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Original scientific paper

GENETIC DIVERSITY IN *Cola acuminata* AND *Cola nitida* USING RAPD PRIMERS

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Cola species constitute an important non–timber forest product. Besides the food value, *Cola* is rich in numerous phytochemicals, making it more important for its use in both African traditional medicine and potentials in industrial pharmacopoeia. Knowledge about genetic diversity is essential for conservation. In this paper, we reported genetic variability of *Cola acuminata* and *C. nitida* germplasm across the *Cola* – producing states (the rain forest and derived savannah zones) in Nigeria using Random Amplified Polymorphic DNA (RAPD) markers. Fifteen primers which gives an average of 6.5 bands per primer were selected for both species. *C. acuminata* exhibited a higher level of variation with 71.5% of the detected markers being polymorphic (223 polymorphic alleles), whereas *C. nitida* presented 58.3% variation with 182 polymorphicalleles. Inter-population differentiation was measured as Jaccard's similarity coefficient. The mean similarity index amounted to 42.5% in *C. acuminata* and 46.7% in *C. nitida* respectively. Results reveal the genetic structure of both species and conservation strategies are suggested.
Keywords: *Cola*, conservation, biodiversity, forest resources, random amplified polymorphic DNA (RAPD), molecular markers.

INTRODUCTION

Cola acuminata (P.Beauv.) Schott and Endl and *Cola nitida* (Vent.) A. Chev. both belong to the plant family Sterculiaceae, and are rich in antioxidant micronutrients, caffeine, crude protein, carbohydrates and other phytochemicals (BLADES, 2000; ODEBUNMI *et al*., 2009). Novel extracts from different parts of the plants have been reported to possess several medicinal properties (GRIEVE, 2011; SONIBARE *et al*., 2009) and these have considerable potentials for the

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development of new pharmaceuticals (KUBATA *et al.,* 2005). As an important non–timber forest product, *Cola* is one of the numerous candidate species both for domestication and inclusion in multi-strata systems. An update of the genetic diversity of *Cola* present across the country, as a precursor to further breeding and improvement of this tree crop has been presented by ADENUGA *et al.,* (2012). The preservation of genetic diversity both within and among natural populations is a fundamental goal of conservation biology (KEIPER and MCCONCHIE, 2000).

Over the years, the methods for detecting and assessing genetic diversity have extended from analysis of discrete morphological traits to biochemical and molecular traits (MUTHUSAMY, 2008). Polymerase Chain Reaction (PCR) based markers using arbitrary primers, such as Random Amplified Polymorphic DNA (RAPD), have been widely used for investigating genetic relatedness and diversity in plant populations and cultivars (LI and NELSON, 2001; PAREDES *et al.,* 2002; SAINI *et al.,* 2010; MUHAMMAD and MUHAMMAD, 2014; MILADINOVIĆ *et al.*, 2018). Genetic discoveries can often provide novel, conservation-relevant insights and strategies (ABDELHAMID *et al*., 2014). ONOMO *et al.,* (2006) reported isoenzyme variability germplasm of three species of *Cola* in Cameroon. To our knowledge RAPD markers were not yet employed to analyze genetic diversity in *Cola* species in spite of the several advantages it has. RAPD offers a simple and efficient means for cultivar identification (SANDRA *et al*., 2014) through genetic polymorphism and construction of linkage maps (SCHULMANN, 2007) and for genetic improvements (RASHID *et al*., 2016; LAKIC *et al*., 2018).

In the present study, we investigated genetic diversity and relationship among *Cola acuminata* and *Cola nitida* through RAPD analysis. This research work represents a reference point for further research on *Cola* species as well as for improved conservation.

MATERIAL AND METHODS

Plant Materials

Fresh young leaves were harvested from 30 to 35 days–old *Cola* seedlings from eleven *Cola acuminata* and ten *C. nitida* accessions which were randomly collected from different farms located across the producing States in Nigeria (Tables 1(a) and 1(b)).

