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Research Paper

Diversity analysis of selected coffee genotypes using microsatellites and random amplified polymorphic DNA

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Abstract. Two molecular marker systems, RAPD (Random Amplified Polymorphic DNA) and SSR (Simple Sequence Repeats) were used to identify genetic relationship among 24 coffee accessions. The amplification fragments were scored for presence (1) and absence (0) and the data subjected to analysis using STATSTICA software version 8. A total of 79 bands were detected by 10 RAPD and 50 bands were detected by 13 SSR primers. The polymorphism detected by both markers ranged from 33 to 100% for SSR and 50 to 100% for RAPD with average polymorphism of 65 and 81%, respectively. The genetic dissimilarity index among the genotypes ranged from 0.06 to 1 for both SSR and RAPD primers. Using the Unweighted Pair Group Method with Arithmetic Average (UPGMA) analysis, RAPD and SSR markers clustered the genotypes according to the three different species namely; *C. eugenioides*, *C. canephora* and *C. arabica*. This study confirmed the low genetic diversity in Arabica coffee genotypes evaluated with average dissimilarity index of 0.5. The study also widened the information on genetic diversity of coffee germplasm available for breeding programmes in Kenya unlike previous work which focused on commercial cultivars and donors of resistance to diseases.

Keywords: Microsatellites, RAPD, Diversity, Polymorphism.

INTRODUCTION

Coffee is an important agricultural commodity in global trade. It is grown in over 80 countries and it provides income to about 25 million Coffee farming families around the world (Pare, 2002). Coffee belongs to the genus *Coffea* in the Rubiaceae family and is mostly grown in the tropical and subtropical regions (Berthaud and Charrier, 1988). The genus *Coffea* consists of about 100 species out of which *C. arabica* L. (Arabica coffee) and *C. canephora P*. (Robusta coffee) are the two most cultivated and of economic significance (Davis et al., 2006).

Coffee arabica is predominantly autogamous (Wrigley, 1995) and it is the only *Coffea* species that is polyploidy (tetraploid) with chromosome number (2n = 4x = 44) (Clarindo and Carvalho, 2008). Molecular analysis and cytogenetic analysis established that *C. arabica* is an allotetraploid formed by hybridization between the diploids *C. Canephora* and *C. eugenioides* (Lashermes *et al*., 2011). *C. arabica* has a very narrow genetic base which is contributed by its autogamous nature (Vossen, 1985). The narrow diversity observed in *C. arabica* is believed to be a consequence of its reproductive biology, origin and evolution (Anthony *et al*., 2001a). The remaining species are diploid with 2n=2x=22 chromosomes and are generally self-incompatible.

Through variety improvement, commercially cultivated cultivars in Kenya include Ruiru 11, Batian, K7, SL 34 and SL 28. Coffee accessions from Guatemala, Tanzania, South and Central America, Ethiopia, Angola, Sudan, India and Kenya are conserved ex-situ at Kenya Agricultural and Livestock Research Organization -Coffee Research Institute (KALRO-CRI) in Ruiru, Kenya in germplasm field plots. In spite of the commercial importance of the genus *Coffea*, the relationship at DNA level between the majorities of coffee species has not been fully explored and understood. Understanding the

genetic relationship between coffee species is not only important for the genetic improvement program but also for conservation of potential populations and for understanding taxonomic ambiguity.

Genetic diversity within and among species can be estimated using biochemical, morphological and molecular markers. Diversity studies using morphological techniques in plants is limited due to delays associated with phenotypic expression of various growth stages of the plant and also the influence of environmental factors (Weising *et al*., 2005). Use of biochemical method (isozymes), was developed. However, isozyme markers were found to be inappropriate for determining the genetic variation in *C. arabica* due to fact that analysis of isozymes in *C. arabica* failed to reveal the amount of polymorphism detected using morphological markers (Bustamanate and Polanco, 1999).

