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RESEARCH ARTICLE

Elucidation of the cola genetic diversity by using inter-simple sequence repeat markers

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Abstract

Kola (*Cola spp*.) is a valuable tree crop with significant nutritional, medicinal, and industrial importance. This study employed Inter-Simple Sequence Repeat (ISSR) markers to assess the genetic diversity of 20 Nigerian kola accessions. The analysis revealed a high degree of polymorphism, with primers generating 5 to 17 alleles each. The average Polymorphic Information Content (PIC) was 0.749, indicating high marker informativeness, while the gene diversity was 0.767. A UPGMA dendrogram clustered the accessions into five distinct groups, with a similarly coefficient of 0.72 confirming substantial genetic variation. These results demonstrate that ISSR markers are highly effective for the genetic characterization of kola, revealing phylogenetic relationships and identifying variability that can be harnessed for cultivar development and crop improvement.

Keywords: Accessions, polymorphic, dendrogram, clusters, tef, ISSR

Introduction

Kola nuts (*Cola spp*.) are economically significant for their high caffeine content, which ranges from 1.84 to 2.56 in the food industry, particularly in the manufacturing of energy in the food industry, particularly in the manufacturing of energy drinks. Burdock *et al.*, 2009 and Adedayo *et al.*, 2019 suggested that Beyond their commercial use, kola nuts hold substantial value in traditional medicine for treating ailments such as asthma, whooping cough, migraines, and indigestion, and are also used as a fertility regulator(Esimone *et al.*, 2007). Their therapeutic potential is further recognized in the pharmaceutical industry, where they are investigated for drug development against cardiovascular diseases(Madingou *et al.*, 2012). Generally, kola nut contains protein, starch, phenol, niacin, and riboflavins which are good for ones health live healthy. Proximate results reported by Dewole *et al.*, (2017) showed that the moisture content of kola nutwere in respective range of 9.73 to 9.81%, ash 2.72 to 2.21%, fat 3.02 to 2.20%, protein 19.14 to 15.24%, crude fiber 7.30 to 4.18% and carbohydrate 58.09 to 66.45% (Nyamien *et al.*, 2014). This property makes them a key ingredient kola nut contains considerable quantity of glucose compared to other stimulants like cocoa and coffee, and is three times greater in starch than cocoa but with relatively little fat. Substantial quantity of starch and glucose of kola nut have been reported to boost physical energy and suppresses hunger (Decker, 2017). Kola nut also contains higher quantities of phenolic constituents than many fruits

which play an important role in determining colour and flavour and have an impact on metabolic processes. Other uses of kola nuts include; cosmetics and textiles (Jayeola and Akinwale, 2002). In addition, Kola nut plays an important role in African society for cultural and customary ceremonies such as births and weddings (Durand *et al.,* 2015). In spite of the health and socio- economic importance of Cola, sterility, self and cross incompatibilities, unpleasantly tall trees, low nut yield and longer gestation period are among the problems faced by growers of this tree crop. For its improvement and advancing its research attention, accurate genetic diversity study among the existing Cola germplasm in Nigeria is prerequisite to assist in selecting suitable parents for breeding programmes. A number of projects have been embarked upon, including the collection of Cola from different farmers field with no distinguishing features. These are maintained as field gene banks with the view to effectively incorporate them in breeding programmes. Molecular characterization, which highlights the amount of genetic diversity and relationship among various groups of different accessions, is required for a direct and more reliable selection and distinguishing of plant germpalsm accessions (Ramesh, 2017; Singh and Kumar, 2017). To assess the genetic diversity of the germplasm established, inter simple Sequence Repeat (ISSR) marker system was employed. There has been no report of usage ISSR marker system for Cola spp diversity studies despite the several advantages of the marker system that has seen it utilized for tropical crops like Cacao and Coffee. Inter simple Sequence Repeat (ISSR) offers simple, fast and efficient techniques for selection of suitable parent(s) for a successful Cola breeding programme.

Materials and methods

Twenty accessions of Cola spp in a new germplasm collection at the Headquarters of Cocoa Research Institute of Nigeria, Ibadan, Nigeria, were used in this study (Table 1). Fresh young leaf samples of each of the selected twenty accessions of Cola were harvested into well labeled sample bags and tightly closed. The samples were placed on ice pack and immediately conveyed to biotechnology laboratory for DNA extraction and genetic profiling.