Table 1. Ecogeographical and collection parameters of Cola accessions sampled:

1 (a). *Cola acuminata*

Cultivar No	State	Longitude/Latitude
AN-A1	Anambra	06º 06'N 06º 42'E
BN-A1	Benue	07º 48'N 06º 46'E
CR-A1	Cross River	04º 57'N 08º 20'E
ED-A1	Edo	06º 20'N 05º 31'E
EK-A1	Ekiti	07º 10'N 05º39'E
EN-A1	Enugu	06º 30'N 07º 30'E
KG-A1	Kogi	07º 47'N 06º 45'E
OD-A1	Ondo	07º 32'N 05º 40'E
OG-A1	Ogun	06º 47'N 03º 58'E
OS-A1	Osun	08º 01'N 04º 11'E
OY-A1	Oyo	07º 51'N 03º 56'E

1 (b). *Cola nitida*

Cultivar No	State	Longitude/Latitude
AN-N1	Anambra	06º 06'N 06º 42'E
BN-N1	Benue	07º 48'N 06º 46'E
CR-N1	Cross River	04º 57'N 08º 20'E
ED-N2	Edo	06º 20'N 05º 31'E
EK-N1	Ekiti	07º 10'N 05º39'E
KG-N2	Kogi	07º 47'N 06º 45'E
OD-N1	Ondo	07º 32'N 05º 40'E
OG-N3	Ogun	06º 47'N 03º 58'E
OS-N1	Osun	08º 01'N 04º 11'E
OY-N2	Oyo	07º 51'N 03º 56'E

DNA Extraction and PCR

DNA was extracted from the fresh young leaf samples of *Cola* spp using the protocols developed by ADESOYE *et al*. (2014).

The Polymerization Chain Reaction (PCR) in a 10 µL reaction volumes reaction mixture contained 10 ng genomic DNA template (in 3 µL), 0.5 µL of 1× PCR buffer, 0.5 µL of 2.5 mM dNTPs, 0.4 µL of $MgCl_2$, 0.5 µL primer, 0.2U Taq DNA Polymerase (Bioline) and 3.72 µL ddH_2O in a final 10 µL volume. A total of fifty arbitrary decamer oligonucleotides primers (kits B, H and T from Operon Technology) were screened for polymorphic bands, of which fifteen were optimized for uniform, clearly visible and dense bands (Table 2).

Table 2. Characteristics of the Oligonucleotide primers applied in RAPD analysis across the accessions of C. nitida and C. acuminata

Primer Code	No. of bands	No. of banding patterns	Range of frequency of polymorphic bands	Nucleotide sequence
OPB 01	3	2	0.8049 – 1.0000	5' – GTTTCGCTCC – 3'
OPB 02	6	9	0.2546 – 0.7454	5'–TGATCCCTGG – 3'
OPB 03	6	5	0.2023 – 0.7385	5'–CATCCCCCTG–3'
OPB 04	7	10	0.1181 – 0.8819	5'–GGACTGGAGT– 3'
OPB 05	5	8	0.1548 – 0.7559	5' –TGCGCCCTTC– 3'
OPH 01	6	11	0.2441 – 0.8452	5' –GGTCGGACAA– 3'
OPH 02	9	15	0.1823 – 0.8165	5' –TCGGACGTGA– 3'
OPH 03	8	11	0.3333 – 0.8165	5' –AGACGTCCAC– 3'
OPH 04	11	21	0.2254 – 0.9487	5' –GGAAGTCGCC– 3'
OPH 05	8	10	0.3015 – 0.9045	5' – AGTCGTCCCC– 3'
OPT 01	5	9	0.6000 – 1.0000	5' –GGGCCACTCA– 3'
OPT 02	8	11	0.0513 – 1.0000	5' –GGAGAGACTC– 3'
OPT 03	8	6	0.3675 – 0.9535	5' –TCCACTCCTG– 3'
OPT 04	8	9	0.2929 – 1.0000	5' –CACAGAGGGA–3'
OPT 05	5	6	0.0286 – 0.9714	5' –GGGTTTGGCA– 3'