A variety of molecular techniques have been developed to estimate genetic variation at both interspecific and intraspecific levels in a number of plant species. DNA based markers have in a recent past been developed for genetic analysis studies in plants. DNA-based techniques are in use in different coffee genetic studies. These include the conventional Restriction Fragment Length Polymorphism method (Crouzillat *et al*., 2004) and the different PCR-based methods such as RAPD (Orozco-Castillo *et al*., 1994), AFLP (Anthony *et al*., 2001b) and microsatellite (SSRs) markers (Lashermes *et al*., 1995). Molecular markers are of significant importance for genetic improvement in perennial crops like coffee. It allows selection of desirable genotypes at seedling stage on a large number of breeding lines, reduce the number of backcross cycles required to restore the quality of the recurrent parent and for simultaneous improvement of different traits (Lashermes *et al*., 2000b). DNA-based markers offer consistent results regardless of cropping conditions, type and age of sampled tissue (Sakiyama, 2000), thus making it suitable for coffee research, since coffee is a perennial crop with a long juvenile period.

MATERIALS AND METHODS

Plant materials

A total of 24 coffee genotypes consisting of C. arabica, C. canephora and C. eugenioides (Table 1) were analysed in this study. The coffee trees of these genotypes are available in the commercial fields, experimental sites and field gene bank at Coffee Research Institute located at Ruiru-Kenya. The study was conducted at Kenya Agriculture & Livestock Research Organization-Coffee Research Institute (KALRO-CRI).

Genomic DNA extraction

Young disease-free leaves were picked from mature coffee trees at KALRO-CRI. The DNA of the genotypes (Table 1) was extracted according to the method described by Diniz *et al*. (2005), using mixed alkyl trimethyl ammonium bromide (MATAB).

Amplification of coffee genomic DNA using RAPDS

The method described by Lashermes *et al*. (1996b) and modified by Agwanda *et al*. (1997) was used for RAPD analysis.Ten arbitrary decamer oligonucleotides were used. A total of 4 ng of each DNA sample was used in PCR reactions for RAPD markers amplification. A reaction mix was prepared to include: 2.5 ul of buffer (10x), 2.5 ul of $MgCl_2$ (25 mM), 3.5 ul of dNTPs (500 uM), 2.0 ul RAPD primer (10 µM), 0.5 ul of Taq polymerase (5u / µl). Reaction was incubated in a thermocycler set for the following amplification conditions using the primers as described in Table 2: an initial denaturation at 95°C for 5 min, followed by loop 1 (45 cycles) at 94°C for 1 min to denature, 34°C for 1 min to anneal the primer to the template DNA and extention at 72°C for 1 min 30 s. A final extention period at 72°C for 10 min to complete the PCR and final products were held at 4°C. The RAPD products were electrophoresed in 1.8% agarose gel and then visualized in a UV trans-illuminator after staining in ethidium bromide solution.

Amplification of coffee genomic DNA using SSR

Methodology described by Combes *et al*. (2000) was used for SSR analysis. A total of 100 ng of each DNA sample was used in PCR reactions for amplification of SSR markers. A reaction mix was prepared to include: 2.5 ul of buffer (10x) , 2.5 ul of $MgCl_2$ (25 mM), 3.5 ul of dNTPs (500 µM), 2 ul of SSR (10 µM) Reverse and forward primer, 0.5 ul of Taq polymerase (5u/µl). Reaction was incubated in a thermocycler set for the following amplification conditions using the primers described in Table 3: an initial denaturation at 95°C for 5 min, followed by loop 1 (35 cycles) at 94°C for 1 min to denature, 60°C for one minute to anneal the primer to the template DNA, and extention at 72°C for 1 min. A final extention period at 72°C for 10 min to complete the PCR and final products were held at 4°C.

Data analysis

The SSR and RAPD amplified bands were scored for the presence (1) or absence (0) of amplified products to create a binary matrix. The total number of bands, the distribution of bands across all species, the polymorphic bands and average number of bands per primer were calculated using STATSTICA software version 8. Genetic dissimilarities indices was estimated using Pearson

Table 1. Status and sources of coffee germplasm used in the study.