Table 1: List of the accessions received from Cocoa Research Institute of Nigeria (CRIN)

Sr. No.	Accessions	Collection site	Sr. No.	Accessions	Collection site
1	CN1	Ibadan	11	CN11	Ibadan
2	CN2	Ibadan	12	CN12	Ibadan
3	CN3	Ibadan	13	CN13	Ibadan
4	CN4	Ibadan	14	CN14	Ibadan
5	CN5	Ibadan	15	CN15	Ibadan
6	CN6	Ibadan	16	CN16	Ibadan
7	CN7	Ibadan	17	CN17	Ibadan
8	CN8	Ibadan	18	CN18	Ibadan
9	CN9	Ibadan	19	CN19	Ibadan
10	CN10	Ibadan	20	CN20	Ibadan

DNA extraction

Collected leaf samples were prepared for DNA extraction by adding approximately 100 mg of silica gel dried tissues into an extraction tube. Two steel balls each were added into the tube to enable grinding. The dried tissue was ground into fine powder by vortexing at a high speed for 5 minutes. 750 µl of pre-heated plant extraction buffer was added. Tubes were incubated at 650 C for 20 mins, stirred occasionally by inverting the tubes to homogenize the sample. The tubes were removed from the incubator and allowed to cool for 2 mins. 200 µl of ice-cold 5 M Potassium acetate were added. The tubes were again incubated for 20 minutes to precipitate protein. 500 µl of chloroform Isoamylalcohol (24:1) was added and mixed gently to further precipitate protein and lipids. The tubes were then centrifuged at 10000 rpm for 10 mins and the supernatant was transferred into freshly labelled tubes. 2/3 volume of ice-cold Isopropanol was added, mixed gently and incubated in -80^{o} c for 15 mins to precipitate the DNA and then Centrifuged at 10000 rpm for 10 min to pellet the DNA. The supernatant was gently decanted until the last drop. 400 µl of 70% ethanol was added to wash the DNA pellet and centrifuged at 10000 rpm for 10 min. Supernatant was gently decanted until the last drop and the pellet air dried (until ethanol smell was no longer perceived). 60 ul of TE was added to re-suspend the DNA. 2 ul of RNase was added and it was incubated at 37oC for 30-40 minutes.

Confirmation of extracted DNA using agarose gel electrophoresis and PCR

Successful extraction of DNA from leaf tissue was determined through agarose gel electrophoresis. 1g of agarose powder was measured into 100 mL 1xTAE in a microwavable flat bottom flask. It was placed in the microwave for 3-5 min until the agarose until agarose powder completely dissolved (but did not over boil the solution, to prevent evaporation of the buffer, which would alter the final percentage of agarose in the gel). The agarose solution was allowed to cool down to about 60°C (about when one could comfortably keep your hold the flask with the hand), about 5 mins. 10 µL EZ vision DNA stain was added. EZ vision binds to the DNA and allows one to visualize the DNA under ultraviolet (UV) light. The agarose was poured into a gel tray with the well comb in place. The gel was allowed to remain at room temperature for 20-30 mins, until it has completely solidified. Loading buffer was added to each of the DNA samples. Once solidified, the agarose gel was placed into the gel box (electrophoresis unit). Gel box was filled with 1xTAE (or TBE) until the gel is submerged. The molecular weight ladder was loaded carefully into the first lane of the gel. The samples were carefully loaded into the additional wells of the gel. The gel was run at 80-150 V for about 1-1.5 hours. The power was turned off; the electrodes were disconnected from the power source, and the gel was carefully removed from the gel box. The gel was visualized under UV transilluminator. Presence of DNA band indicated presence of DNA. The PCR mix was made up of 12.5 µL of Taq 2X Master Mix from New England Biolabs (M0270); 1 µL each of 10 µM forward and reverse primer respectively; 2 µL of DNA template and then made up with 8.5µL Nuclease free water. PCR Protocol 5 mins, followed by 36 cycles of denaturation at 940c for 30 seconds anneling at 55oc for 30seconds and elongation at 72oc for 45seconds followed by a final elongation step at 72oc for 7 minuts and hold temperature at 10oc forever.

