

Full Length Research Paper

Genetic diversity among commercial coffee varieties, advanced selections and museum collections in Kenya using molecular markers

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Molecular markers have effectively been applied to study genetic diversity and as markers of particular traits. This study assessed the diversity of twenty four (24) coffee genotypes using 10 Random Amplified Polymorphic DNA (RAPD) primers and 2 microsatellites (M24 and Sat 235). A total of 35 polymorphic bands were generated by the RAPD primers. The bands were scored for presence (1) and absence (0) of amplified products. The data was subjected to cluster analysis using R statistical software and a dendrogram constructed using Unweighted Pair Group Method with Arithmetic Average (UPGMA). The genotypes separated into three main clusters. *C. eugenioides* clustered alone in the first cluster while un-introgressed Arabica genotypes dominated the second cluster. The third cluster comprised of *Coffea canephora* (Robusta) and introgressed genotypes, Ruiru 11, Hibrido de Timor and Catimor. Robusta and *C. eugenioides* were the most distantly related and generated most of the diversity observed. The similarities observed among un-introgressed Arabica genotypes attest to the narrow genetic diversity within *Coffea arabica*. The divergence observed among introgressed genotypes could be utilized in future breeding programmes.

Key words: Coffee accessions, RAPD, microsatellites, diversity, Kenya.

INTRODUCTION

Coffee belongs to the genus *Coffea* sub-genus *Coffea*, family *Rubiaceae* and is mostly present in tropical and subtropical regions of the world (Davis et al., 2006). The species which are most cultivated world-wide are *Coffea arabica* (Arabica coffee) and *Coffea canephora* (Robusta coffee). *C. arabica* is the only tetraploid species in the genus and is self-fertile, while other species are diploid and generally self-incompatible (Clarindo and Carvalho, 2008). French Missionaries introduced coffee into Kenya around 1900 AD (Mwangi, 1983). In Kenya, the commercial varieties recommended for cultivation include K7, SL 28 and SL 34. However, they are susceptible to

diseases such as Coffee Berry Disease (CBD), Coffee Leaf Rust (CLR) and Bacterial Blight of Coffee (BBC). In view of the constraint to economic coffee production, development and cultivation of disease resistant varieties is a desirable effective and viable option.

The coffee trees conserved *ex-situ* at Coffee Research Station (CRS) Ruiru in germplasm field plots has many *C. arabica* accessions from Ethiopia, Sudan, Kenya, Tanzania, Angola, India, Reunion, Portugal, South and Central America (Kathurima et al., 2009). In spite of its significance, this *Coffea* collection has not been evaluated for genetic variability mainly at DNA level. Some of these conserved genotypes have been used as progenitors in a main breeding program at CRS (Walyaro, 1983).

The coffee breeding programme successfully transferred resistance to CLR and Coffee Berry Disease

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CBD from *C. canephora*, via the interspecific hybrid referred to as Hibrido de Timor (HDT, Timor Hybrid) (*C. arabica* x *C. canephora*) (Nyoro and Sprey, 1986). The cultivar developed from this breeding regime (Ruiru11) is suitable for all coffee growing areas because it is resistant to CBD and CLR (Opile and Agwanda, 1993). Recently, five coffee lines coded Cr8, Cr22, Cr23, Cr27 and Cr30 have been under evaluation at CRF with the prospect of releasing them as commercial varieties. The recently released variety, Batian, is a selection of these lines. Their unique features include tall stature, true breeding and resistance to both CBD and CLR (Gichimu and Omondi, 2010a).