Genotype	Status	Source
Rume sudan	Gene bank accession	Sudan
Arabusta	Gene bank accession	Kenya
Canephora	Gene bank accession	Uganda
BA (India selection)	Gene bank accession	India
Ruiru 11 cd 93	Advanced selection	Kenya
Blue Mountain	Gene bank accession	Kenya
Typica	Gene bank accession	Kenya
SL28	Gene bank accession	Kenya
Mundo novo	Commercial variety	Brazil
Robarbica	Gene bank accession	Kenya
Catura	Gene bank accession	Kenya
Hybrido de timor	Gene bank accession	Portugal
Drought resistant (dr)	Gene bank accession	Tanzania
Catimor (line 86)	Breeders material	Colombia
Devermachy	Gene bank accession	Kenya
Ruiri11 Cd 80	Advanced selection	Kenya
Ruiru11Cd50	Advanced selection	Kenya
Sarchmore	Gene bank accession	Costa Rica
Bourbon	Gene bank accession	Kenya
Mokka	Gene bank accession	Brazil
Colombia	Gene bank accession	Guatamala
Batian	Advanced selection	Kenya
C.eugeniodes	Gene bank accession	Kenya
Erecta	Gene bank accession	Kenya

dissimilarity. Cluster analysis was performed using Un-weighted Pair Group Method with Arithmetic Averages (UPGMA) (Bigirimana *et al.*, 2013) using STATSTICA software version 8.

RESULTS AND DISCUSSION

Genetic diversity of 24 Coffee accessions as revealed by RAPD primers

Ten RAPD primer combinations were used to amplify DNA. Among the ten RAPD primers tested, all primers showed amplification and produced clear bands that could be scored . The total number of fragments observed among the coffee genotypes based on the 10 RAPD primers was 79. The number of amplified bands per primer varied from 3.0 to 12.0. The total number of polymorphic fragments produced was 65. The average bands produced by the 10 primers was 7.9 and recording an average polymorphic bands of 6.5. The distribution of bands revealed by ten RAPD primers is shown in Table 4.

To estimate the genetic diversity in the evaluated germplasm, amplified data from RAPD marker system was used for calculation of genetic distance matrices generated by the Pearson's dissimilarity index. The highest values for genetic distances were obtained between Sarchmore and SL28 and between Sarchmore and Batian with ascore of 1 implying that these varieties were genetically quite distinct. The lowest genetic distance values for the 24 genotypes was between Sarchmore and Robarbica with ascore of 0.07 implying high genetic resemblance of the two genotypes. The pattern of genetic relationships among genotypes was assessed using Unweighted Pair Group Method with Arithmatic Average (UPGMA) method of cluster analysis. Dendrogram representing most probable genetic relationship between cultivars is presented in Figure 1. The genotypes separated into three main clusters namely: *C. arabica*, *C. canephora* and *C. eugenioides*.

Genetic diversity of 24 coffee accessions as revealed by SSR primers

Thirteen SSR primer combinations were used to amplify DNA. Among the 13 SSR primers tested, all the primers showed amplification and produced clear bands that could be scored. The total number of fragments observed among the coffee genotypes based on the 13 SSR primers was 50. Due to polyploidy nature of *Coffee arabica*, the determination of heterozygosity and homozygosity is challenging. In addition, available software

Table 2. RAPD primers used for PCR analysis of 24 coffee accessions.

Primer	Sequence
OPN-18	5-'GGT GAG GTC A-3'
OPL-18	5'-ACC ACC CAC C-3'
OPM-04	5'-GGC GGT TGT C-3'
OPI-07	5'-CAG CGA CAA G-3'
OPJ-19	5'-GGA CAC CAC T-3'
OPY-10	5'-CAA ACG TGG G-3'
OPX-20	5'-CCC AGC TAG A-3'
OPY-15	5'-AGT CGC CCT T-3'
OPI-20	5'-AAA GTG CGG G-3'
OPX-16	5'-CTC TGT TCG G-3'

Table 3. SSR primers used for PCR analysis of 24 coffee accessions.

