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RFLP- Based Gel Electrophoregram of DNA Primers for Acha (Fonio) Characterisation From Nigeria

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Abstract

The evaluation of the molecular diversity of Acha (*Digitaria sp.*) was carried out using Microsatelite primer combinations specially designed for*Digitaria exilis.* The amplifiedmicrosatellite fragments were also used in the other species of *Digitaria* namely; *Digitaria barbinodis* and *Digitaria iburua.*DNA extraction was carried and Agarose Gel Electrophoresis was conducted on the restricted amplified DNA extracts using microsatellite primers developed for *Digitaria exilis*. RFLPs clearly suggest a distinct separation of the three species of Acha at the molecular level (*D. iburua, D. exilis* and *D. barbinodis)* demonstrating the extent of their genetic differences at the DNA level.

Keywords: *Gel, electrophoresis, RFLP, Acha*

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Introduction

Acha or Fonio (*Digitaria exilis* Kippis Stapf, and *D. iburua* Kippis Stapf a very important neglected but underutilized crop in West and Central Africa resemble each other in some remarkable ways, as reported by Porteres (1976), and Haq and Ogbe, (1995). However, *D. iburua* appears to have taller plant height, wider stem girth, wider leaf width, longer leaf length, more number of days to maturity and larger seed size (Kwon-Ndung, et al., 2001; Dachi and Gana, 2012).

Kuta et al., (1996) have elucidated the great prospects of the use of RAPD techniques in breeding of Fonio. DNA polymorphisms are important markers in genetic analyses and are increasingly detected by using genome resequencing (Qi et al., 2006). The DNA polymorphisms are ubiquitous genetic variations among individuals and include single nucleotide polymorphisms (SNPs), insertions and deletions (indels), and other larger rearrangements (Feuk et al., 2006; Sharp et al., 2006; Mitchell-Olds and Schmitt, 2006). They can have phenotypic consequences and also serve as molecular markers for genetic analyses, facilitating linkage and association studies of genetic diseases, and other traits in humans (Stankiewicz and Lupski, 2002; Hurles et al., 2008; Stankiewicz and Lupski, 2010), animals, plants, (Johanson et al., 2000, Michaels et al., 2003; Koonneef et al., 2004; Krieger et al., 2010) and other organisms. Using DNA polymorphisms for modern genetic applications requires low-error and high-throughput analytical strategies. This is crucial in the subsequent strategies to improve the status of the genetic potential of the crop in Nigeria.

Materials and Methods

One gram (1g) of seeds from the different accessions was germinated in petri-dishes overlaid beneath with filter paper. These were kept moist by the addition of water during the period of germination and seedling development. These seedlings were harvested whole after 10 days and used for the DNA extraction.

DNA extraction was carried out using the protocol described by Yin *et al*. (2011) at the biotechnology laboratory of the National Veterinary Research Institute in Vom, Plateau state. The following solutions and reagents were used in this process:

a. Extraction buffer: 2% CTAB; 100mM Tris-HCL, pH 8.0; 1.5M NaCl; 2% polyvinylpyrrolidone (PVP)-40; 20mM ethylene diamine tetraacetic acid (EDTA), pH 8.0; 1% β-mercaptoethanol. 50µg/ml proteinase K was added immediately before use. Others included:
b. 8M Lithium Chloride (LiCl),
c. Chloroform,
d. 75% Ethanol,
e. 100% Ethanol,

f. RNase-free water.

Freshly harvested seedlings of the Acha were crushed in a mortar with a pestle until a very soft paste was made. This was to enable the disruption of cells from the plant tissue. 100mg of the sample paste was transferred into 10ml centrifuge tubes. To each sample 5ml of extraction buffer was added, and mixed thoroughly by vortexing and incubated at 65^0C for 20 minutes with occasional swirling. Samples were cooled to room temperature and then, 3ml of Chloroform was added to each tube. This was then vortexed and incubated on ice for 5 minutes. The mixture was then centrifuged at 8000 rpm for 20 min at 4^0C to pellet the cellular debris, protein, and polysaccharides. The supernatant was transferred to a new tube. 1.5ml of 8M Lithium Chloride was added to each tube and gently mixed and incubated at -80^0C for 2 hours. These were again centrifuged at 8000 rpm at 4^0C for 20 minutes. The solution was decanted and the pellet was washed with 75% ethanol and centrifuged at 10000 rpm at 4^0C for 10 min. The pellet was air dried for 10 min before dissolving in 100µl RNase free water.

A 1% agarose gel was prepared. 1µl of 6X gel loading dye was added to 2µl of each DNA sample before loading the wells of the gel. The dye was added to allow for the monitoring of the extent to which the samples would have migrated during electrophoresis, so that it could be halted at the appropriate stage. One well was loaded with uncut, good quality λDNA as molecular weight standards. The submarine electrophoretic gel was run at 100V for 30 minutes till the dye had migrated one-third of the distance in the gel. The extracts were checked for DNA on a 1% agarose gel before storing at -20^0C until needed. The DNA was then visualized using a BIO-RAD Gel Doc™ XR+ molecular imager.

The reagent mix and cycling program was also adopted from Barnaud *et al*. (2012) with some slight modifications. The Cycling Program was as follows:

Initial denaturation	94^0C for 5 min	
Denaturation	94^0C for 30 sec	
Annealing	58^0C for 90 sec	35 cycles
Extension	72^0C for 90 sec	
Final extension	72^0C for 10 min	

The amplification was carried out on a GeneAmp® PCR system 9700 (Applied Biosystems). The PCR products were electrophoresed on a 2% agarose gel stained with ethidium bromide using a SIGMA PS 2000-2 machine and the gel viewed in a BIO-RAD Gel Doc™ XR+ molecular imager. The gel was run at 100 V for 30 minutes.