According to Jump et al. (2008) there is heavy reliance on plant genetic diversity for future crop security in agriculture and industry. However, they observed that genetic diversity for natural populations receives less attention. Like it is for many crops, evaluation of the genetic diversity and available resources within the genus *Coffea* is an important step in coffee breeding (Cubry et al., 2008). As new coffee varieties are continuously being developed through hybridization, there is a need to determine the level and sources of genetic variation within and between new and existing coffee varieties (Gichimu and Omondi, 2010a). Genetic consistency within varieties is also essential to quality assurance for any agricultural product. Hue (2005) reported that morphological variability in coffee plantations is adverse to the product quality. Reduced genetic diversity is also reported to compromise the ability of populations to evolve to cope with environmental changes and thus reducing their chances of long-term persistence (Frankham et al., 2002). Determination of genetic diversity/consistency is therefore important not only in coffee but also to other crops. A variety of techniques have been utilized to measure genetic variation of coffee species. For instance, Walyaro (1983) successfully determined the diversity of eleven coffee genotypes using morphological characteristics. Gichimu and Omondi (2010b) also determined the morphological diversity among some newly developed and existing commercial cultivars in Kenya. However, morphological markers are reportedly inefficient because they are generally dominant traits, they often exhibit epistatic interactions with other genetic traits and can also be influenced by the environment (Weising et al., 2005).

Lashermes et al. (1996a) reported that genetic factors are more accurately tested by molecular markers. In the recent past, detection of genetic variation at DNA level has been made possible by the advent of molecular markers. Several DNA analysis techniques have been used to assess genetic diversity of coffee. The techniques differ in technical requirements, cost, sequence specificity and repeatability. RAPD, Restriction Fragment Length Polymorphism (RFLP) and Amplified Fragment Length Polymorphism (AFLP) do not require prior genetic sequence analysis for primer design and

offer genome wide scanning. On the other hand, Microsatellites (Simple Sequence Repeat- SSRs) and Sequence characterised Amplified Regions (SCARs) are based on sequence specific primers with limited transferability across species but are more repeatable. In this study, RAPD were the main ones used due to their ease of use and availability of primers. In addition, SSRs that have been applied in our laboratory in the past were used. RAPD markers have been used extensively to study the genetic diversity and relationships among *Coffea* species (Lashermes et al., 1996a; Masumbuko et al., 2003; Aga et al., 2003; Tshilenge et al., 2009; Mishra et al., 2011). Microsatellites have also been applied in coffee to identify *C. arabica*, *C. canephora* and related species (Combes et al., 2000). They have also been used to investigate polymorphisms among wild and cultivated *C. arabica* accessions (Rovelli et al., 2000; Anthony et al., 2002; Baruah et al., 2003; Moncada and Couch 2004) and to analyze the introgression of DNA fragments from *C. canephora* and *C. liberica* into *C. arabica* (Lashermes et al., 2000, Lashermes, et al., 2010; Prakash et al., 2002; Gichuru et al., 2008). In Kenya, Agwanda et al., (1997) identified RAPD markers associated with CBD resistance which could be used to select against the genetic background of CBD resistance donors. Introgressed *C. canephora* fragments were identified in lines of HDT and cv Catimor and subsequently characterised as markers or candidate markers for disease resistance (Gichuru 2007; Gichuru et al., 2008). These genotypes are used as donors of resistance to CBD and CLR in Kenya. Omondi et al. (2009) reported SSR polymorphism between a disease resistance donor, Rume Sudan, and a susceptible cultivar, SL28. Most of the studies conducted focused on disease resistance. However, this study utilized RAPD and microsatellites to assess overall genetic diversity of museum genotypes, commercial varieties and upcoming coffee varieties in Kenya.

MATERIALS AND METHODS

Plant materials

Twenty four coffee genotypes comprising of one breeder's materials Catimor (Line 90), four commercial varieties, five advanced coffee selections and fourteen non-commercial accessions were used in this study (Table 1). The coffee trees of these genotypes are available in the commercial fields, experimental sites and museum plots at Coffee Research Station (CRS), Kenya.

Genomic DNA extraction

Disease-free leaves were picked from second and third nodes from the growing tips of the coffee branches for DNA extraction. Genomic DNA was extracted from the fresh leaf material by the method of Diniz et al. (2005) with minor modifications using mixed alkyltrimethylammonium bromide (MATAB).

