua*) in Nigeria, using microsatellite (ISSR) markers.

MATERIALS AND METHODS

Plant material. Seeds of eleven genotypes of two cultivated species of acha (*D. exilis* and *D. iburua*) were collected from different sources in Nigeria (Table 1). The seeds were grown in a screen house to raise young plants for the molecular characterisation. Two weeks after germination, young unfolding leaves of

TABLE 1. Genotype name, common name, and source of *D. exilis* and *D. iburua* used for the study

Genotype	Name	Common name	Source [†]
Gwabi	<i>D. iburua</i>	Black acha	NCRI
Kereep	<i>D. exilis</i>	White acha	NCRI
Agyong	<i>D. exilis</i>	White acha	NCRI
Gopanto	<i>D. iburua</i>	Black acha	NCRI
Ndai	<i>D. exilis</i>	White acha	NCRI
Nopiya	<i>D. iburua</i>	Black acha	NCRI
Bauchi	<i>D. iburua</i>	Black acha	Field
NGB00629	<i>D. exilis</i>	White acha	NACGRAB
Niger	<i>D. iburua</i>	Black acha	Market
Kebbi	<i>D. exilis</i>	Black acha	Field
Kebbi-2	<i>D. iburua</i>	Black acha	Field

[†]NCRI = National Cereal Research Institute, Badeggi Niger state Nigeria

[†]NACGRAB = National Centre for Genetic Resources and Biotechnology, Ibadan, Nigeria

each genotype were harvested into separate labelled sample bottles for DNA isolation.

DNA isolation and polymerase chain reaction. Genomic DNA was isolated from 100 mg of the leaf sample using Zymo Plant Mini Kit (Zymo Research, USA). The quality of the DNA was checked on 0.8% agarose gels and the DNA concentrations estimated using a Nanodrop 8000 spectrophotometer (Nanodrop Technologies, USA). A total of 20 Inter-Simple Sequence Repeat (ISSR) primers were initially used for the Polymerase Chain Reaction (PCR). The final reaction volume was 20 µl in thin wall PCR tube (Axygen; USA). Each reaction mixture consisted of 1.0 µl (50 ng) of gDNA, 1.0 µl of primer (10 pmol ml⁻¹), 10.5 µl of 1X PCR mix (3 mM MgCl₂, 0.2 mM dNTPs, one unit of *Taq* DNA Polymerase (Fermentas, Life Sciences) and 7.5 µl nuclease free water. The PCR was performed on Thermal cycler (Applied Biosystems, USA) following the PCR conditions of Animasaun *et al.* (2015). The PCR products and loading dye were electrophoresed on 1.5% agarose gel stained with ethidium bromide using 1X TAE buffer. Fragment sizes of the amplified products were anticipated from the gel by comparison with standard molecular weight

marker (ThermoFisher, USA). The amplified product was visualised as a single compact band under UV light and documented by gel documentation system (SIGMA-3-16KL).

Data analysis. Reproducible and clear bands were scored as 1 for presence and 0 for absence across the lanes in reference to the standard marker. The data were subsequently used to construct a dendrogram using the unweighted pair group method of arithmetic averages (UPGMA). All the computations were carried out using the NTSYpc (Numerical Taxonomy and Multivariate Analysis System) Software Version 2.

RESULTS

The concentrations of genomic DNA extracted from the genotypes of acha ranged from 123.81-186.03 g µl⁻¹, the OD (Optical Density) at 260-280 nm was between 1.51 and 1.72. Out of the twenty ISSR primers tested, thirteen ISSR markers produced remarkable and reproducible fragments. A total of 95 bands were generated by 13 primers with average of 7.30 loci per primer (Table 2). About 53% of the amplified loci were polymorphic and 46.6% were monomorphic bands. Figure 1 showed

TABLE 2. Amplification and polymorphism pattern of ISSSR markers used in the diversity study of accessions of *Digitaria* spp.

SN	Marker code	Sequence (5'-3')	TNA	TNL	NML	NPL	P (%)
1	ISSR1	GAGAGAGAGAGACC	51	6	3	3	50.00
2	ISSR2	CTCTCTCTCTCTCTAC	64	6	2	4	66.67
3	ISSR3	CACACACACACAAG	62	7	4	3	42.90
4	ISSR4	CAGCACACACACACA	66	6	4	2	33.33
5	ISSR5	GTGTGTGTGTGTCC	79	10	4	6	60.00
6	ISSR6	CTCTCTCTCTCTCTGC	67	7	3	4	57.14
7	ISSR7	GAGAGAGAGAGAGG	73	8	4	4	50.00
8	ISSR8	AGCACGAGCAGCAGCGG	35	7	2	5	85.71
9	ISSR9	CACACACACACACAAT	67	8	5	3	37.50
10	ISSR10	AGCACGAGCAGCAGCGT	70	10	3	7	70.00
11	ISSR11	GTGTGTGTGTGTGTTG	43	5	3	2	40.60
12	ISSR12	GTGTGTGTGTGTGTCT	43	6	4	2	33.30
13	ISSR13	GCTGAGAGAGAGAGAGA	70	9	6	3	66.67