Ten microsatellite primers developed by Barnaud *et al*. (2012) were synthesized and used for amplification on the DNA extracts. These markers had been developed for *Digitaria exilis*. The primers used are shown in Table 1:

Table 1. *List of selected specific microsatellite primers used in the work*

Locus	Forward primers (5′ – 3′)	Reverse primers (5′ – 3′)	Size (bp)
De-01	CTAACTCCTTCTCCCTCACC	TGGCACTGACACAGTAAC	257
De-04	CATTTTCCCGAAGACAGAGG	GACCTTGTGGCACCCATC	258
De-06	AGGAATGGCCTCAATACAT	AGAAAGCAGTTGGATTGGT	207
De-08	TTGGTGGATATTGGAATTATG	TTTACCCAACGCATAGGTAG	206
De-10	TCTTTTGTTTCTGGGATG	ACTTGAGACCTGCAAAGA	203
De-14	CGAGACCTGATTTGTTTAGC	CAAGTCTTTGATTTCCGTCT	199
De-17	GTAACGAACATCGGGTGA	CTGATGGCAAGGATGTGT	201
De-19	CATCTTCGAGGTTCTTGGT	AGCAGTGATTCGGTAGGAC	164
De-22	ATCGAGAGTTCAGTGAGTCC	GATCATCAAACCATTTACCC	193
De-26	AATACATTTTCCCCTTCGTC	GGATCTCGTTCATGTGCTAT	181

Results and Discussion

Plates 1 to 10 show the electrophoregram of the selected accessions' genomic DNA. The accessions genomic DNA were loaded as follows: GeneRuler 100 bp DNA Ladder Lane 1, P1 (*D. iburua*) Lane 2, P4 (*D. barbinodis*) Lane 3, P6 (*D. exilis*) Lane 4, P15 (*D. exilis*) Lane 5, P24 (*D. iburua*) Lane 6, P27 (*D. exilis*) Lane 7, K1 (*D. exilis*) Lane 8, K2 (*D. exilis*) Lane 9, K4 (*D. iburua*) Lane 10, T3 (*D. exilis*) Lane 11, and T4 (*D. exilis*) Lane 12 (Table 2).

Table 2. *List of Selected Accessions Used for the molecular analyses*

GEL WELLS	Accession Code	Source	Species
LANE 2	P1	PLATEAU	*Digitaria iburua*
LANE 3	P4	PLATEAU	*Digitaria barbinodis*
LANE 4	P6	PLATEAU	*Digitaria exilis*
LANE 5	P15	PLATEAU	*Digitaria exilis*
LANE 6	P24	PLATEAU	*Digitaria iburua*
LANE 7	P27	PLATEAU	*Digitaria exilis*
LANE 8	K1	KADUNA	*Digitaria exilis*
LANE 9	K2	KADUNA	*Digitaria exilis*
LANE 10	K4	KADUNA	*Digitaria iburua*
LANE 11	T3	BAUCHI	*Digitaria exilis*
LANE 12	T4	BAUCHI	*Digitaria exilis*

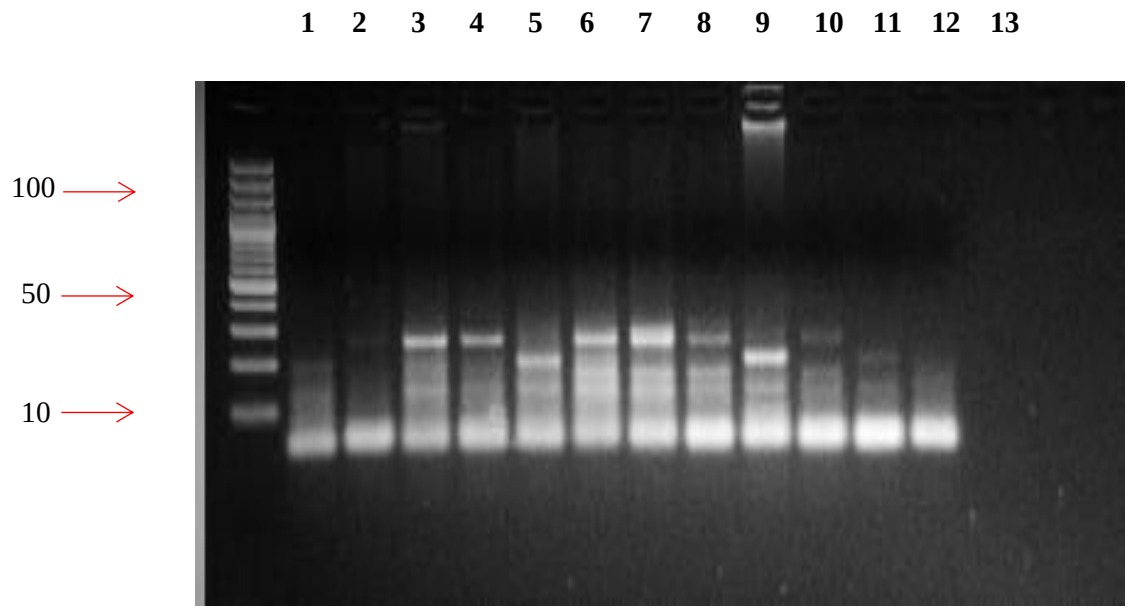

Plate 1. Gel electrophoresis picture of primer De 01 on the 11 accessions

Lane 1: 100 bp marker; Lane 2: P1(*Digitaria iburua*); Lane 3 P4 (*Digitaria barbnodis*); Lane 4 P6 (*Digitaria exilis*); Lane 5 P15 (*Digitaria exilis*); Lane 6: P 24 (*Digitaria iburua*); Lane 7: P27 (*Digitaria exilis*); Lane 8: K1(*Digitaria exilis*); Lane 9: K2 (*Digitaria exilis*); Lane 10: K4 (*Digitaria iburua*); Lane 11: T3 (*Digitaria exilis*); Lane 12: T4 (*Digitaria exilis*); Lane 13: negative control (using nuclease free water)