Table 1. Status and Sources of coffee germplasm used in the studies.

S/no.	Genotypes	Status	Source
1	Marsabit	Museum accession	Wild from Northern Kenya
2	Geisha 11	Museum accession	Kitale, Kenya
3	Columnaris	Museum accession	Puerto Rico
4	Grafts	Museum accession	Not identified
5	Moca	Museum accession	Yemen
6	N39	Museum accession	Lyamungu Tanzania
7	<i>C. eugenoides</i>	Museum accession	Nandi Forest, Kenya
8	Harar	Museum accession	Ethiopia
9	Ennareta	Museum accession	Ethiopia
10	Laurina	Museum accession	LA Reunion
11	Hibrido De Timor	Museum accession	Portugal
12	Pretoria	Museum accession	Guatemala
13	K7	Commercial variety	Kenya
14	SL34	Commercial variety	Kenya
15	SL 28	Commercial variety	Kenya
16	Blue Mountain	Museum accession	Guatemala
17	Robusta	Museum accession	Uganda
18	Cross 8 (Cr 8)	Advanced Selection	Kenya
19	Cross22 (Cr 22)	Advanced Selection	Kenya
20	Cross 23 (Cr 23)	Advanced Selection	Kenya
21	Cross30 Cr 30)	Advanced Selection	Kenya
22	Cross 27 (Cr 27)	Advanced Selection	Kenya
23	Catimor - Line 90	Breeders' material	Colombia
24	Ruiru11-Line 5	Commercial variety	Kenya

Amplification of coffee genomic DNA

The method of Lashermes et al. (1996b) and modified by Agwanda et al. (1997) was used for RAPD analysis. Twenty one (21) arbitrary decamer oligonucleotides (Operon) were pre-selected and a subset showing clear amplifications were selected for analysis of the full set of study genotypes (Table 1). Amplification was carried out in a Eurogene thermocycler. The amplification program started with one cycle of initial denaturation at 94°C for 5 min followed by 45 cycles of 1 min at 94°C (denaturation), 1 min at 35°C (annealing), and 2 min at 72°C (elongation). The final extension was done at 72°C for 7 min to ensure that the primer extension reaction was completed. The RAPD products were electrophoresed in 1.8% (w/w) agarose gel and then visualised in a UV trans-illuminator after staining in ethidium bromide solution. Two microsatellites were selected based on results by Gichuru (2007) and Omondi et al. (2009). The DNA was amplified using two micro-satellites primers, Sat 235 (with forward sequence of TCGTTCTGTCATTAATCGTCAA and reverse sequence of GCAAATCATGAAAATAGTTGGTG) and M24 (with forward sequence of GGCTCGAGATATCTGTTTATAG and reverse sequence of TTAATGGGCATAGGGTCC) by the methodology described by Combes et al. (2000) but visualized in 2.3% agarose gel.

Band scoring and analysis

The bands were scored for presence (1) and absence (0) in the various genotypes. The data was organized into a matrix and

subjected to cluster analysis using R statistical software. A dendrogram was constructed using dissimilarity matrix calculation function and unweighted pair-group method using arithmetic averages (UPGMA) (Venables et al., 2006). The R command 'g clus' was used to reorder the genotypes within a cluster keeping them contiguous to each other. The cluster dendrogram constructed was used to estimate the genetic diversity among the 24 genotypes indicating how closely related or different they were. These methods have been used in similar studies.

RESULTS

Among the twenty one (21) RAPD primers tested, 16 primers showed amplification out of which 14 produced clear bands that could be clearly scored (Plate 1). The total number of fragments observed among the coffee genotypes based on the 14 RAPD primers was 83 (Table 2). The number of bands produced per primer ranged from 2 to 12. Ten out of the 14 primers generated 35 polymorphic fragments. The other four primers did not show any polymorphism. Robusta and *C. eugenoides* gave rise to most of the diversity observed while the Arabica accessions variously shared bands with these two species. The two microsatellites tested also showed varying polymorphism amongst the genotypes (Plate 2).