TNA = Total number of allele; TNL = Total number of loci, NML = Number of monomorphic loci, NPL = Number of polymorphic loci, %P = Percentage polymorphism

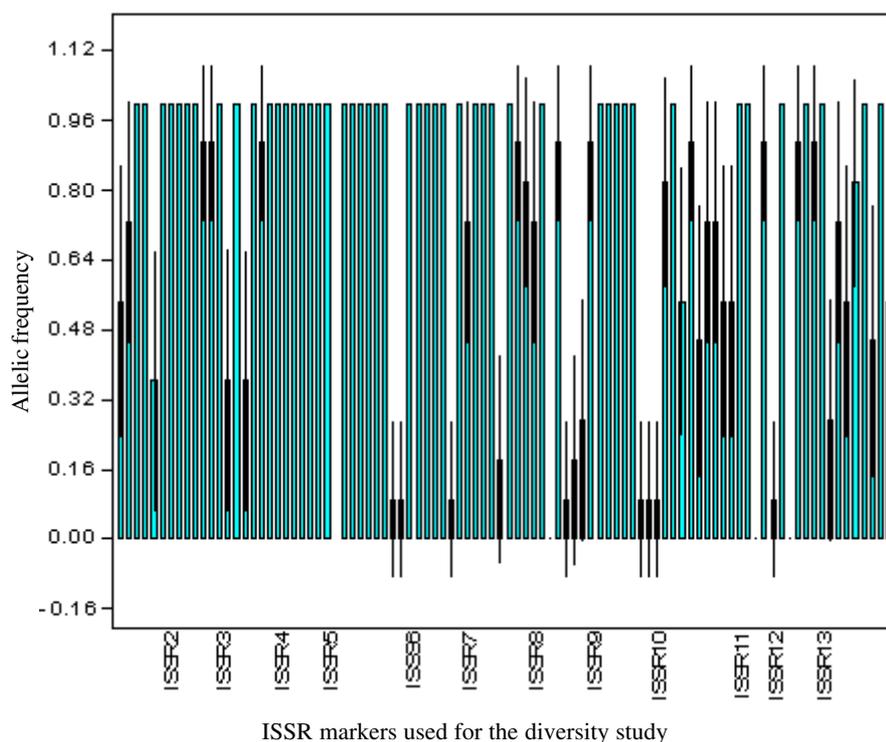


Figure 1. Allelic frequency amplification of the thirteen ISSR primers on eleven cultivated Nigerian acha genotypes.

the frequency of amplification by each of the primer. Primers ISSR5 and ISSR10 had highest allelic frequency of 79 and 70, respectively followed by ISSR13; which occurred in nine loci but marked by 70 alleles. ISSR8 had the least allelic frequency but interestingly had the highest polymorphism. The frequency of null allele was highest in primer ISSR11.

The Principal Coordinate (PCo) analysis of the bands and allelic fragments revealed that nine axes (9 markers) contributed significantly and accounted for 96.38% of the macrosatellite variations observed in the acha genotypes (Fig. 2). The first four most efficient markers; ISSR 8, 10, 2 and 13 cumulatively accounted for over 50% (59.24%) of the variation. The PCo showed a genotype of *D. exilis* and *D. iburua* each in quadrants I. Quadrant II consisted of three *exilis* and one *iburua* members and the trend was similar for genotypes coordinate's quadrants III and IV;

however, more genotypes (4) were placed in quadrant II (Fig. 2).

The genetic similarity among the eleven genotypes of acha (fonio millet) revealed average relationship. At similarity index of 0.68, the dendrogram as defined by the thirteen ISSR markers revealed two major groups; A and B (Fig. 3). Group A consisted of two *D. exilis* (white acha) genotypes; Kebbi-1 and Kereep which were similar at 0.78 index. The second group (group B) had two subgroups (B1 and B2). The B1 had two clusters, whereas cluster B1(i) had two *D. exilis* members that were 84% similar; B1(ii) comprised of six genotypes of five *D. iburua* and a *D. exilis*, with the latter separated from the other five *D. iburua* members at 0.80 index scale. The other five members were more similar, but the closest genotypes of this cluster were Kebbi-2 and Bauchi. In contrast with the B1 sub-group, the B2 had only one genotype, Gwabi (*D. iburua*) a black fonio

