



Characterization of Juvenile Kola (*Cola* spp.) in Nigeria Using Inter Simple Sequence Repeat (ISSR) Markers

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Abstract

Cola spp., one of the most important tree crops, has great nutritional, medicinal, and industrial value. The implementation of molecular markers has demonstrably expedited breeding programs aimed at cultivating diverse crop varieties. These markers facilitate efficient selection for desirable traits, thereby reducing the time and resources required for traditional breeding methodologies. Consequently, the development and deployment of improved crop cultivars have been substantially accelerated through this technology. Molecular marker techniques are considered to be the most suitable means for estimating genetic diversity. This study undertook a molecular characterization of 40 Kola accessions sourced from germplasm collections. The objective was to assess the extent of genetic diversity present within this sample, providing insights into the germplasm's genetic structure. Inter simple sequence repeat (ISSR) markers were used to assay 40 accessions of Kola collected from the Cocoa Research Institute of Nigeria (CRIN) Ibadan germplasm. The results demonstrated that the ISSR markers were highly polymorphic and generated alleles ranging from nine to twenty-seven. The polymorphic information content was highest for UBC834, and the mean average was 0.832. The observed high gene diversity (0.946) suggests substantial genetic variation within the studied population. UPGMA dendrogram analysis of ISSR markers corroborated this finding, revealing a similarity coefficient range of 46–100%, further confirming the presence of considerable variability. These results collectively indicate a genetically diverse population structure. The results revealed that ISSR markers are useful for genetic characterization as they provide information on the interspecies phylogenetic status of the accessions and reveal genetic variability that could be exploited for varietal delineation and used to improve this species in Nigeria.

Keywords: Kola, Genetic diversity, Allele, Inter simple sequence repeat (ISSR)

1. Introduction

Economically, Kola is a significant commodity crop that contributes to local livelihoods and agricultural diversification. The cultivation and trade of Kola products create employment opportunities and encourage smallholder farming, thus enhancing food security and community resilience. Furthermore, as the global demand for caffeine and specialty beverages continues to rise, Kola holds promise for expanding markets and fostering economic growth within the region [1] [2]. The kola nut, derived from the fruit of the Kola tree, holds a significant place in both cultural traditions and nutritional value. Morphologically, these seeds exhibit three distinct colors: white, red, and pink. Each hue not only contributes to its visual appeal but also signifies varying levels of caffeine and theobromine, compounds that are essential for its stimulating properties. In terms of nutritional composition, the kola nut is rich in key chemical elements, including water, fat, ash, fiber, carbohydrates, and proteins. Kola nuts help to reduce the sensations of hunger and tiredness [3]. Kola nuts are characterized by a significant caffeine concentration, typically ranging from 1.84 to 2.56%.

This inherent stimulant property has historically contributed to their utilization in various cultural and commercial contexts. The presence of this substantial caffeine level distinguishes kola nuts from other botanical products [4]. The burgeoning interest in natural remedies has propelled nuts and their extracts into prominence across Europe and North America. Beyond their traditional culinary applications, these resources are increasingly utilized in alternative medicinal practices and as components in diverse industrial products, including soft drinks, confectionery, animal feed, liquid soaps, and dyes [1] [2] [5]. Numerous medicinal properties have been ascribed to the kola nut, including its purported efficacy in treating infections, dermatological conditions, ulcers, and oral discomfort. Furthermore, anecdotal evidence suggests its use in alleviating morning sickness, intestinal ailments, headaches, depression, and diminished libido, as well as respiratory and gastrointestinal disorders [6] [7]. Kola nuts are traditionally purported to provide diverse health benefits, such as digestive aid, hangover relief, support for menstrual regulation and labor complications [8] [7]. Kola nut trees, indigenous to the tropical forests of West Africa, hold considerable cultural importance.

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Beyond their botanical characteristics, these trees are integral to the social, religious, and ceremonial practices of numerous indigenous communities within the region. The kola nut itself thus serves as more than just a product of the forest, functioning as a symbolic element deeply embedded in local traditions [7] [9]. It is utilized at weddings, child naming ceremonies, chief installation ceremonies, funerals, and sacrifices offered to many African mythological gods [7] [9].