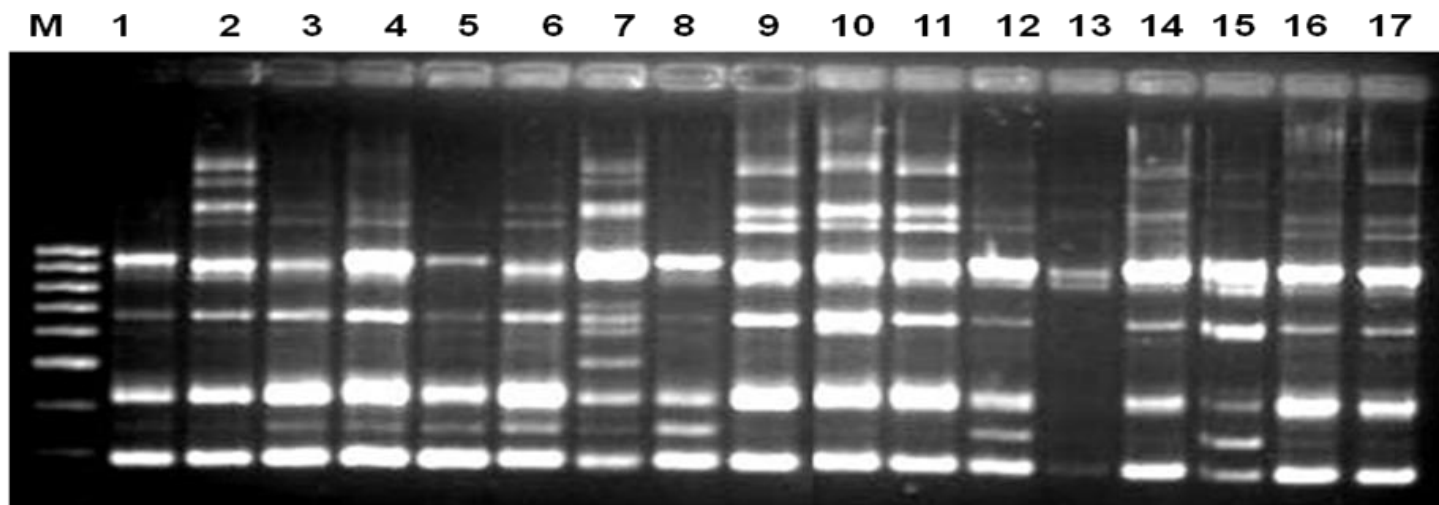


Plate 1. A panel of RAPD profiles generated by primer Y-10 in coffee populations. M is a 100 base pair marker while lanes 1–17 are coffee accessions.

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

S/no.	Primer	Total bands	Polymorphic bands	% polymorphic
1	OPI-07 - CAGCGACAAG	12	10	83
2	OPJ-19 - TGAGCCTCAC	9	4	44
3	OPK-03 - CCAGCTTAGG	5	2	40
4	OPE-05 - TCAGGGAGGT	4	1	25
5	OPE-08 - TCACCACGGT	2	0	0
6	OPE-18 - GGA CTGCAGA	8	1	13
7	OPF-15 - CCAGTACTCC	4	0	0
8	OPF-17 - AACCCGGGAA	5	0	0
9	OPG-03 - GAGCCCTCCA	4	4	100
10	OPG-05 - CTGAGACGGA	4	1	25
11	OPN-18 - GGTGAGGTCA	7	2	29
12	OPM-04 - GGCGGTTGTC	4	0	0
13	OPX-20 - CCCAGCTAGA	7	6	86
14	OPY-10 - CAAACGTGGG	8	4	50
		83	35	42