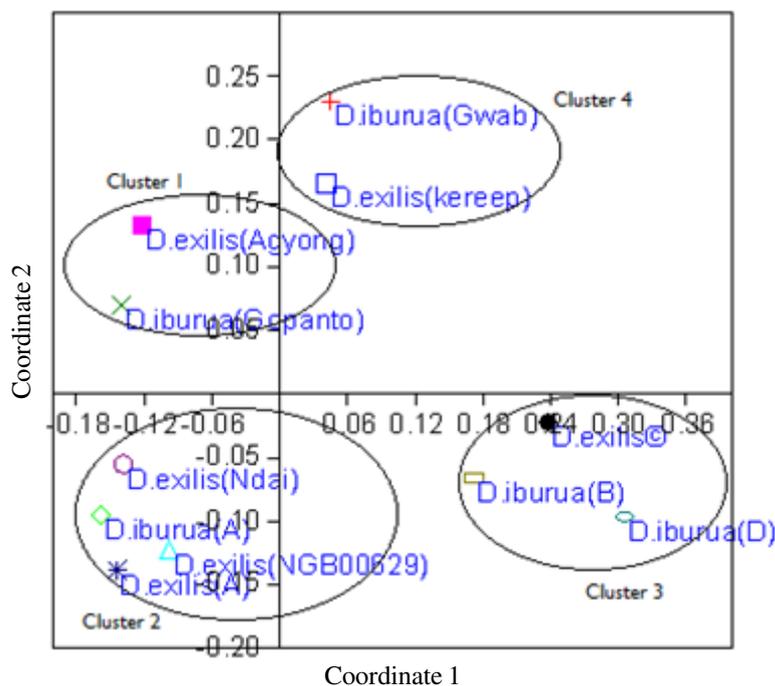


Figure 2. Principal coordinate (PCo) of loci and allelic fragments partitioning of the cultivated acha genotypes in Nigeria based on ISSR marker.

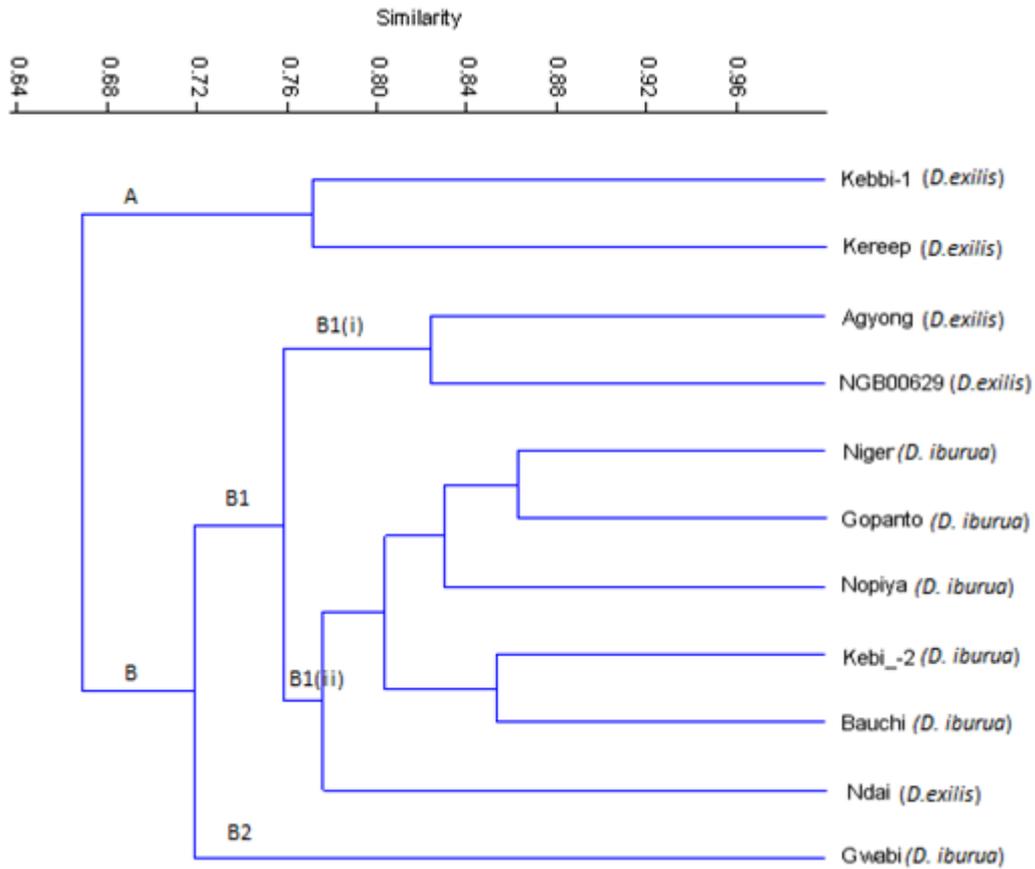


Figure 3. Dendrogram of genetic relationship among the genotypes of white (*D. exilis*) and black (*D. iburua*) acha cultivated in Nigeria.