Locus	Reverse prime	Forward primer
Sat11	CCACACAACTCTCCTCATTC	ACCCGAAAGAAAGAACCAA
Sat32	CTGGGTTTTCTGTGTTCTCG	AACTCTCCATTCCCGCATTC
Sat207	CAATCTCTTTCCGATGCTCT	GAAGCCGTTTCAAGCC
Sat227	ATCCAATGGAGTGTGTTGCT	TGCTTGGTATCCTCACATTCA
Sat235	GCAAATCATGAAAATAGTTGGTG	TCGTTCTGTCATTAAATCGTCAA
Sat240	GGTAAATCACCGAGCATCCA	TGCACCCTTCAAGATACATTCA
Sat255	GGGAAAGGGAGAAAAGCTC	AAAACCACACAACTCTCCTCA
Sat283	GTGTGTGATTGTGTGTGAGAG	GCACACACCCATACTCTCTT
Sat254	AAGTGTGGGAGTGTCTGCAT	ATGTTCTTCGCTTCGCTAAC
Sat229	TTTAATGGCATAGGTCC	GGCTCGAGATATCTGTTTAG
M24	TTCCTCCATGCCATATTG	TTCTAAGTTGTTAAACGAGACGCTTA
Sat172	TCAAAGCAGTAGTAGCGGATG	ACGCAGGTGGTAGAAGAATG
Sat262	GCCGGGAGTCTAGGGTTCTGTG	CTGCGAGGAGGAGTTAAAGATACCAC

only supports diploid species analysis. Therefore, microsatellite data was formatted as dominant data in which each allele was treated as a locus and scored as present (1) and absent (0) (Medini *et al*., 2005; Montemurro *et al*., 2005). The UPGMA algorithm was used for grouping all cultivars based on their genetic distances. Dendrograms representing most probable genetic relationship between cultivars are presented in Figure 2. The cluster dendogram constructed was used to estimate genetic diversity in 24 coffee accessions.

A total of 50 alleles were amplified among 24 coffee genotypes using 13 SSR primers. Of these amplified alleles, 33 were polymorphic. The number of amplified alleles per primer varied from 2.0 to 6.0 with an average of 3.8 alleles. The distribution of bands across the three coffee species is shown in Table 5.

To estimate the genetic diversity in the evaluated germplasm, amplified data from SSR marker system was used to compute genetic distance matrices generated by the Pearson's dissimilarity index. The highest values for genetic distances were obtained between *C. canephora* and *C. eugenioides* and betweeen *Canephora* and Batian with a score of 1 implying that these varieties were genetically quite distinct. The lowest values for genetic distance were recorded between Sarchmore and Robarbica and between Sarchmore and Colombia with a score of 0.06 implying that these varieties were genetically fairly similar.

Analysis of genetic diversity is very important for breeding and conservation programs. Molecular markers offer an approach to unveil the genetic diversity among different species and cultivars based on nucleic acid polymorphisms. Extent of distribution, areas sampled and plant characteristics such as mode of reproduction, breeding behaviour and generation time are some of the important parameters that determine the level of genetic variability revealed in a species (Bhat *et al*., 1999). Diversity in genetic resources is the basis for genetic improvement. Genetic resources will have little value unless it is efficiently conserved and properly utilised. Its efficient utilisation as well as conservation depends on the availability of reliable genetic diversity information. The knowledge of the genetic diversity is important for efficient management of germplasm and utilisation of

Table 4. Amplification products generated by RAPD primers.