All reaction preparation was carried out on ice. The PCR amplification protocol was performed in a thermo-cycler machine (GeneAmp® PCR System 9700) programmed for an initial cycle of 3 minutes at 94°C followed by 45 cycles of 20 seconds at 94°C, 20 seconds at 37°C, and 40 seconds at 72°C. After all cycles were completed, the reactions were held at 72°C for 7 minutes and slowly cooled down to 4°C. Then 2% agarose gel in 1 X buffer Tris-borate EDTA (TBE) stained with 30 µl ethidium bromide 10 mg/ml per 300 ml of gel volume was prepared. 4 µL of loading dye was added to each PCR tube and spin down at 5000 rpm for 10 seconds, while 7 µL of the samples (RAPD-PCR products) and 4 µL of 100 bp DNA ladder were loaded into the gel combs. The electrophoresis was run at 100V constant voltage for 4 h, after which it was drained and viewed using the GDS–8000 of UVP® Bioimaging Systems M–20 and photographs taken.

Amplified bands were recorded as present (1) or absent (0) for each accession and the pairwise Jaccard's similarity coefficient was generated from the binary matrix obtained, with the aid of SIMQUAL (Similarity for Qualitative Data) routine. The similarity coefficients were then used to generate dendrograms using the Unweighted Pair Group Method with Arithmetical Mean (UPGMA) employing Sequential, Agglomerative, Hierarchical, and Nested clustering (SAHN) with the aid of NTSYS-pc program version 1.8.

RESULTS

For both *C. acuminata* and *C. nitida*, the fifteen random primers selected produced amplification products that varied from 170 to 1650 bp and the number of bands per primer ranged from 3 (OPB 01) to 11 (OPH 04) with a mean of 6.9 bands per primer (Table 2). Typical banding patterns for primer OPH02 and OPT04 are depicted in Figure 1 and 2 respectively. However, there was a wide variation in the range of polymorphic bands produced by the primers.

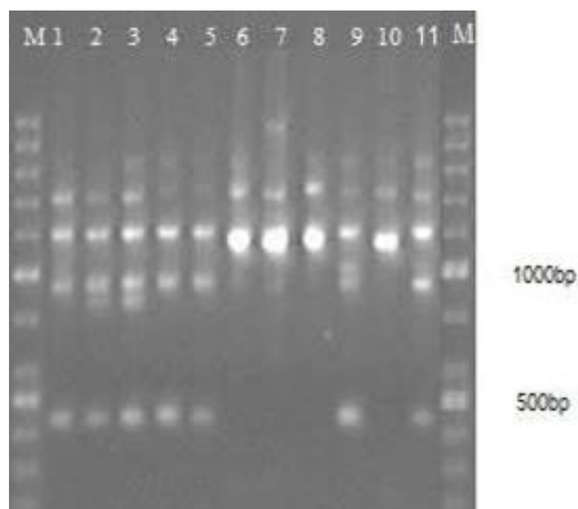

Fig. 1. RAPD amplification products generated from genomic DNA of *Cola acuminata* accessions obtained with primer OPT04

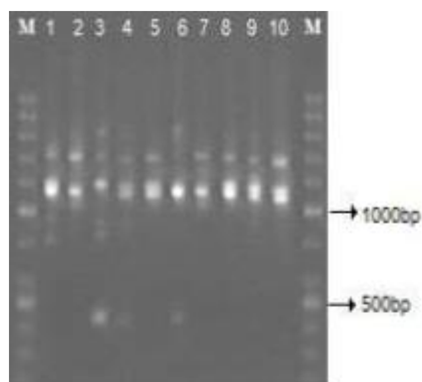

Fig. 2. RAPD amplification products generated from genomic DNA of *Cola nitida* accessions obtained with primer OPH02

Primers such as OPT 05 showed a large variation ranging from 0.0286 to 0.9714 in the frequency of polymorphic bands. On the other hand, a few primers, such as OPB01 and OPT01 showed a narrow range in the frequency of polymorphic bands ranging from 0.8049 to 1.0000 and 0.6000 to 1.0000, respectively (Table 2). Jaccard's similarity coefficient among the genotypes ranged from 35.4% (AN-A1 and KG-A1) to 65.9% (AN-A1 and OS-A1) in *C. acuminata* with a mean of 42.5% (Table 3). Whereas in *C. nitida*, it ranged from 47.4% (AN-N1 and OY-N2) to 65.9% (KG-N2 and OD-N1) with a mean of 46.7% (Table 4).