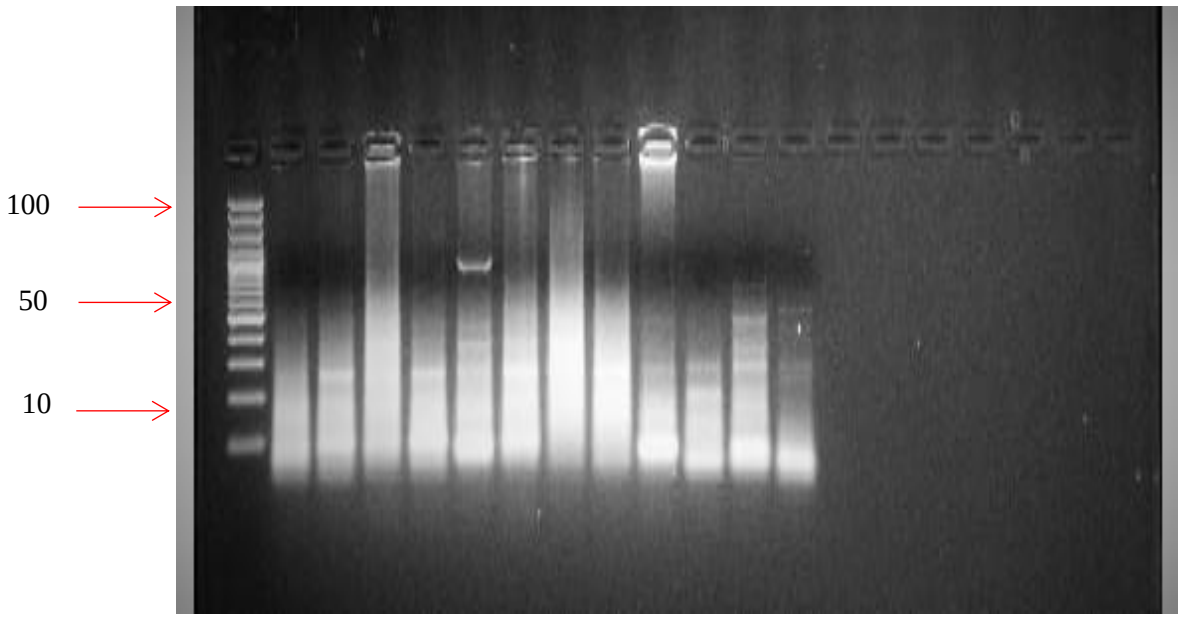

Plate 2: Gel electrophoresis picture of primer De 04 on the 11 accessions.

Lane 1: 100 bp marker; Lane 2: P1(*Digitaria iburua*); Lane 3 P4 (*Digitaria barbnodis*); Lane 4 P6 (*Digitaria exilis*); Lane 5 P15 (*Digitaria exilis*); Lane 6: P 24 (*Digitaria iburua*); Lane 7: P27 (*Digitaria exilis*); Lane 8: K1(*Digitaria exilis*); Lane 9: K2 (*Digitaria exilis*); Lane 10: K4 (*Digitaria iburua*); Lane 11: T3 (*Digitaria exilis*); Lane 12: T4 (*Digitaria exilis*)

The pattern observed from Plate 1, primer De 01, showed that accession P1 in Lane 2 (*D. iburua*), accession P4 Lane 3 (*D. barbinodis*) and accession T4 Lane 12 (*D. exilis*) did not amplify using the primer. The samples, based on their morphology were identified as *Digitaria iburua* Lane 1 *D barbinodis* Lane 2 and *D. exilis* Lane 11. Lanes 3 to 10 were however, amplified. The 257 bp fragment was obtained. The bands in Lanes 5 P24 (*D. iburua*) and 10 were lesser than the others amplified. This was to be expected as the primers were designed to amplify microsatellite fragments in *D. exilis*. Even though Lane 11 is *D. exilis*, there was no evidence of amplification.

For De 04 primer of Plate 2, Lane 2 was not amplified while Lanes 3 to 12 were amplified. For lane 6, P 24 (*D. iburua*) another band of approximately 1Kb size was observed suggesting a slight genetic variation in the accessions.

For primer De 06 in Plate 3, Lanes 2 (*D. iburua*), 6 P24 (*D. iburua*), and 10 were not amplified. This suggests that they do not have the target regions for the primer to amplify. Lanes 3 P4 (*D. barbinodis*), 4 P6 (*D. exilis*), 5 P15 (*D. exilis*), 7 P27 (*D. exilis*), 8 K1 (*D. exilis*), 9 K2 (*D. exilis*), and 11 T 3 (*D. exilis*) were all amplified. Again, even though lane 3 was (*D. barbinodis*), it nevertheless was amplified. Interestingly, lanes 3 and 9 had double bands. This suggests that the 'gene' is double copy (single copy genes amplify single bands while multicopy gene amplify more than one band) because the target area/gene on the genome is not one. Lane 6 P24 (*D. iburua*) has a band with an approximate weight of 850Kbp.

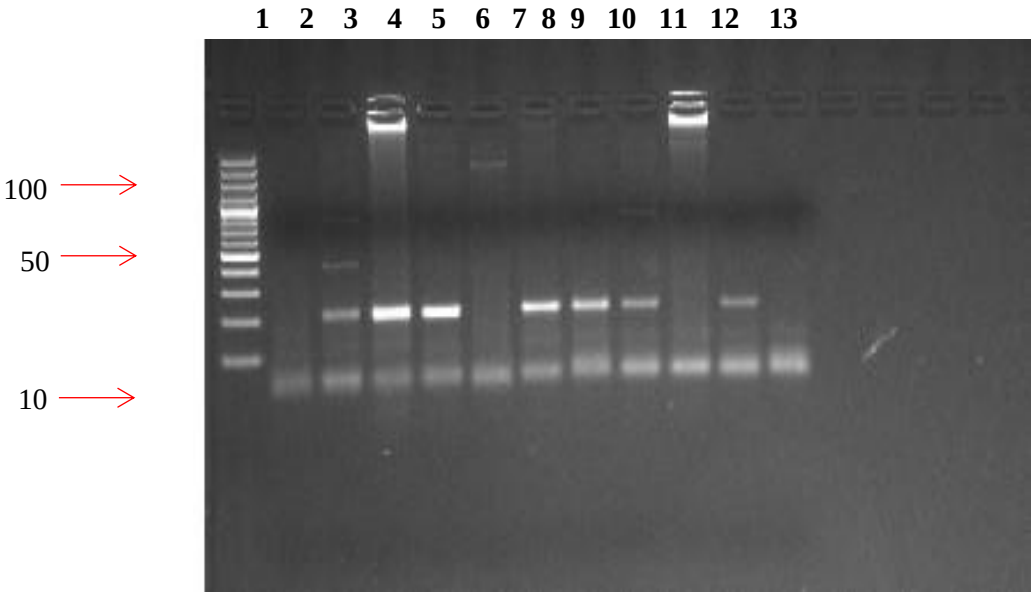

Plate 3. Gel electrophoregram of primer De 06 on the 11 accessions

Lane 1: 100 bp marker; Lane 2: P1(*Digitaria iburua*); Lane 3 P4 (*Digitaria barbnodis*); Lane 4 P6 (*Digitaria exilis*); Lane 5 P15 (*Digitaria exilis*); Lane 6: P 24 (*Digitaria iburua*); Lane 7: P27 (*Digitaria exilis*); Lane 8: K1(*Digitaria exilis*); Lane 9: K2 (*Digitaria exilis*); Lane 10: K4 (*Digitaria iburua*); Lane 11: T3 (*Digitaria exilis*); Lane 12: T4 (*Digitaria exilis*)