Primer	Number of bands	Polymorphic bands	% Polymorphism
OPI-07	3	3	100
OPX-20	8	6	75
OPJ-19	9	8	88
OPY-15	10	8	80
OPI-20	8	7	88
OPX-16	8	6	75
OPY-10	6	3	50
OPM-04	8	8	100
OPN-18	7	4	57
OPL-18	12	12	100
Total	79	65	
Range	3-12	3-12	50-100
Average	7.9	6.5	81

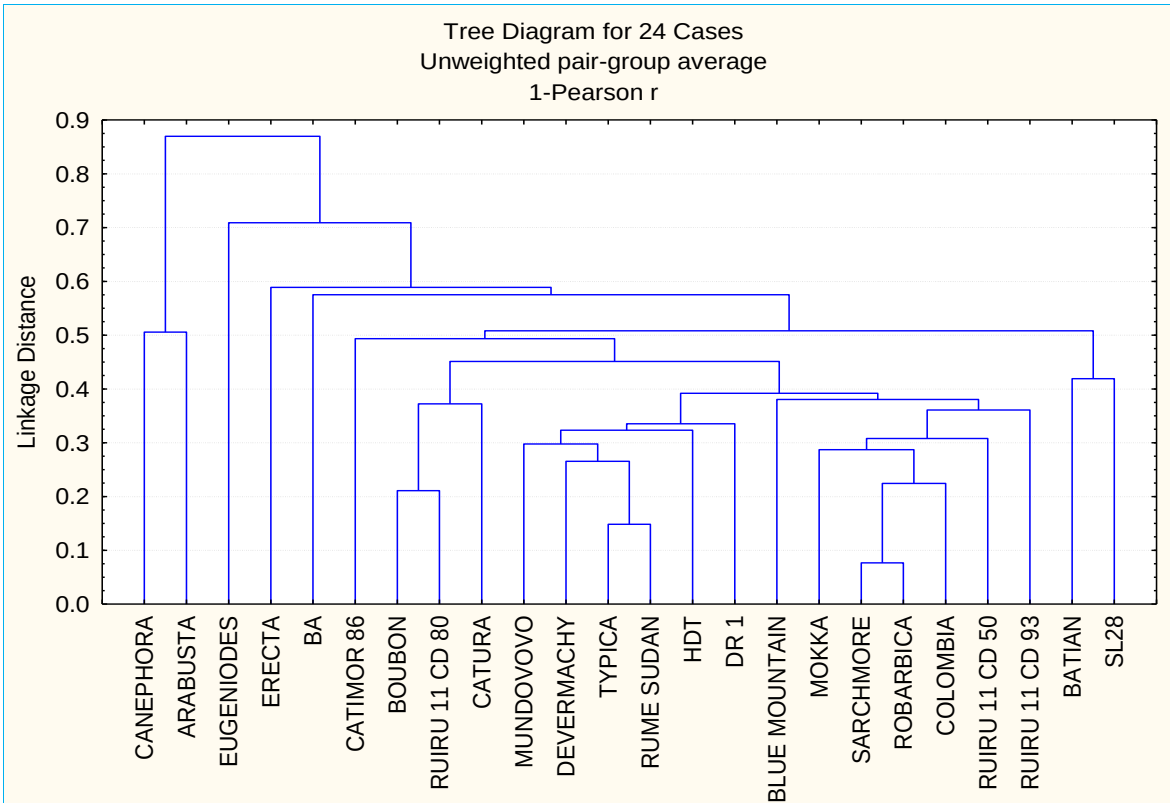

Figure 1. Dendrogram of 24 coffee genotypes based on genetic distance obtained from RAPD markers using the UPGMA method.

material in breeding programmes.

In this study, two markers, RAPD and SSR were simultaneously used to investigate the genetic diversity among 24 coffee accessionss consisting of (1) *C. canephora*, (1) *C. eugenioides* and (22) arabica genotypes. However, Results of the present study using both SSR and RAPD demonstrated the presence of low genetic variation within *C. arabica* genotypes as compared to the diploid species. Furthemore, the results did indicate high genetic similarity between *C. arabica* and *C. eugenioides* as compared to *C. canephora* thus supporting the previous research which confirmed *C. eugeniodes* as the maternal parent of *C. arabica*.