M is 100 base pair marker while 1 to 13 are coffee samples. The lower band (arrowed) is a marker for a Robusta genomic fragment present in HDT and its derivatives. A cluster dendrogram constructed using polymorphic bands was used to estimate the genetic diversity of the twenty four coffee accessions (Figure 1). The genotypes were separated into three main clusters. *C. eugenioides* clustered alone in the first cluster, Arabica accessions dominated the second cluster while the third cluster contained Robusta, Ruiru 11, Hibrido de Timor and Catimor. The R command 'gclus' which was used to reorder the genotypes within and among clusters and

keeping them contiguous to each other depicted Robusta and *C. eugenioides* as the most distantly related. Except for *C. eugenioides*, the maximum dissimilarity index observed was 0.10.

DISCUSSION

Molecular markers have been widely applied in studying the diversity of coffee. Use of different DNA technologies in such studies are expected to generate similar genetic relationships but have different degree of separation and

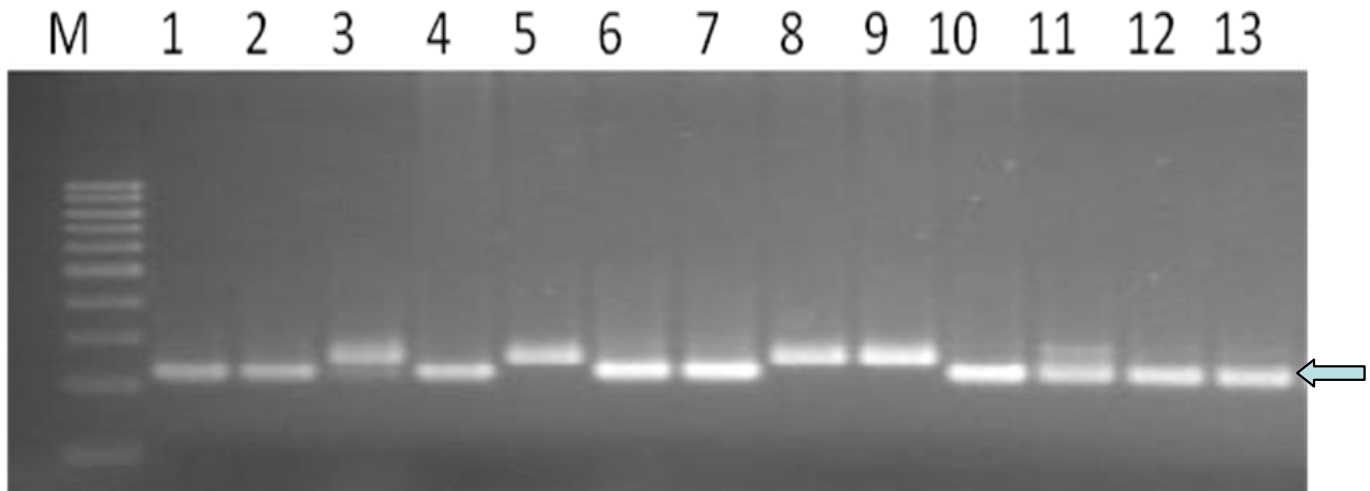


Plate 2. An example of a polymorphic band pattern generated by Sat 235 on the coffee genotypes.

reliability depending on the numbers of primers used and polymorphic bands generated. The results demonstrate that RAPD markers were able to reveal variability among the coffee accessions tested. This agrees with other researchers Lashermes et al. (1993); Agwanda et al. (1997); Anthony et al. (2001, 2002); Aga et al. (2003); Masumbuko et al. (2003) who reported the successful use of RAPD in genetic characterization in *Coffea* species. Although, Hibrido de Timor (HDT), Ruiru 11 and Catimor 90 clustered together with Robusta, it was apparent from the general analysis that the coffee accessions clustered according to the three different species namely Eugenioides, Robusta and Arabica. Thus, for rapid improvement in breeding work, widening of the existing genetic base through interspecific hybridisation is desirable. Similar observation was made by Lashermes et al. (1993) and Agwanda et al. (1997). A study of genetic diversity based on RAPD marker demonstrated high diversity in the spontaneous and sub-spontaneous hybridized materials of Ethiopia (Anthony et al., 2002).