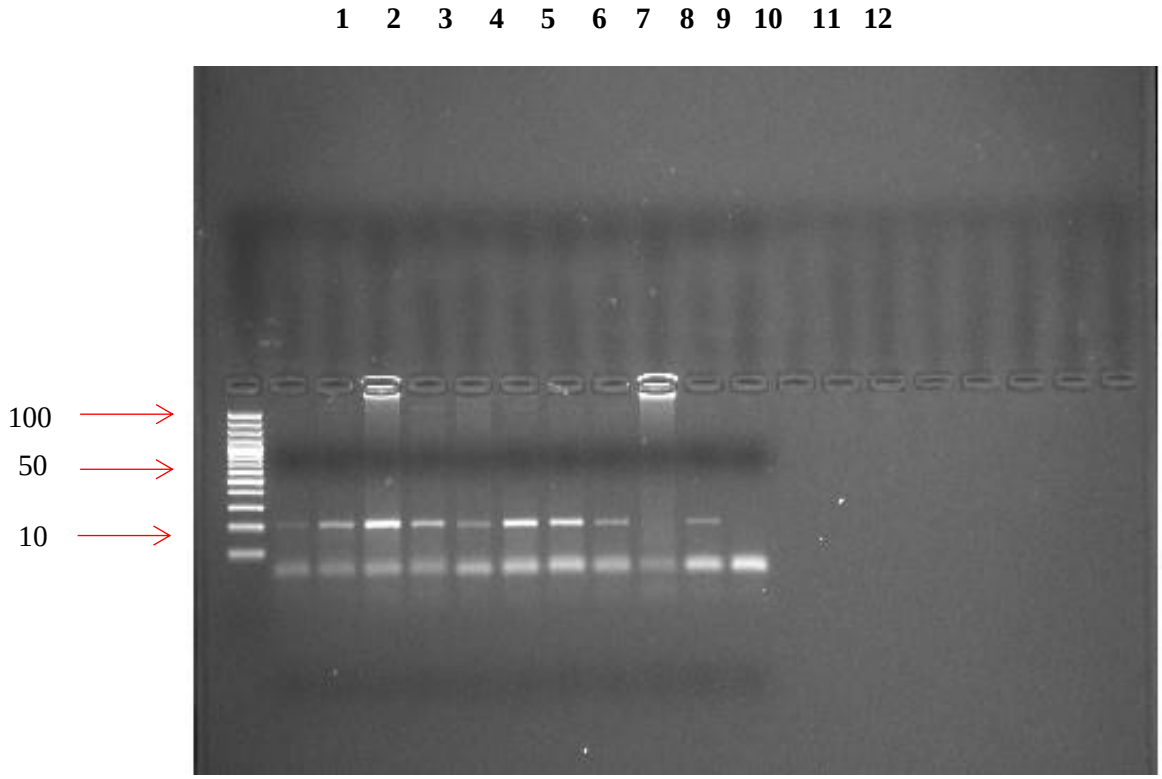

Plate 4: Gel electrophoresis picture of primers De 08 on the 11 accessions
Lane 1: 100 bp marker; Lane 2: P1(*Digitaria iburua*); Lane 3 P4 (*Digitaria barbnodis*); Lane 4 P6 (*Digitaria exilis*); Lane 5 P15 (*Digitaria exilis*); Lane 6: P 24 (*Digitaria iburua*); Lane 7: P27 (*Digitaria exilis*); Lane 8: K1(*Digitaria exilis*); Lane 9: K2 (*Digitaria exilis*); Lane 10: K4 (*Digitaria iburua*); Lane 11: T3 (*Digitaria exilis*); Lane 12: T4 (*Digitaria exilis*)

For De 08 in Plate 4, all lanes had similar band sizes across the accessions except for lanes 10 K4 (*D. iburua*) and 12 T4 (*D. exilis*). This suggests that lanes 2 to 9 and 11 had the target area while lanes 10 and 12 did not. This primer amplified Lane 2, (*D. iburua*) and Lane 3 (*D. barbinodis*) even though it was designed to amplify sections in *D. exilis*.For De 10 primer in Plate 5, Lanes 2 P1 (*D. iburua*), 6 P24 (*D. iburua*) and 9 K2 (*D. exilis*) did not amplify. Very faint bands were observed for Lanes 3 P4 (*D. barbinodis*), 4 P6 (*D. exilis*), 5 P15 (*D. exilis*), 7 P27 (*D. exilis*) and 8 K1 (*D. exilis*) and then 10 K4 (*D. iburua*) and 11 T3 (*D. exilis*) at a 100bp level.

In De 14 in Plate 6, no definitive fragments were observed in all the lanes, even though lanes 5 P15 (*D. exilis*) and 6 P24 (*D. iburua*) were observed to have very faint bands at the 100bp or even slightly lower. However, lanes 3 P4 (*D. barbinodis*), 4 P6 (*D. exilis*), and 7 P27 (*D. exilis*) gave double bands of unequal sizes greater or equal to 1Kb.

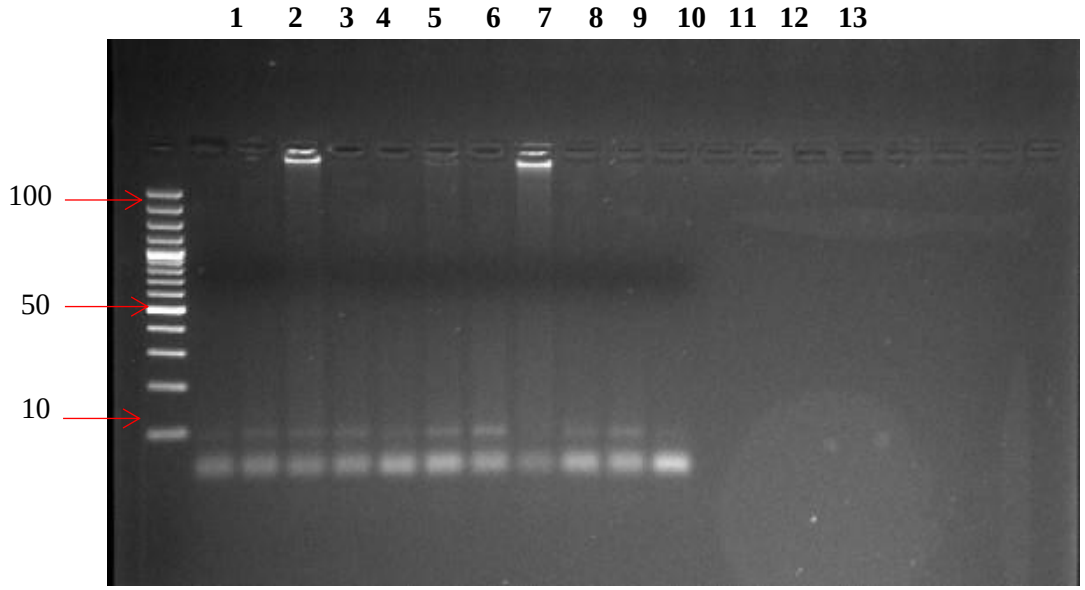

Plate 5. Gel electrophoregram of primer De 10 on the 11 accessions

Lane 1: 100 bp marker; Lane 2: P1(*Digitaria iburua*); Lane 3 P4 (*Digitaria barbnodis*); Lane 4 P6 (*Digitaria exilis*); Lane 5 P15 (*Digitaria exilis*); Lane 6: P 24 (*Digitaria iburua*); Lane 7: P27 (*Digitaria exilis*); Lane 8: K1(*Digitaria exilis*); Lane 9: K2 (*Digitaria exilis*); Lane 10: K4 (*Digitaria iburua*); Lane 11: T3 (*Digitaria exilis*); Lane 12: T4 (*Digitaria exilis*)