Table 3. Jaccard's similarity coefficients of C. acuminata based on RAPD markers

	AN-A1	BN-A1	CR-A1	ED-A1	EK-A1	EN-A1	KG-A1	OD-A1	OG-A1	OS-A1	OY-A1
AN-A1											
BN-A1	49.4										
CR-A1	51.3	45.1									
ED-A1	45.5	54.4	35.9								
EK-A1	42.7	57.6	45.3	52.2							
EN-A1	44.3	57.9	41.2	46.2	55.4						
KG-A1	35.4	48.7	44.1	42.3	55.6	49.3					
OD-A1	62.5	59.3	52.4	50.0	56.1	56.3	45.2				
OG-A1	52.6	60.0	47.3	48.5	61.7	59.4	42.4	55.7			
OS-A1	65.9	46.5	52.5	45.5	44.3	48.1	40.1	55.1	56.9		
OY-A1	58.1	60.9	51.9	46.8	55.6	51.7	51.9	54.3	56.8	53.0	

Table 4. Jaccard's similarity coefficient matrix of C. nitida based on RAPD markers

	AN-N1	BN-N1	CR-N1	ED-N2	EK-N1	KG-N2	OD-N1	OG-N3	OS-N1	OY-N2
AN-N1										
BN-N1	61.3									
CR-N1	59.5	51.7								
ED-N2	64.7	59.5	50.6							
EK-N1	63.3	58.6	54.3	57.0						
KG-N2	55.9	61.0	53.4	60.0	54.2					
OD-N1	60.3	54.6	53.8	61.1	60.2	57.5				
OG-N3	56.1	62.8	46.8	65.5	57.7	65.9	63.7			
OS-N1	58.6	59.2	43.8	65.6	57.5	58.3	59.4	61.2		
OY-N2	47.4	59.3	49.3	52.8	54.1	52.2	49.3	59.1	51.0	

The dendrograms (Figure 3 and 4 respectively) showing genetic interrelationship among individual accessions of *C. acuminata* and *C. nitida* populations based on UPGMA clustering of Jaccard's similarity values (at coefficient of 0.5), showed two main clusters. Individual populations of *C. acuminata* from AN-A1, OS-A1, OD-A1 and CR-A1 formed a cluster, while those from BN-A1, OY-A1, EK-A1, OG-A1 and EN-A1 are seen together, whereas those from ED-A1 and KG-A1 stood out distinctly. AN-A1 and OS-A1 are closely related at 0.65 and both are related to ON-A1 at 0.60 while EK-A1 and OG-A1 (0.62) are related to BN-A1 and OY-A1 (0.61) at a coefficient of 0.58.