Using 13 SSR primers, a total of 50 alleles were amplified among 24 coffee genotypes. Of these amplified alleles, 33 were polymorphic. The number of amplified

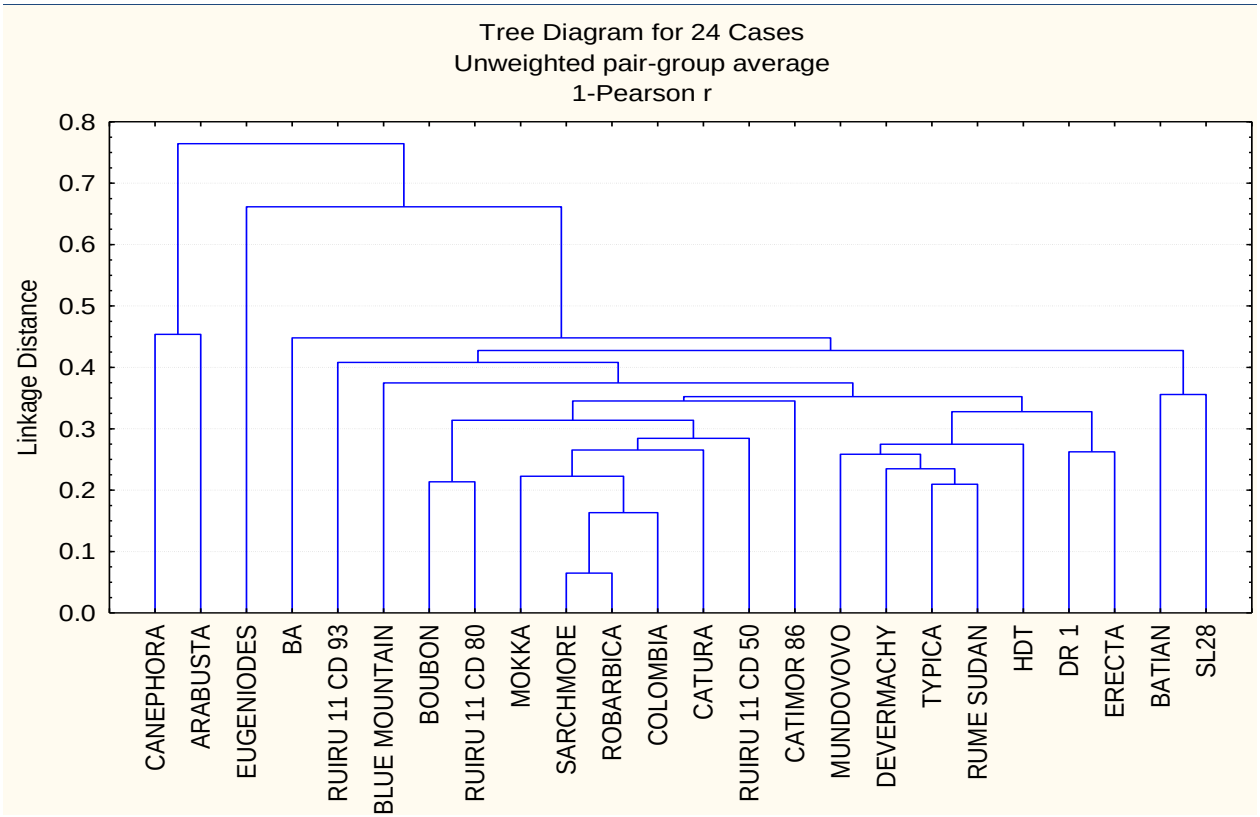

Figure 2. Dendrogram of 24 coffee genotypes based on genetic distance obtained from SSR markers using the UPGMA method.

Table 5. Amplification products generated by SSR primers.