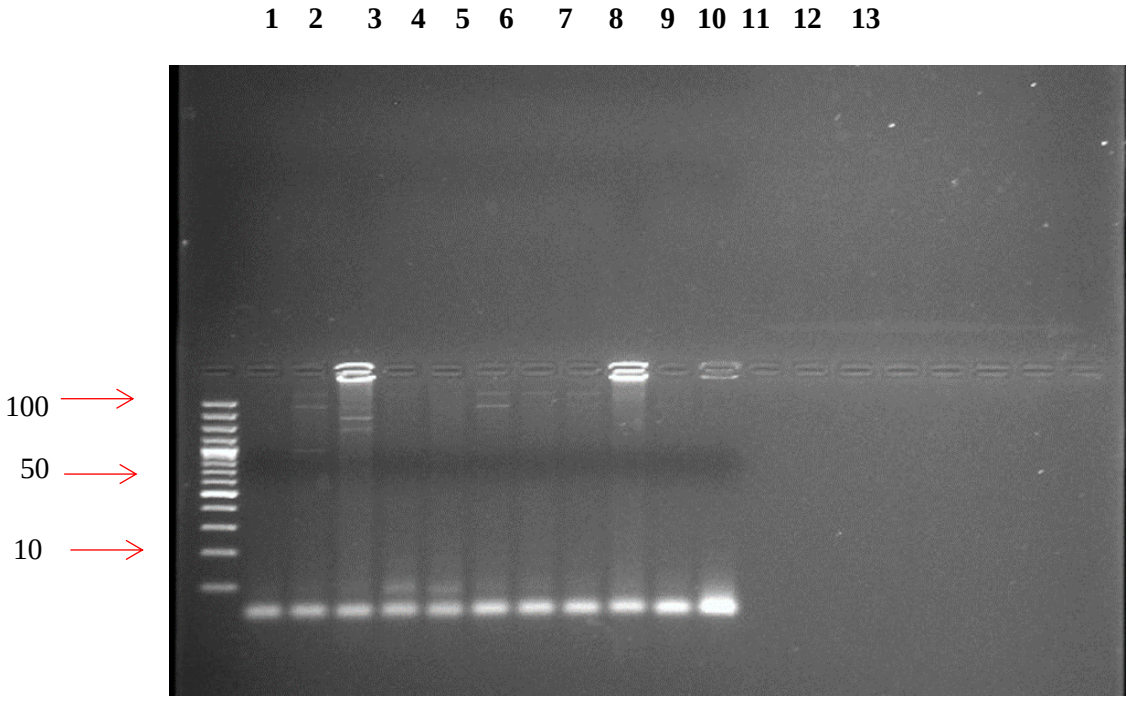

Plate 6. Gel electrophoregram of primers De 14 on the 11 plant extracts

Lane 1: 100 bp marker; Lane 2: P1(*Digitaria iburua*); Lane 3 P4 (*Digitaria barbnodis*); Lane 4 P6 (*Digitaria exilis*); Lane 5 P15 (*Digitaria exilis*); Lane 6: P 24 (*Digitaria iburua*); Lane 7: P27 (*Digitaria exilis*); Lane 8: K1(*Digitaria exilis*); Lane 9: K2 (*Digitaria exilis*); Lane 10: K4 (*Digitaria iburua*); Lane 11: T3 (*Digitaria exilis*); Lane 12: T4 (*Digitaria exilis*)

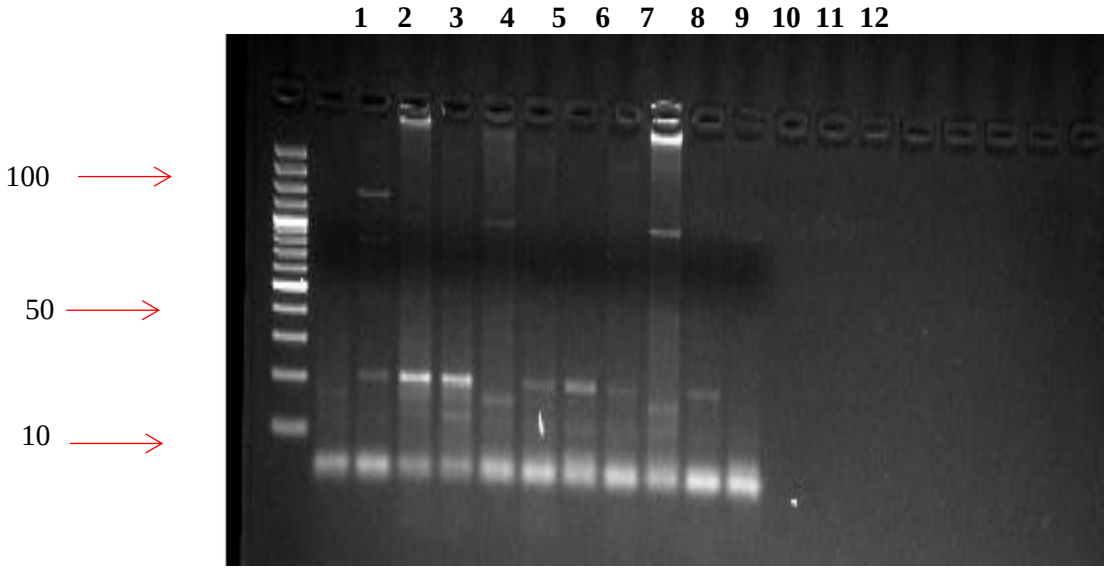

Plate 7. Gel electrophoregram of primer De 17 on the 11 plant extracts

Lane 1: 100 bp marker; Lane 2: P1(*Digitaria iburua*); Lane 3 P4 (*Digitaria barbnodis*); Lane 4 P6 (*Digitaria exilis*); Lane 5 P15 (*Digitaria exilis*); Lane 6: P 24 (*Digitaria iburua*); Lane 7: P27 (*Digitaria exilis*); Lane 8: K1(*Digitaria exilis*); Lane 9: K2 (*Digitaria exilis*); Lane 10: K4 (*Digitaria iburua*); Lane 11: T3 (*Digitaria exilis*); Lane 12: T4 (*Digitaria exilis*)