DNA scoring and analysis

PCR product was resolved using agarose gel electrophoresis as described above and visualized under UV light. Gel images were taken, and the alleles (DNA bands) were

scored in binary codes; clearly visible bands were assigned 1 for presence, 0 for absence and m for missing data. The percentage of polymorphism for each marker was calculated as the number of polymorphic bands over the total number of bands scored for the marker and multiplied by 100 (Martos et al., 2005). The Polymorphic

Information Content (PIC), a measure of variability for each locus was calculated across the assay of units by the formula of Roldan-Ruiz *et al.*, (2000): PICi= 2fi(1 - fi) where fi is the frequency of the amplified allele (band present), and (1 fi) is the frequency of the null allele (band absent) of marker i. To estimate the level of genetic diversity, genetic similarities were evaluated using Nei and Li/Dice similarity index (Nei and Li, 1979) with the aid of the NTSYSpc software, version 2.11 (Rolf, 1998). A dendrogram was generated from the similarity matrix using the UPGMA (Unweighted Pair Group Method of Analysis using arithmetic averages) in NTSYSpc program.

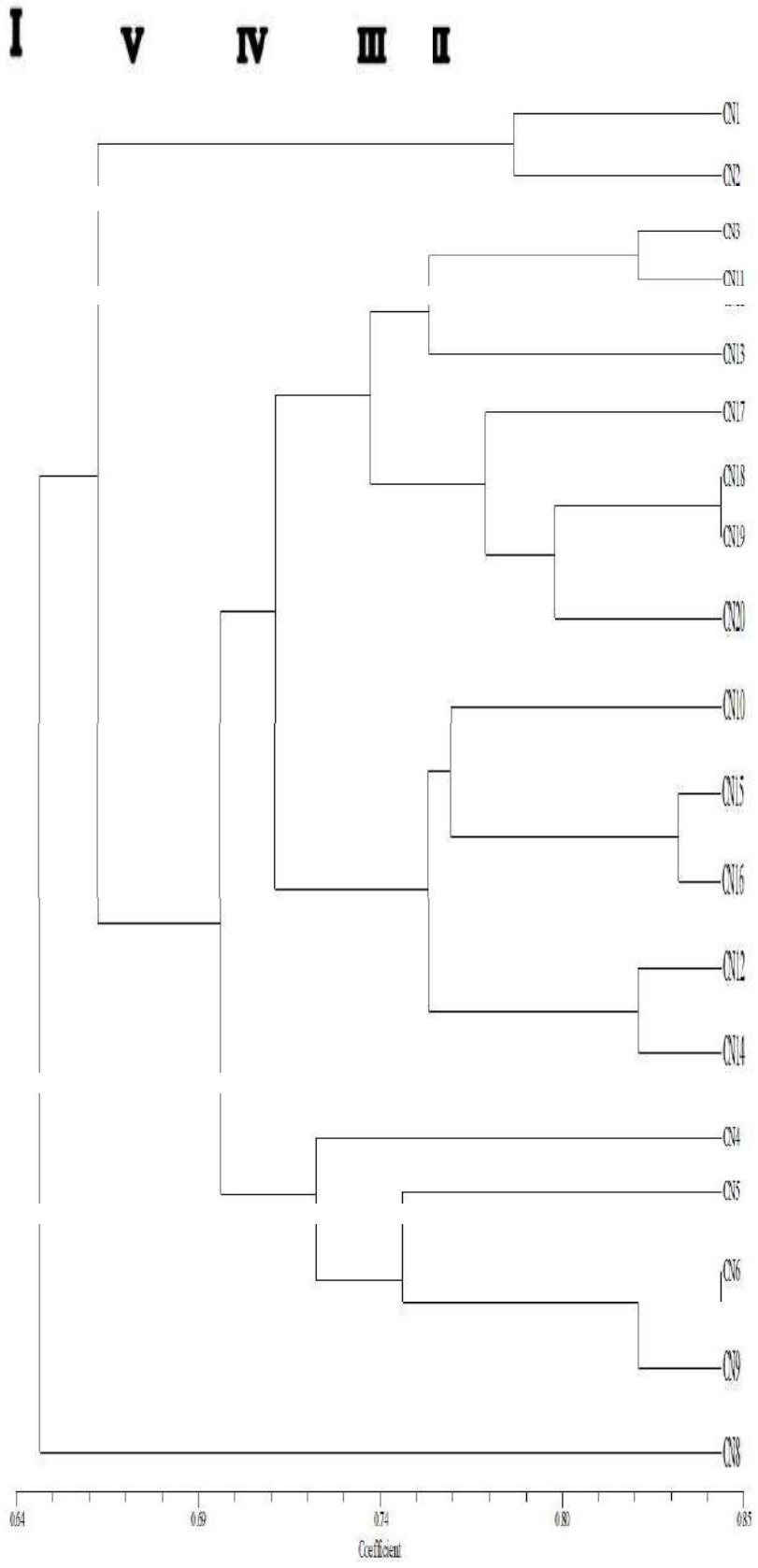
Results and discussion

Number of alleles, gene diversity, and polymorphic information content of Cola ISSR markers are presented on Table 2. The 10 ISSR markers exhibited extensive polymorphism among the Cola accessions. The major allele frequency ranged from 0.100 to 0.700, with a mean of 0.360. Genetic diversity of the primers used ranged from 0.490 to 0.935 with a mean of 0.767, where marker UBC811 revealed the highest genetic diversity. The allele number per ISSR marker ranged from 5 to 17, with a mean of 10. Polymorphism Information Content (PIC) for Cola varied from 0.470 (UBC888) to 0.931 (UBC811) with a mean of 0.749 (Table 2). Figure 1 is a dendrogram showing overall genetic dissimilarity (variation) among the 20 accessions of Cola as revealed based on ISSR markers. At the 100% level of dissimilarity, all the accessions were distinct from one another. All the accessions formed a single cluster at 0.64 level of dissimilarity, indicating some degree of similarities among them. Five distinct clusters were formed from the analysis of the pooled ISSR marker data at the similarity coefficient of 0.72. Cluster I had only one accession CN8, indicating that CN8 is the most distinct among all the accessions. Cluster II and III had five accessions each II (CN9, CN7, CN6, CN5, CN4) and III (CN14, CN12, CN16, CN15, CN10) while Cluster IV had the largest number of accessions 7 (CN20, CN19, CN18, CN17, CN13, CN11, CN3), Cluster V composed of two accessions CN2 and CN.

Table 2: Number of alleles, gene diversity, and polymorphic information content PIC of Cola ISSR markers