Primer	Number of alleles	Polymorphic alleles	% polymorphism
Sat254	6	6	100
Sat 235	3	3	100
Sat11	2	1	50
Sat32	2	1	50
Sat207	3	3	100
Sat227	6	5	83
Sat240	3	2	66
Sat255	4	2	50
Sat283	6	2	33
M24	4	3	75
Sat229	4	2	50
Sat 172	3	1	33
Sat262	4	2	50
Total	50	33	
Range	2-6	1-6	33-100
Average	3.8	2.5	65

alleles per primer varied from 2.0 to 6.0 with an average value of 3.8 alleles and recording average polymorphic bands of 2.5 percent. Polymorphism ranged from 33 to 100%, with a mean of 65% polymorphism. These results are relatively similar to previous results of Anthony *et al*. *(*2002) who reported an average number of 4.7 alleles per primer using only 6 primers in arabica coffee collections containing 4 Typica, 5 Bourbon and 10 sub-

spontaneous derived accessions. Using 34 SSRs, Moncada *et al.* (2004) reported an average of 2.5 and 1.9 amplified alleles per primer in 11 wild coffee genotypes and 12 cultivated Arabica coffee respectively, with the number of alleles ranging from 1 to 8. Maluf *et al*. (2005) also reported an average number of 2.87 alleles in 28 cultivated Arabica lines using 23 SSRs. Teressa *et al*. (2010) reported a total of 209 alleles for 32 SSR markers across 133 Arabica accessions. Out of 209 alleles, 200 alleles were polymorphic for all samples. The number of observed alleles per SSRs varied from 2 to 14 with an average of 6.5 alleles for all arabica collection. The reason for such difference could be due to the sample size and the type of coffee genotypes used in the previous studies as compared to the present study. The other reason could be the number of SSRs used and their genome coverage (Teressa *et al*., 2010). The number of alleles produced in this study using 13 SSR primers was low as compared to RAPD. This illustrated the inadequacy of the SSR primers to scan different parts of the genome and to pinpoint genetic differences between coffee genotypes. Hence, a wider range of informative SSR primers need to be adopted for successful fingerprinting of *C. arabica* genotypes.

The UPGMA method of cluster analysis classified genotypes into four main clusters.The first main cluster contained genotypes of *Canephora* and Arabusta, the second cluster contained Eugenioides and the third cluster BA. Eugenioides andCanephora were genetically the most divergent genotypes from all other genotypes with dissimilarity of 1.01. The fourth main cluster contained 20 *C. arabica* genotypes .

The number of amplified bands in RAPD primers varied from 3.0 to 12.0. The total number of polymorphic fragments produced was 65. The average bands produced by the ten primers was 7.9 and recording average polymorphic bands of 6.5. Percent polymorphism ranged from 50 to 100%, with a mean of 81% polymorphism. Relatively, similar results were obtained from previous work. Lashermes *et al*. (1993) used 23 primers and reported 118 amplified fragments with number of fragments ranging from 1 to 8 and average of 4.3 fragments per primer. Agwanda *et al*. (1997) reported average of 8 fragments in *C. arabica* with number of fragments ranging from 1 to 15. Terezinha *et al*. (2002) used 52 primers on 40 coffee species and reported the average number of polymorphic bands as 6.69 per primer among all genotypes, and 1.27 among Arabica coffee genotypes. Anthony *et al*. (2002a) who reported an average number of 4.7 alleles per primer using only 6 primers in Arabica coffee collections containing 4 Typica, 5 Bourbon and 10 subspontaneous derived accessions. Aga *et al*. (2003) used 12 RAPD primers to assess genetic diversity among 144 genotypes representing 16 *C. arabica* populations. The number of polymorphic bands detected with each primer ranged from 2 to 9 with a mean of 6.2 bands per primer. Banding patterns ranged in percentage polymorphism from 37 to 73% with an overall mean of 56% for the populations analyzed. Moncada *et al*. (2004) reported an average of 2.5 and 1.9 amplified alleles per primer in 11 wild coffee genotypes and 12 cultivated Arabica coffee, respectively, with the number of alleles ranging from 1 to 8. Tshilenge *et al*. (2009) established high variability in the Congolese *C. canephora* using 7 primers. Kathurima *et al*. (2012) using 14 RAPD primers analysed 24 coffee genotypes and reported 83 amplified fragments with arange of 2 to 12 fragments per primer and 35 polymorphic fragments. Lal *et al*. (2013) used a total of 10 RAPD primers to check the genetic variation in 16 different accessions of *C. rosés*. Out of these 10, 4 primers gave satisfactory and reproducible bands. A total of 266 bands were observed. From these total bands, 234 were polymorphic while 32 bands were monomorphic. This resulted in total polymorphism of 87.96%. Bigirimana *et al*. (2013) using 6 primers reported a total bands of 19, ranging between 2-5 and average of 3.2. The reason for such difference could be due to the small sample size and the type of coffee genotypes used in the previous studies as compared to the present study.