Considering that the coffee genotypes evaluated in this study originated from different countries (Kenya, Puerto Rico, Tanzania, Ethiopia, Reunion, Portugal, Yemen, Guatemala and Colombia), the similarities observed among Arabica genotypes, attests to the narrow genetic diversity among cultivated Arabica coffee reported in other studies (Lashermes et al., 1993). Comparatively, higher genetic diversity has been reported among wild coffee populations than within cultivated genotypes (Anthony et al., 2000; Aga et al., 2003; Masumbuko et al., 2003; Masumbuko and Bryngelsson 2006; Maluf et al., 2005). Close genetic proximity was observed among the existing traditional commercial varieties in Kenya, namely SL28, SL34 and K7. These results are in agreement with the work of Agwanda et al. (1997) and Hue (2005) which revealed high genetic similarity between Kenyan

traditional commercial varieties. In this study, the accession Marsabit which is a wild accession from Northern Kenya clustered with K7 which confirmed the findings of Lashermes et al. (1996b) that cultivar K7 was closely related to an accession collected in Marsabit Mountain.

Hibrido de Timor (HDT), Catimor Line 90 and Ruiru 11, were found to be genetically divergent from the rest of the varieties and bearing close relationship to Robusta coffee. Similar observations were made by Agwanda et al. (1997) and Lashermes et al. (1996b). Different lines of HDT have been used worldwide to breed coffee varieties that are resistant to different pathogens. As would be expected, different accessions of HDT derivatives have different levels of introgressed *C. canephora* genome (Lashermes et al., 2000; Silveira et al., 2003). This could explain the close relationship observed between HDT, Ruiru 11 and Catimor Line 90 to Robusta. On the other hand, the cultivar Ruiru 11 is a composite F1 hybrid between lines of the variety Catimor, (as the female parent), and male selections most of which have HDT in their pedigree. The breeding programmes to development the male parents involved backcrossing and selfing at various selection stages which affected the amount of Robusta genome passed on to the next generation. This can explain the wide range of diversity observed between HDT and its derivatives (Catimor Line 90, Ruiru 11 line 5, Cr8, Cr22, Cr23, Cr27 and Cr30).

The objective of this study was to widen the information on genetic diversity of coffee germplasm available for breeding programmes in Kenya since previous work was biased to commercial cultivars and donors of resistance to diseases. The study confirms the low genetic diversity in the Arabica coffee genotypes evaluated with dissimilarity of less than 5% (Figure 1). However, there is diversity that can be exploited in breeding programmes (Plates 3 and 4) and the possibility of identifying DNA