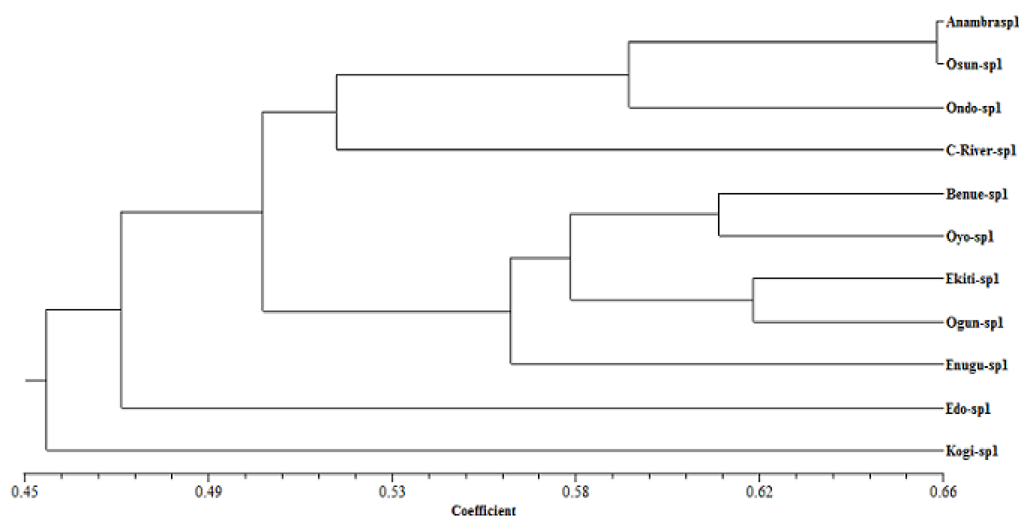

Fig 3. UPGMA dendrogram showing genetic relationships among *C. acuminata* germplasm using Jaccard's similarity index

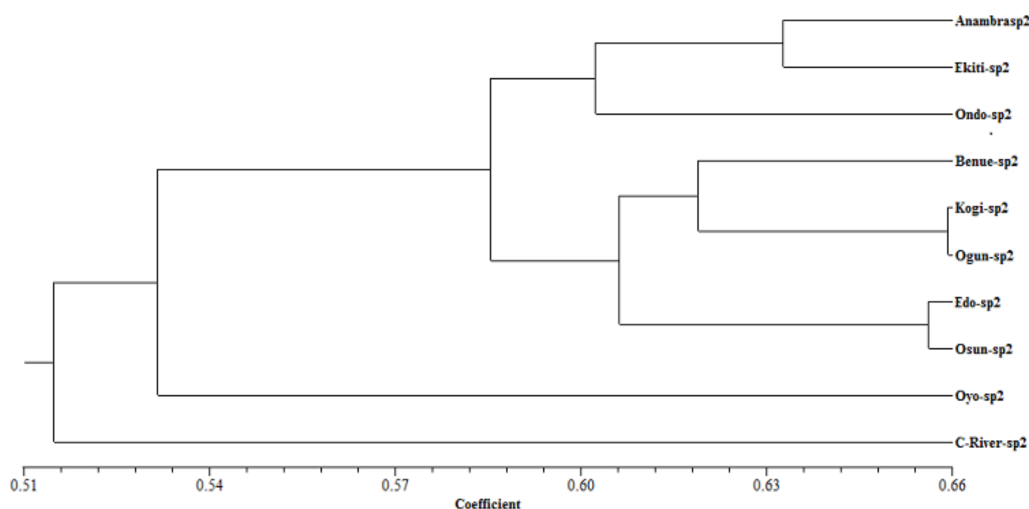

Fig. 4. Dendrogram showing relationships among *C. nitida* germplasm based on UPGMA analysis of Jaccard's similarity coefficient RAPD data.

For *C. nitida* accessions, at a coefficient of 0.58, individual populations from Anambra, Ekiti, Ondo, Benue, Kogi, Ogun, Edo and Osun formed a cluster, and all cluster with Oyo at 0.53. Kogi and Ogun closely related at 0.66, whereas Edo and Osun are similar at 0.65. Cross-River populations were the most diverse, as it stood out at 0.516 from the rest of the accessions (Figure 4).

DISCUSSION

The percentage of the polymorphic loci within the individuals in this study was 58.33% (182 polymorphic loci) in *C. acuminata* and 71.47% (233 polymorphic loci) in *C. nitida*. Both studied *Cola* species showed genetic variation as revealed by polymorphism of bands in the selected primers used. ONOMO *et al*. (2006) also reported polymorphism and intra-population variation in both *C. acuminata* and *C. nitida* germplasm in Cameroon using isoenzyme traits. DNA based molecular markers such as RAPDs have been reported to reveal genetic diversity in forest trees and crops (HEIDER *et al*., 2007; ABDELHAMID *et al*., 2014).