Kola cultivation, while vital for both health and economic stability, presents significant challenges to producers. These challenges include reproductive barriers such as sterility and incompatibilities in pollination, exacerbated by undesirable agronomic traits such as excessive tree height, diminished nut production, and extended maturation timelines. These factors collectively hinder kola production efficiency and profitability. To improve and advance its research attention, accurate genetic diversity studies of the existing Kola germplasm in Nigeria are needed to assist in selecting suitable parents for subsequent breeding programs. Several projects, including the collection of Kola accessions from different farmer fields in different locations in Nigeria, have been conducted, although with no distinguishing features. These are maintained as field gene banks with the aim of effectively incorporating them in breeding programs. Molecular characterization, which highlights the genetic diversity and relationships among various groups of different accessions, is required for direct and more reliable selection of Kola accessions. Intersimple sequence repeat (ISSR) markers represent a valuable tool for discerning genetic variation within crops and tree species. Recent application of this technology to Kola accessions within the Institute's germplasm collection has facilitated the assessment of existing genetic diversity. Such characterization is crucial for comprehending the patterns of diversity and, ultimately, enhancing desirable traits through targeted breeding strategies.

2. Materials and Methods

2.1 Plant Materials and Sample Collection

This study utilized forty Kola accessions maintained in a newly established germplasm collection at the Cocoa Research Institute of Nigeria Headquarters in Ibadan (Table 1). Fresh, young leaf samples were collected from each of the selected accessions and carefully preserved in appropriately labeled and sealed bags for subsequent analysis. Following collection, samples were immediately transferred on ice to the bioscience laboratory at the International Institute of Tropical Agriculture (IITA), Ibadan. Upon arrival, DNA extraction and subsequent genetic profiling were conducted utilizing the Intersimple Sequence Repeat (ISSR) marker procedure.

Table 1: Plant materials used in the research

S/N	Accessions	Collection site
1	CN1	CRIN Germplasm
2	CN2	CRIN Germplasm
3	CN3	CRIN Germplasm
4	CN4	CRIN Germplasm
5	CN5	CRIN Germplasm
6	CN6	CRIN Germplasm
7	CN7	CRIN Germplasm
8	CN8	CRIN Germplasm
9	CN9	CRIN Germplasm
10	CN10	CRIN Germplasm
11	CN11	CRIN Germplasm
12	CN12	CRIN Germplasm
13	CN13	CRIN Germplasm
14	CN14	CRIN Germplasm

15	CN15	CRIN Germplasm
16	CN16	CRIN Germplasm
17	CN17	CRIN Germplasm
18	CN18	CRIN Germplasm
19	CN19	CRIN Germplasm
20	CN20	CRIN Germplasm
21	CN21	CRIN Germplasm
22	CN22	CRIN Germplasm
23	CN23	CRIN Germplasm
24	CN24	CRIN Germplasm
25	CN25	CRIN Germplasm
26	CN26	CRIN Germplasm
27	CN27	CRIN Germplasm
28	CN28	CRIN Germplasm
29	CN29	CRIN Germplasm
30	CN30	CRIN Germplasm
31	CN31	CRIN Germplasm
32	CN32	CRIN Germplasm
33	CN33	CRIN Germplasm
34	CN34	CRIN Germplasm
35	CN35	CRIN Germplasm
36	CN36	CRIN Germplasm
37	CN37	CRIN Germplasm
38	CN38	CRIN Germplasm
39	CN39	CRIN Germplasm
40	CN40	CRIN Germplasm

CRIN = Cocoa Research Institute of Nigeria

2.2 DNA Extraction

Leaf samples underwent a DNA extraction protocol commencing with mechanical disruption via vortexing in the presence of steel balls and silica gel. Subsequently, a preheated extraction buffer was applied, followed by incubation and homogenization. Protein precipitation was achieved through the addition of potassium acetate and chloroform isoamyl alcohol, with centrifugation separating the supernatant. DNA precipitation was then induced using isopropanol and a subsequent cold incubation, followed by ethanol washes and pellet air-drying. Finally, the DNA pellet was resuspended in ultrapure water, treated with RNase to remove RNA, and incubated.

2.3 Electrophoresis of DNA

DNA extraction from leaf tissue was successfully confirmed via agarose gel electrophoresis. A 1% agarose gel was prepared by dissolving agarose powder in 1x TAE buffer, followed by the addition of EZVision DNA stain after cooling. DNA samples, combined with loading buffer, were then electrophoresed alongside a molecular weight ladder. Post-electrophoresis, the gel was visualized under UV transillumination, with the presence of distinct DNA bands indicating successful DNA extraction.