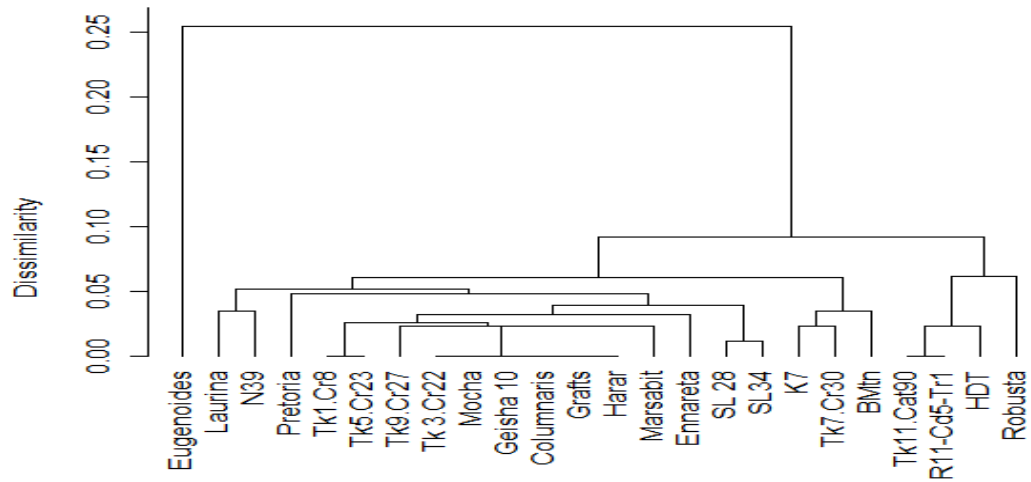


Figure 1. Cluster dendrogram illustrating genetic diversity among twenty four coffee genotypes.

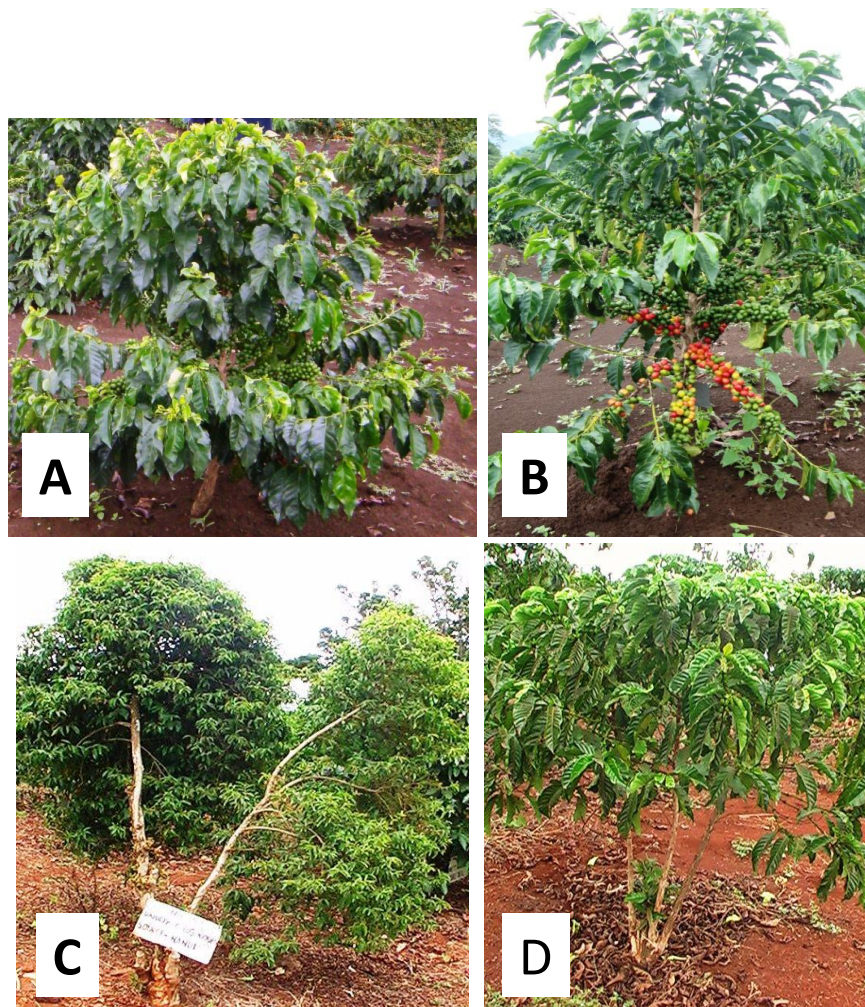


Plate 3. Differences in the canopies of various coffee varieties and species; (A) the compact Ruiru II, (B) the tall advanced selection, (C) the bushy *C. eugenioides*, (D) the robust *C. canephora*.