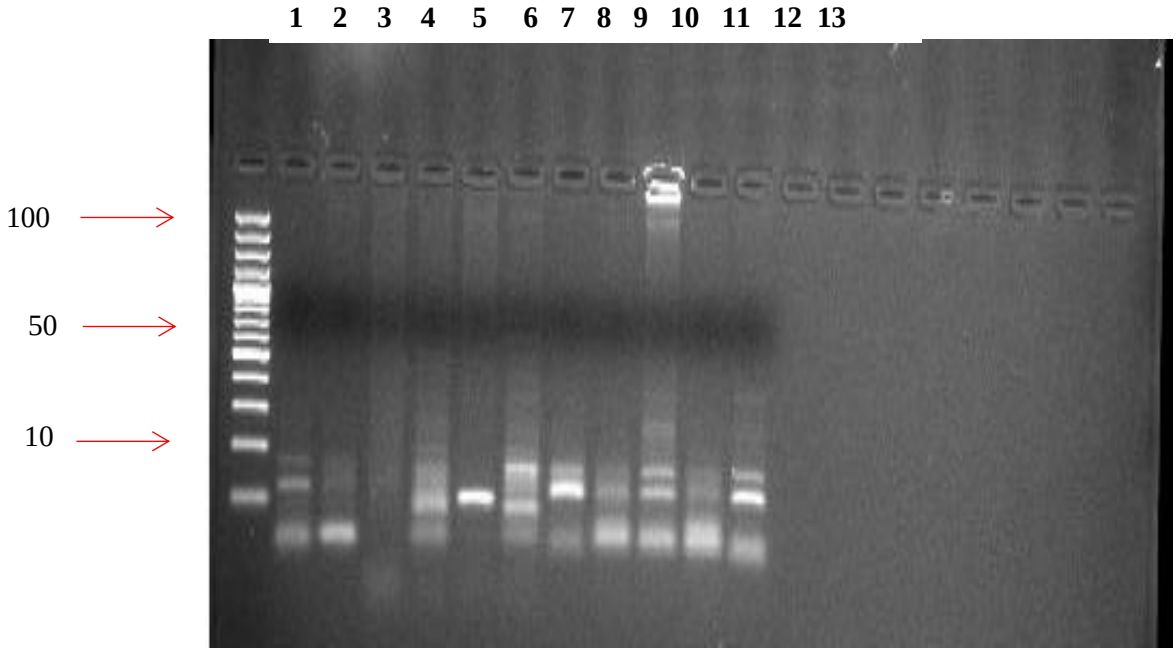

Plate 8. Gel electrophoregram of primer De 19 on the 11 accessions

Lane 1: 100 bp marker; Lane 2: P1(*Digitaria iburua*); Lane 3 P4 (*Digitaria barbnodis*); Lane 4 P6 (*Digitaria exilis*); Lane 5 P15 (*Digitaria exilis*); Lane 6: P 24 (*Digitaria iburua*); Lane 7: P27 (*Digitaria exilis*); Lane 8: K1(*Digitaria exilis*); Lane 9: K2 (*Digitaria exilis*); Lane 10: K4 (*Digitaria iburua*); Lane 11: T3 (*Digitaria exilis*); Lane 12: T4 (*Digitaria exilis*)

De 17 primer in Plate 7 amplified all the lanes, with the exception of lane 2 P1 (*D. iburua*) and 12, suggesting that the target region is found in all the accessions studied. Lanes 3 P4 (*D. barbinodis*), 6 P24 (*D. iburua*), and 10 were slightly different from the rest because they had more than once of the target region which was evident from the number of band sizes. A 201 bp and greater than 1 Kb were observed.

Primer De19 in Plate 8 amplified all lanes except lane 4. Lanes 2 and 12 which were not amplified with De 01 to De 18 were amplified by this primer. Double bands were observed for lanes 2 P1 (*D. iburua*), 5 P15 (*D. exilis*), 7 P27 (*D. exilis*), 8 K1 (*D. exilis*), 9 K2 (*D. exilis*), 10 K4 (*D. iburua*), 11 T3 (*D. exilis*) and 12 T4 (*D. exilis*), even though lanes 9 and 11 were not so definitive.