2.4 Polymerase chain reaction

The polymerase chain reaction (PCR) was performed utilizing a mixture of Taq 2X Master Mix, forward and reverse primers, DNA template, and nuclease-free water, combined in specified volumes. The thermocycler program commenced with an initial denaturation, followed by 36 cycles of denaturation, annealing, and elongation, each with designated temperatures and durations. A final elongation step was implemented, succeeded by a holding temperature of 10°C.

2.5 DNA scoring and analysis

Following PCR amplification, amplified fragments were separated via agarose gel electrophoresis and visualized with UV illumination.

Alleles were scored binarily based on band presence (1), absence (0), or missing data (m). Polymorphism percentage was determined by dividing the number of polymorphic bands by the total number of scored bands, multiplied by 100 [10]. Polymorphic information content (PIC) was calculated using the formula [11]

$$PIC_i = 2f_i(1 - f_i),$$

Where f_i is the frequency of the amplified allele (band present) and $(1 - f_i)$ is the frequency of the null allele (band absent) of marker i .

Genetic similarities were assessed using Nei and Li/Dice indices [12] within NTSYSpc software [13], from which a UPGMA dendrogram was constructed to illustrate genetic relationships.

3.0 Results

Extracted DNA obtained from 40 Kola accessions showed sharp and clear bands. The DNA bands did not indicate smearing (degraded DNA) as observed via agarose gel electrophoresis (Plate 1), and the genomic DNA was a satisfactory PCR template. Table 2 details the characteristics of polymorphic Inter-Simple Sequence Repeat (ISSR) markers employed in the molecular analysis of 40 Kola accessions. The ten ISSR markers demonstrated considerable polymorphism within the accessions; however, the major allele frequency was observed to range from 0.100 to 0.500. The genetic diversity of the primers used ranged from 0.640 to 0.946, where UBC834 revealed the highest genetic diversity (Table 2). Inter-simple sequence repeat (ISSR) markers exhibited a variable number of alleles, ranging from 9 to 27. The polymorphism information content (PIC) values for Kola displayed a similar range, fluctuating between 0.584 for UBC866 and 0.944 for UBC834. The average PIC value observed across all markers was 0.832 (Table 2).

Figure 1 is a dendrogram showing overall genetic dissimilarity (variation) among the 40 accessions of Kola as revealed based on ISSR markers. All the accessions were distinct from one another at the 100% level of dissimilarity. The dendrogram showed that the first linkage was formed between CN34 and CN35 at a 0.97 dissimilarity level, indicating that these two linkages were the closest neighbors (most similar) among all the accessions. All the accessions formed a single cluster at the 0.46 level of dissimilarity, indicating some degree of similarity among them. Four distinct clusters were formed from the analysis of the pooled ISSR marker data, for which the similarity coefficient was 0.68. Cluster I had the largest number of accessions and had two sub clusters. Sub cluster A included twelve accessions (CN32, CN30, CN29, CN33, CN37, CN40, CN39, CN38, CN35, CN34, CN25, and CN24), while eight accessions were in subcluster B (CN27, CN26, CN31, CN28, CN23, CN36, CN22, and CN21). Cluster II included only one accession, CN8, indicating that CN8 was the most distinct among all the accessions. Cluster III was composed of seventeen accessions, five of which were grouped into sub cluster A (CN9, CN7, CN6, CN5, and CN4), and the remaining twelve accessions formed sub cluster B (CN14, CN12, CN16, CN15, CN10, CN20, CN19, CN18, CN17, CN13, CN11, and CN3). Cluster IV included accessions CN1 and CN2 (Figure 1).

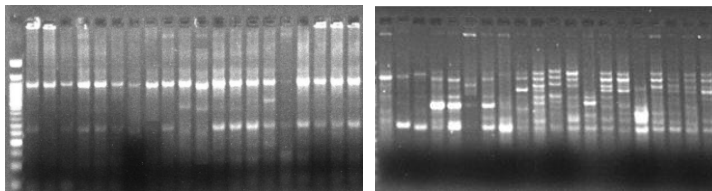


Plate 1: DNA of 40 accessions of Kola visualized via agarose gel electrophoresis.