The clustering pattern reflects the geographical positioning of collecting sites with the exceptions of accessions from Ekiti and Anambra states which showed close relation even though both States are far apart. However, this can be explained by the fact that there is bilateral relationship between the two States in the aspect of trading and farming which might facilitate some gene flow. As such, the accession might have a common origin due to long distance dispersal.

The notable genetic differentiation which was found among accessions may be attributed to a very limited gene flow as a result genetic distance (ISLAM *et al*., 2014; COLLINS *et al*., 2015). Breeding system and seed dispersal can also affect differentiation of genetic diversity. Outcrossing and sexual~~ly~~ reproducing plants are usually more diverse than clonal plants or species with mixed – mating systems, such as in *Senna multijuga* (RIBEIRO and LOVATO, 2014).

The level of geographical distribution of the sampled sites deviates from the clustering pattern in both species of *Cola*. This suggests that a probable isolation-by-distance model does not apply.

RAPD analyses of other tree species have been shown to portray wide range of genetic variation. Relations among three Piper species have been compared using similarity indexes from morphological investigations and RAPD analysis (RACHANEE *et al*. 2002). The genetic variability among *Artemisia capillaris* populations in Malaysia was reported by HASAN *et al*. (2009). While ASH *et al*. (2003) showed that there is distinct genetic variability within the *Cartahmus lanatus* populations in Australia. The significant levels of genetic differentiation observed in both species studied suggest that they should be considered as separate units for the purpose of conservation.

Areas that host populations of high diversity should be targeted more intensively for further collecting activities and for potential *in situ* conservation. Small populations deserve conservation efforts and intensive sampling of greater areas for *ex situ* safe - guarding. The application of biomolecular techniques for studying genetic diversity between and among natural populations in conservation biology provides an appropriate focus for conservation management and monitoring.

CONCLUSION

The results of this study indicated that RAPD analyses provide an effective tool for the analyses of genetic diversity in *Cola* species. The RAPD method developed need to be adapted and optimized in order to establish a sound database of genetic diversity in *Cola*. The method of collection could be modified from seed collection to a more elaborate processing of leaf materials directly which presents a challenge under field conditions. As a consequence of overexploitation of natural resources, genetic erosion is occurring in Nigeria, all options to meet future requirements should therefore be explored especially in the Southern part of the country, which revealed greater genetic diversity. The conservation of genetic diversity of species with high potential as food and medicinal crops such as *Cola* is highly recommended.

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GENETIČKI DIVERZITET *Cola acuminata* I *Cola nitida* PRIMENOM RAPD PRAJMERA

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Izvod

Cola vrste predstavljaju važan ne-drvni šumski proizvod. Pored prehrambene vrednosti, Cola je bogata brojnim fitohemikalijama, što ga čini važnijim za upotrebu kako u afričkoj tradicionalnoj medicine, tako za industrijsku farmakopeju. Poznavanje genetičkog diverziteta je od velikog značaja za konzervaciju. U radu smo prikazali genetičku varijabilnost germplazme *Cola acuminate* i *C. nitida*, kroz njihove proizvodne oblasti (kišne šume i savane) u Nigeriju, korišćenjem RAPD markera. Za obe vrste, odabrano je po 15 prajmera koji su dali u proseku 6.5 traka po prajmeru. *C. acuminate* je pokazala veći stepen varijacije sa 71.5% (223) polimorfnih alela, dok je *C. nitida* imala 58.3% varijacije, sa 182 polimorfna alela. Međupopulacijska diferencijacija izmerena je pomoću *Jaccard*-ovog koeficijenta sličnosti, koji je iznosio 42.5% u *C. acuminate* i 46.7% kod *C. nitida*. Rezultati su utvrdili genetičku strukturu za obe vrste I predložene su strategije za konzervaciju.

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