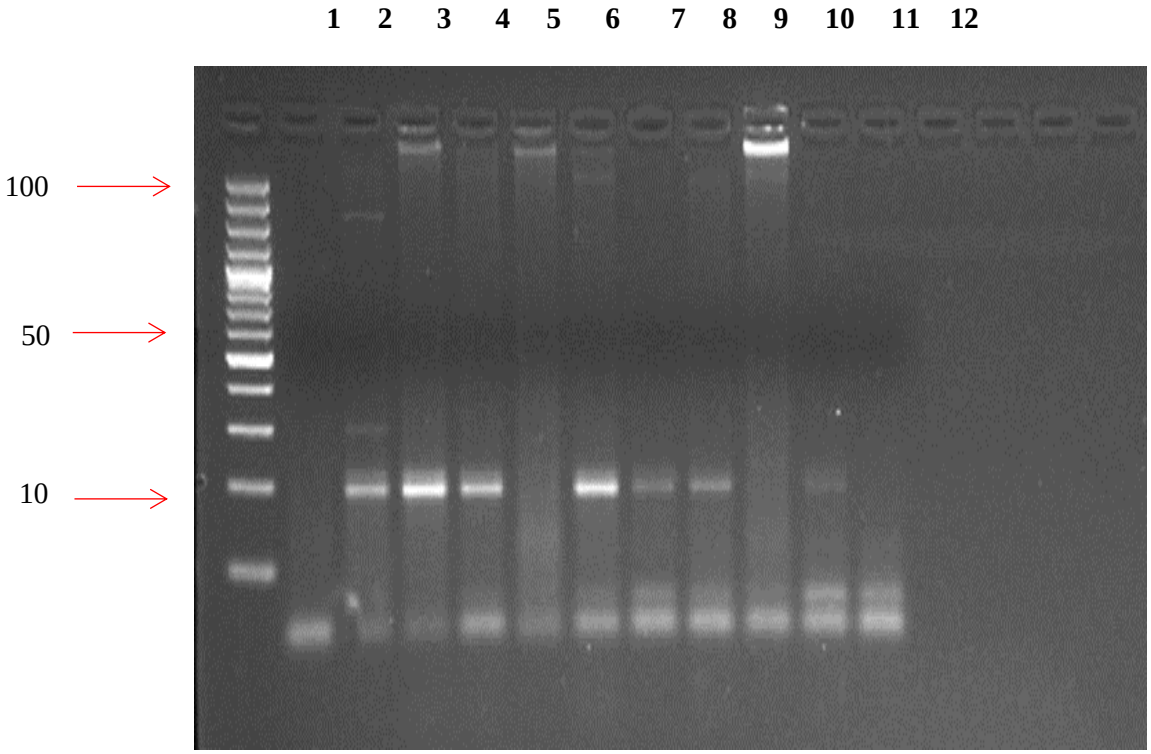

Plate 9. Gel electrophoregram of primers De 22 on the 11plant accessions
Lane 1: 100 bp marker; Lane 2: P1(*Digitaria iburua*); Lane 3 P4 (*Digitaria barbnodis*); Lane 4 P6 (*Digitaria exilis*); Lane 5 P15 (*Digitaria exilis*); Lane 6: P 24 (*Digitaria iburua*); Lane 7: P27 (*Digitaria exilis*); Lane 8: K1(*Digitaria exilis*); Lane 9: K2 (*Digitaria exilis*); Lane 10: K4 (*Digitaria iburua*); Lane 11: T3 (*Digitaria exilis*); Lane 12: T4 (*Digitaria exilis*)

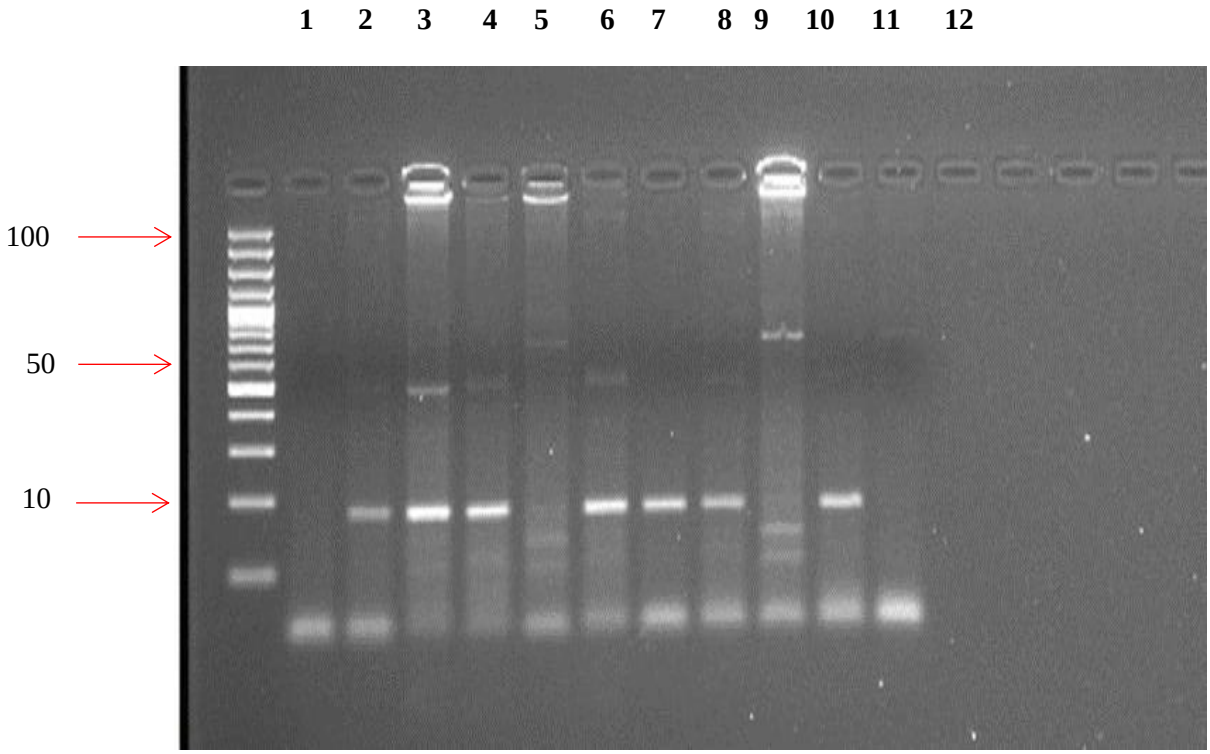

Plate 10: Gel electrophoregram of primer De 26 on the 11accessions
Lane 1: 100 bp marker; Lane 2: P1(*Digitaria iburua*); Lane 3 P4 (*Digitaria barbnodis*); Lane 4 P6 (*Digitaria exilis*); Lane 5 P15 (*Digitaria exilis*); Lane 6: P 24 (*Digitaria iburua*); Lane 7: P27 (*Digitaria exilis*); Lane 8: K1(*Digitaria exilis*); Lane 9: K2 (*Digitaria exilis*); Lane 10: K4 (*Digitaria iburua*); Lane 11: T3 (*Digitaria exilis*); Lane 12: T4 (*Digitaria exilis*)

For primer De 22 in Plate 9, lanes 2 P1 (*D. iburua*), 6 P24 (*D. iburua*), and K4 (*D. iburua*) were not amplified. Lanes 3 P4 (*D. barbinodis*), 4 P6 (*D. exilis*), 5 P15 (*D. exilis*), 7 P27 (*D. exilis*), 8 K1 (*D. exilis*), 9 K2 (*D. exilis*), T3 (*D. exilis*), and T 4 (*D. exilis*) were all amplified, with double bands observed in lanes 3 P4 (*D. barbinodis*), 8 K1 (*D. exilis*), 9 K2 (*D. exilis*), and 11 T3 (*D. exilis*).

For primer De 26 in Plate 10, amplification was observed in lanes 3 P4 (*D. barbinodis*), 4 P6 (*D. exilis*), 5 P15 (*D. exilis*), 7 P27 (*D. exilis*), 8 K1 (*D. exilis*), 9 K2 (*D. exilis*), 10 K4 (*D. iburua*) and 11 T 3 (*D. exilis*), with double bands in 4 P6 (*D. exilis*), 7, and 10. This primer failed to amplify in lanes 2 P1 (*D. iburua*), Lane 6 P24 (*D. iburua*), and Lane 12 T4 (*D. exilis*).