Table 2: Characteristics of polymorphic ISSR markers used in the molecular analysis of 40 Kola accessions

Markers	Major Allele Frequency	No of alleles	Gene Diversity	PIC
UBC888	0.350	10	0.786	0.759
UBC881	0.350	19	0.843	0.833
UBC866	0.500	9	0.640	0.584
UBC818	0.450	12	0.735	0.708
UBC808	0.150	17	0.919	0.913
UBC840	0.200	15	0.890	0.881
UBC834	0.100	24	0.946	0.944
UBC811	0.200	27	0.933	0.930
UBC810	0.250	13	0.871	0.860
UBC2	0.225	23	0.909	0.903
Mean	0.278	16.9	0.847	0.832

PIC=Polymorphic Information Content

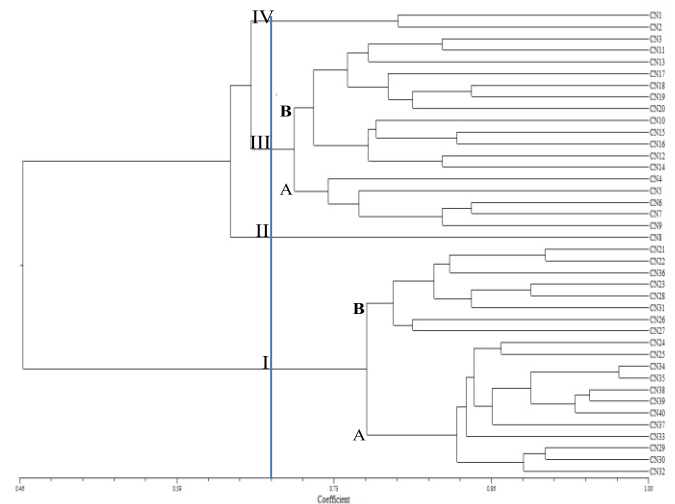


Figure 1: Molecular dendrogram showing genetic dissimilarity among the forty accessions of Kola based on ISSR markers

4.0 Discussion

Inter Simple Sequence Repeat (ISSR) is a Polymerase Chain Reaction-based technique employing highly polymorphic markers valuable for diverse genetic analyses. Its applications encompass the study of genetic diversity, phylogeny, gene tagging, genome mapping, and evolutionary biology. The ISSR method has proven effective in assessing the breadth of genetic variation within numerous crop species, notably *Camellia sinensis* [14], *Jatropha curcas* [15], and *Juglans regia* [16]. This technique provides useful information for the exploitation of available genetic variability. The number of alleles and the high genetic diversity (0.946) observed in this study confirmed that significant genetic variability occurred among the kola accessions. [17] reported that polymorphic information content (PIC) marker values greater than 0.5 are considered highly informative. The average PIC value of 0.832 obtained in the present study is highly informative, suggesting that the ISSR marker employed in the study was very useful for diversity studies in Kola. All ten polymorphic ISSR primers used in this study were useful for the delineation of accessions showing high allelic variation in the DNA of the kola accessions. These observations are similar to those reported by [15], where ten primers were used to distinguish 48 accessions of *Jatropha curcas*; likewise, a report by [16], where eleven primers were used to distinguish 11 accessions of *Juglans regia*; and a report by [18], in which eleven primers were used to distinguish 34 accessions of *Ziziphus spina-christi*. UPGMA analysis generated a dendrogram that effectively partitioned the germplasm collection. The resultant classification revealed four primary clusters, further subdivided into four secondary clusters,

and subsequently exhibiting several additional sub-clusters within these hierarchical groupings.

Based on the dendrogram's depiction of genetic divergence and diversity indices, accessions exhibiting significant distance are optimally suited as parental candidates for hybridization. Utilizing such disparate accessions maximizes the potential for generating novel genetic combinations and enhancing desirable traits in progeny. Augmenting genetic diversity through this approach is expected to intensify selective pressures, ultimately fostering a more productive index. This should manifest as improvements in key agronomic traits, including earlier flowering and fruiting, enhanced fruit yield per hectare, greater nut weight, and overall gains in other yield-related components.

5.0 Conclusions

The results obtained showed that valuable genetic diversity is present within the Kola accessions studied. The ISSR technique used for the study produced many clusters from the forty accessions and revealed variation within the clusters.

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