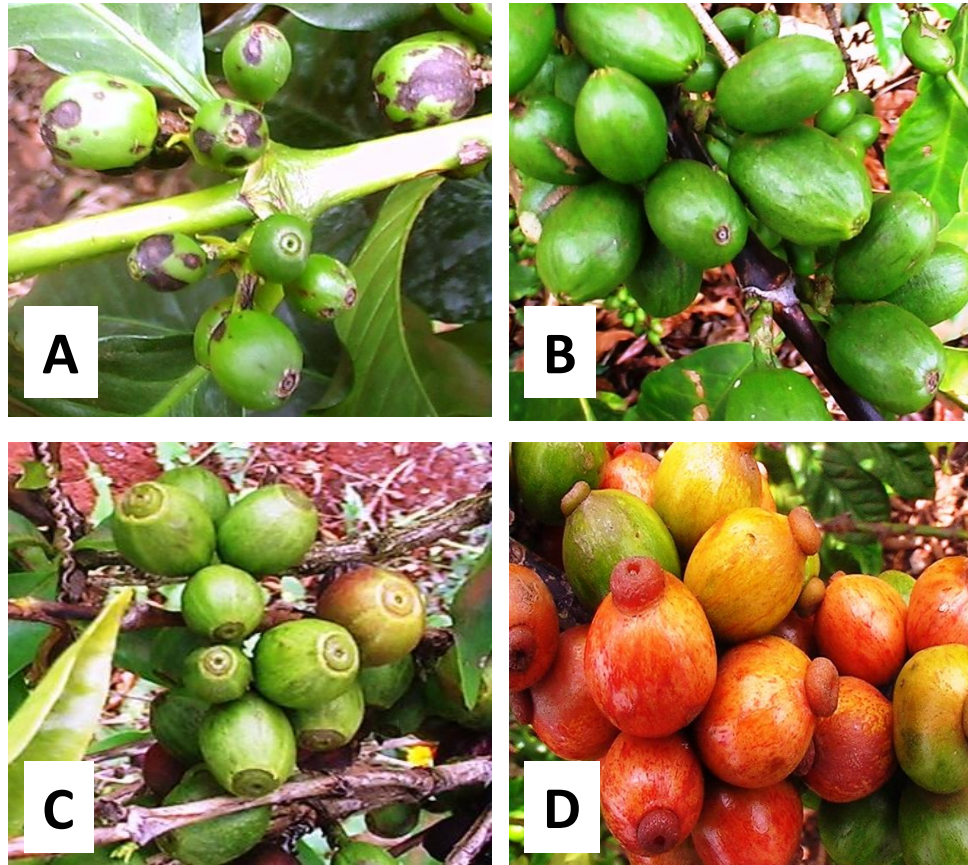


Plate 4. Differences in the berries of various coffee varieties and species; (A) SL 34 showing infection by Coffee Berry Disease, (B) Hibrido de Timor, (C) *C. eugenioides*, (D) *C. canephora*.

markers was demonstrated. It needs to be noted that the material analysed in this study represent a fraction of germplasm conserved in CRS museum plots and breeding materials. Previous breeding has focussed on the introduction of pest resistance into cultivated varieties. In fact the Sat 235 used in this study is linked to a gene of resistance to CBD (Gichuru et al., 2008). Future studies need to explore more germplasm and also attempt to relate the observed diversity to other traits with an objective of identifying parents for various breeding programmes. The traits can range from agronomic and quality related traits (such as caffeine content) to novel utilization of coffee such as aesthetics. The divergence of HDT derivatives is also of importance in breeding programmes. For example, Ruiru 11 progenies comprise of 60 lines (Omondi et al., 2001) and therefore more intensive molecular analysis could help in characterizing them and consequently selecting elite lines.

Conclusion

Generally, the study confirmed the narrow genetic base

reported in *C. arabica* and emphasized the need to widen the existing genetic diversity through interspecific hybridisation. Although, RAPD markers were able to determine variability among the coffee accessions tested, combination of multiple molecular techniques (such as AFLP, RFLP, SSRs, and SCARs) may reveal more accurate estimation of genetic diversity and relate the diversity to qualitative traits in subsequent studies.

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