The pattern observed from the Plates above showed that samples P1, P24, K4 and T4 did not amplify using the primers available. The samples, based on their morphology were identified as *Digitaria iburua* with the exception of accession T4 which was identified as *D. exilis.* This is not unusual as P1, P24 and K4 are *D. iburua* and the primers were designed to amplify microsatellite fragments in *D. exilis.*

The total amplified fragment length polymorphs of the accessions ranged between 6 and 16, with accession P4 (*D. barbinodis*) and accession P15 (*D. exilis*) from Plateau State, having the highest number of polymorphs while accession P1 (*D. iburua*) also from Plateau State and B4 (*D. exilis*) from Bauchi state, having the least number of polymorphs. Other accessions found between these extremes included B3 (*D. exilis*) and K4 (*D. iburua*), K2 (*D. exilis*) and P6 (*D. exilis*), K1 (*D. exilis*) and P24 (*D. iburua*) and P27 (*D. exilis*) with the corresponding number of polymorphs as 11, 12, 13, and 14 respectively.

Out of the ten (10) primers used for the RFLP and microsatellite PCR of the Acha genomic DNA, Primer De 01with molecular weight of 257 base pairs (bp) appeared to express the highest percentage polymorphism of 16 (Table 2). This was followed by De 19 and De 26 with molecular weights of 164 and 181 base pairs respectively, and a percentage of 12.31. De 04 with a molecular weight of 258bp had 11.53%. De 17 and De 22 had 10% each while De 14 had 7.69. De 08 has a percentage polymorphism of 6.92 while De 06 and De 10 had the lowest percentage polymorphism of 6.15

The results of the number of restriction fragment length polymorphs (RFLP) with the estimated molecular weights (bp) are presented in Table 3.The 10 selected RFLP and microsatellite primer pairs yielded a total of 103 scorable bands whose molecular weights ranged in size between 100 and 400 bp. Even though no single primer amplified microsatellite sections in all the accessions, all the primers amplified microsatellite sections in P4 (*D. barbinodis*), P15 (*D. exilis*), and P27 (*D. exilis*) (Table 3). Primer De 01 amplified sections in all the accessions except for B4 (*D. exilis*). Primer De 04 amplified microsatellite sections in all the accessions tested except P1 (*D. iburua*), and K1 (*D. exilis*). Primer De 06 did not amplify any sections in P1, P24, K4 and B4, the first 3 being *D. iburua.* The same B4 which was identified as *D. exilis,* was also, not amplified by primers De 08, De 10, De 14, De 17 and De 26. These primers were expected to amplify fragments in *D. exilis.* Accession P6 (*D. exilis*), was also amplified except b primer De 19. Accession P24 (*D. iburua*), was not amplified by De 06 and De 22. Whereas accession K1 (*D. exilis*) was not amplified by primer De 04, it was amplified by all the other primers. Accession K2 (*D. exilis*) and accession K4 (*D. iburua*), were not amplified by Primers De 10 and De 14. Accessoion B3 (*D. exilis*) also not amplified by De 14 and De 17 (Table 3).

Variations exist in the number of polymorphic bands generated by each primer combination. All the RFLP primer combinations used were suitable to fingerprint the 11 accessions as most of the bands appeared to be present in all the accessions (Table 3).

Table 3. *Sequences of Primers Used for RFLP and Microsatellite PCR of Acha (Digitaria sp) genomic DNA and the Polymorphism Obtained*

S/N	Primers	Mol. Wt. /Size (bp)	Locus Fwd Primers 5′ - 3′	P1	P4	P6	P15	P24	P27	K1	K2	K4	B3	B4	Total
1	De 01	257	CTAACTCCTTCTCCCTCACC	2	1	2	2	2	3	3	3	2	2	0	22
2	De 04	258	CATTTTCCCGAAGACAGAGG	0	1	1	1	3	1	0	1	2	2	3	15
3	De 06	207	AGGAATGGCCTCAATACAT	0	2	1	1	0	1	1	1	0	1	0	8
4	De 08	206	TTGGTGGATATTGGAATTATG	1	1	1	1	1	1	1	1	0	1	0	9
5	De 10	203	TCTTTTGTTTCTGGGATG	0	1	1	1	1	1	1	0	1	1	0	8
6	De 14	199	CGAGACCTGATTTGTTTAGC	0	3	2	1	1	2	1	0	0	0	0	10
7	De 17	201	GTAACGAACATCGGGTGA	1	2	1	3	2	1	1	1	1	0	0	13
8	De 19	164	CATCTTCGAGGTTCTTGGT	2	1	0	1	1	2	2	2	2	1	2	16
9	De 22	193	ATCGAGAGTTCAGTGAGTCC	0	3	1	1	0	1	2	2	0	2	1	13
10	De 26	181	AATACATTTTCCCCTTCGTC	0	1	2	4	2	1	1	1	3	1	0	16
TOTAL				6	16	12	16	13	14	13	12	11	11	6	130
Expected Molecular Weight				100 – 200	100 - 400	150 - 300	150 – 300	150 – 200	180 – 300	180 – 310	180 – 300	180 – 220	250 – 300	190 - 240	

Conclusion

RFLPs appear to suggest a clear separation of the the 3 species (D. iburua, D. exilis and D. barbinodis) demonstrating their genetic differences at the molecular/DNA level. In this study, some of the RFLPs were shared by the 3 species, showing their relatedness. This level of shared genome may suggest some form of evolutionary separation between them. For instance, all the microsatellite primers for the D.exilis used in this study were amplified in D. barbinodis. This was followed by D. exilis andthe least amplification was in, D. iburua, suggesting that though the primers were developedfor D. exilis, it was more of a generic than of species problem.

Phenotypically, *D. iburua* shows divergent traits from *D. exilis* and *D. barbinodis*.RFLPs appear to suggest a clear separation of the the 3 species (*D. iburua, D. exilis* and *D. barbinodis)* demonstrating their genetic differences at the molecular/DNA level. This level of shared genome may suggest some form of evolutionary separation between them. For instance, all the microsatellite primers for the *D. exilis* used in this study were amplified in *D. barbinodis.* This was followed by *D. exilis* and the least amplification was in, *D. iburua*, suggesting that eventhough the primers were developed for *D. exilis*, it was more of a generic than of species problem/consideration.RFLPs appear to suggest a clear separation of the the 3 species (*D. iburua, D. exilis* and *D. barbinodis)* demonstrating the extent of their genetic differences at the molecular/DNA level.

Further work should consider sequencing of RFLP and data used to establish similarity and or dissimilarity among the accessions. Since gene mapping was not part of this study, it is suggested that mapping studies should be conducted to identify specific genes or genomic regions that have an influence on the phenotypic variations so far observed.

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