The UPGMA method of cluster analysis classified genotypes into 5 main clusters.The first main cluster contained genotypes of *Canephora* and Arabusta, the second cluster contained Eugenioides, the third cluster contained Erecta ,the fourth contained BA and the final cluster contained 19 *C. arabica* genotypes. Sarchmore and SL28 were genetically the most divergent genotypes from all other genotypes with dissimilarity of 1.12.

The polymorphism detected by both markers ranged from 33 to 100% for SSR and 50 to 100% for RAPD with average polymorphism of 65% and 81% respectively. These results demonstrated that RAPD were suitable for genetic diversity studies in coffee accessions. Considering that the coffee genotypes evaluated in this study originated from different countries (Kenya, Tanzania, Costa Rica, India, Portugal, Brazil, Sudan, Uganda, Guatemala and Colombia), the similarities (for both SSR and RAPD results) observed among Arabica genotypes, attests to the narrow genetic diversity among Arabica coffee as reported in other studies (Lashermes *et al*., 1993; Kathurima *et al*., 2012). This could be attributed to the allotetraploid origin, reproductive biology, evolution of *C. arabica* and may also be explained by the high level of homozigosity as *C. arabica* is a self-pollinated species (Lashermes *et al*., 1995; 1999). From the general analysis, the 24 coffee accessions clustered according to the three different species namely *C. eugenioides*, *C. canephora* (Robusta) and *C. arabica* (Arabica). This is in agreement with prevoius research. Kathurima *et al*. (2012) reported coffee accessions clustered according to the three different species namely Eugenioides, Robusta and Arabica, Gimase *et al*. (2014) reported coffee genotypes clustered according to the two different species of origin namely; Robusta and Arabica. Thus, for

rapid improvement in breedingwork, widening of the existing genetic diversity through interspecific hybridisation is desirable and by having more introductions especially from the centre of diversity (Antony *et al*., 2001a), initiation of hybridisation programmes to create variability and use of diploid species as a source of desirable genes (Lashermes *et al*., 1995, 1999), diversifying the genetic base and increasing the number of varieties released for production with different genetic composition is vital, which helps to reduce losses due to disease outbreak and other constraints. Similar observation was made by Lashermes *et al*. (1993) and Agwanda *et al*. (1997).

CONCLUSIONS

Results of the present study using both SSR and RAPD demonstrated the presence of low genetic variation within *C. arabica* genotypes as compared to the diploid species (*C. canephora* and *C. eugenioides*. The narrow genetic base in Arabica coffee may also be explained by the high level of homozygosity as *C. arabica* is a self-pollinated species. Thus this call for widening of the existing genetic base through interspecific hybridisation, introducing more accessions in Kenyan cultivated Arabica coffee as well as exploring the wild coffee arabica.

A combination of different markers may provide more reliable information about genetic diversity compared to the use of a single marker because errors presented by one marker could be minimized using other markers. Both markers revealed genetic diversity within and bewteen species, thus indicating their possible utilization for genetic diversity studies. It is recommended that a wider range of informative SSR primers need to be employed for successful fingerprinting of coffee species.

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