TABLE 3. Principal coordinates of loci and allele fragments of acha genotypes based on ISSR markers

Principal coordinates	Percentage of variation		
	Eigenvalue	Individual (%)	Cumulative (%)
Axis 1	4.15	22.38	22.38
Axis 2	2.81	15.18	37.56
Axis 3	2.13	11.52	49.08
Axis 4	1.88	10.16	59.24
Axis 5	1.66	8.96	68.19
Axis 6	1.51	8.17	76.36
Axis 7	1.37	7.36	83.75
Axis 8	1.22	6.59	90.34
Axis 9	1.11	6.04	96.38

millet which is distantly separated from the other *D. iburua* genotypes in group B.

DISCUSSION

Since amplification of DNA fragment by PCR depends on quality of the gDNA among other factors. A good DNA has ratio of OD_{260}/OD_{280} between 1.8 and 2.0, values below and above this range signify protein and RNA contaminations, respectively. Meanwhile, the OD values obtained (1.5-1.72) in this study were good enough for amplification. To corroborate this, Scotti *et al.* (1999) remarked that DNA with low OD values could give a good PCR product, however, this statement is relative and did not distinctively elucidate the degree of impurity the technique could accommodate. In addition, DNA with OD values less than 1.5 was successfully amplified for SSR genetic analysis in accessions of yam (Otoo *et al.*, 2009).

Inter- and intra-specific diversity assessment of germplasm is useful in conservation of genetic resources and prediction of combining ability of breeding materials (Animasaun *et al.*, 2015). Fragment amplification by the ISSR primers revealed inter- and intra-genetic diversity among the genotypes of *Digitaria exilis* and *D. iburua* in Nigeria. This implies that the markers are effective for the genotypes partitioning and identification. The genotypes showed considerable heterologous amplification of the alleles, where about 46% were monomorphic and over 53% were polymorphic. This indicated that the ISSR markers are effective in revealing genetic variations in the genotypes. This is in agreement with earlier studies which demonstrated that microsatellite markers are effective in genotyping and fingerprinting of plant accessions (Ajibade *et al.*, 2000; Animasaun *et al.*, 2015).

A high number of alleles and polymorphism are important for correct estimation of genetic diversity of a germplasm. Since, microsatellite profiles are commonly interpreted in terms of

allele phenotype, the degree of polymorphism correlates to diversity and effectiveness of the markers (Pfeiffer *et al.*, 2011). Although a few of the markers had low allelic frequencies, low number of loci amplification and percentage polymorphism, most of the primers produced higher polymorphism. Significantly low numbers of alleles and gene diversity was recorded in cultivated pearl millet accessions compared with wild accessions using SSR markers (Oumar *et al.*, 2008). However, Azevedo *et al.* (2012) reported high loci amplification and polymorphism in napier grass and millet accessions using microsatellite markers. The present study conform to latter findings as eight (60%) of the primers produced polymorphism above 50%.

The result of cluster analysis revealed that although, *D. exilis* are genetically dissimilar from the *D. iburua*, some degree of intra-specific diversity also existed. The genetic variation in these two species was also earlier reported (Hilu *et al.*, 1997; Adoukonou-Safgadja, 2010). Although authors summations were based on morphological and agronomical studies, the pattern of cluster obtained in this study further support existence of genetic variability among the genotypes. Separation of the genotypes into two major group at genetic similarity index of 0.66, revealed that the genotypes were of two classes, (i) a class of homogenous species (*D. exilis*) and (ii) the other consisting of heterogeneous members (mixture of *exilis* and *iburua*). The genetic relationship that exists between Agyong, NGB00629 and Ndai, which are all *exilis* genotypes, but are clustered with the *iburua* suggested that they are genetically linked. More so, the species are members of the same genus, hence some degree of similarity would be expected, which they may have evolved from a common progenitor. The pattern of cluster in the present study concurred with genetic analysis of the Togo fonio accessions using random amplified polymorphic DNA (Hilu, 1995).

CONCLUSION

Accurate assessment of genetic diversity is important in crop breeding and adequate genetic information would identify potential parental combinations to create segregating progenies with maximum genetic variability for further selection. Findings from the present study showed that ISSR markers are valuable asset for estimating genetic diversity. The polymorphism obtained indicate remarkable inter- and intra-specific variation among the available genotypes of acha in Nigeria. Occurrence of *D. exilis* and *D. iburua* together in a cluster revealed their relatedness and suggesting a common ancestor. It is, however, imperative that more genotypes and wild relatives from diverse sources be evaluated for diversity and phylogeny to provide broader gene pool from where selection can be made for a breeding programme to improve the crop

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