Markers	Major allele frequency	No of alleles	Gene diversity	PIC	Sequence 1- 5
UBC888	0.700	6	0.490	0.470	ACACACACACACA
UBC881	0.150	15	0.915	0.909	GGGTGGGGTGGGGTG
UBC866	0.650	8	0.560	0.545	CTCCTCCTCCTCCTCCTC
UBC818	0.450	10	0.760	0.744	CACACACACACACACAG
UBC808	0.250	11	0.860	0.846	AGAGAGAGAGAGAGAGC
UBC840	0.150	12	0.900	0.892	GAGAGAGAGAGAGAGAT
UBC834	0.200	11	0.875	0.863	AGAGAGAGAGAGAGAGT
UBC811	0.100	17	0.935	0.931	GAGAGAGAGAGAGAGAC
UBC81	0 0.500	5	0.675	0.634	GAGAGAGAGAGAGAGA
UBC2	0.450	5	0.700	0.656	GAGAGAGAGAGAGAGAT
Mean	**0.360**	**10**	**0.767**	**0.749**	

Figure 1: Molecular dendrogram showing genetic dissimilarity among the twenty accessions of Cola based on ISSR marker



ISSRs have been successfully used to estimate the extent of genetic diversity level in a wide range of tree crop species including *Camellia sinensis* (Yogurtcu and Aygun, 2021), Jatropha informatio for the exploitation of available genetic variability. The number of alleles and the high gene diversity (0.767) observed proved that significant genetic variability existed among the kola accessions. Botstein *et al.,* (1980) reported that a marker with PIC value of more than 0.5 is considered as highly informative. The average PIC value of 0.74 obtained is highly informative suggesting that the ISSR marker employed in the study was veryuseful for diversity study in Cola. All the ten polymorphic ISSRs primers used in this study were found useful for the delineation of accessions showing high allelic variation of the kola accessions. These observations are similar to those reported by Arolu *et al.,* (2012) where ten primers were used to distinguish 48 accessions of *Jatropha curcas*, likewise a report by Potter *et al.*, (2002) where eight primers were used to distinguish 48 cultivars of *Juglans regia*, and also a report by Alansi *et al.*, (2016) showed that eleven primers, were used to distinguish 34 accessions of *Ziziphusspina christ* L in *et al.,* (2019) also reported that nineteen primers were used to distinguish 159 accessions of coffee. The grouping of Cola a ccessions based on the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) in this study provides an opportunity for selection of parents for improvement programmes. Based on the information from the dendrogram, accessions that are far from each other by virtue of diversity index are strongly recommended to be used as parent for crossing. This will bring about greater genetic diversity, thus resulting into increase in selection gain. Hence in conclusion, the result obtained showed that a valuable genetic diversity is present within the Cola accessions under study based on the ISSR technique. Cluster analysis grouped the twenty Cola accessions into distinct groups and revealed variation within the clusters.

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