Sciknow Publications Ltd.
Journal of Renewable Agriculture

JRA 2013, 1(6): 106-112
DOI: 10.12966/jra.09.01.2013

Genetic Diversity among Disease Resistant Coffee Varieties and Cultivars in Rwanda Based On RAPD and SSR Markers

J. Bigirimana[1,2,*], Kiarie Njoroge[2], J.W. Muthomi[2], D. Gahakwa[1], N.A. Phiri[3], E.K. Gichuru[4], & D.J. Walyaro[1]

[1] Rwanda Agriculture Board, P.O. Box 5016, Kigali, Rwanda.
[2] Department of Plant Science and Crop Protection, University of Nairobi, P.O. Box 29053 -00625, Nairobi, Kenya.
[3] CABI Africa, P.O. Box 633 - 00621, Nairobi, Kenya.
[4] Coffee Research Foundation, P.O. Box 4 - 00232, Ruiru, Kenya.

*Corresponding author (Email: bigjos2200@yahoo.fr)

Abstract - Understanding the genetic diversity existing in a germplasm collection is important to identify parental combinations with distinct gene sets that can be used in crossing to obtain superior hybrids. This study was carried out to evaluate the genetic diversity in the Rwandan coffee germplasm using both RAPD and SSR markers and to determine any relationship between these two markers. Varieties with resistance to coffee leaf rust and coffee berry disease as well as local cultivars in Rwanda were used for DNA extraction and subjected to PCR amplification. The highest values for genetic distances were obtained between BM 139 and HDT and between Harar and Catimor implying that these varieties were genetically quite distinct. The lowest values for genetic distance were recorded between BM 139 and BM 71 and between Matinho and Rume Sudan implying that these varieties were genetically fairly similar. A Pearson correlation of 55.52% was obtained between the two markers indicating a moderate association between these two analytical procedures. It is recommended that crosses should be made between genetically distant susceptible and resistant varieties to derive hybrids that combine resistance with marked levels of heterosis particularly for yield.

Keywords - DNA Extraction, Heterosis, Genetic Distances, Hybrids

1. Introduction

Coffee is an important agricultural export commodity and accounts for the bulk of the export earnings in more than 50 developing countries of Africa, Asia and Latin America [1]. For Rwanda in particular, it accounts for about 25% of its annual foreign exchange earnings [2]. The major constraints to coffee productivity in Rwanda include prolonged periods of moisture stress, low soil fertility, pests and diseases such as coffee leaf rust (CLR) and coffee berry disease (CBD). CBD for example, can cause up to 80% crop loss if not controlled and conditions are favorable [3]. Preventive control by fungicide sprays can account for 30 – 40% of total production costs and annual economic damage in Africa has been estimated at US$ 300 - 500 million [4]. CLR, which is the major disease on coffee in Rwanda, is estimated to cause crop losses of up 40% annually [5]. Cultivation of resistant varieties is the most viable and effective option for disease management.

Rwanda has introduced coffee accessions from various sources including among others, Ethiopia, Democratic Republic of Congo (DRC), Brazil, Uganda and Kenya. These accessions are currently maintained *ex-situ* in germplasm collection plots. The collections include varieties of several species e.g. *Coffea arabica*, *Coffea canephora* and *Coffea liberica.* Though the commercial coffee varieties grown in Rwanda were selected among these accessions, many others are of no direct commercial value but represent an important source of genetic variation for characteristics such as canopy architecture, pests and disease resistance, yield, quality and other agronomic and industrial traits.

The knowledge of genetic diversity existing in a germplasm collection is useful in the development of new varieties [6]. Determination of genetic diversity helps in identification of parental combinations, in order to use distinct gene sets in crossing to obtain superior hybrids [7]. Determination of genetic diversity among coffee accessions was often based on evaluation of morphometric characters; however, more recently a number of molecular techniques have been used to measure this diversity in coffee. Lashermes et al. [8] used RAPD markers to study the genetic variability and relationships of *Coffea* species and showed consistent relation to the known history and evolution of the *Coffea* species. Aga et al. [9] showed the existence of four different populations for *in-situ* conservation of Ethiopian *Coffea arabica* species

while Masumbuko et al. [10] suggested the widening of the existing genetic base by introducing more accessions in Tanzanian cultivated Arabica coffee.

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 [7, 11]. This study was carried out with the following objectives: (1) to evaluate the genetic diversity in the Rwandan coffee germplasm using RAPD and SSR markers and (2) to determine any relationship between data generated with these two analytical procedures.

2. Material and Methods

2.1. Plant Material

Fourteen genotypes were used in the study. Their characteristics are described in table 1. Seeds of these varieties were germinated in sterile sand, transplanted into polythene bags and then transferred into a nursery. The leaf samples used for DNA extraction were collected from 8 months old seedlings growing in the nursery.

Table 1. Description of Coffee Genotypes Used in the Study

Name	Source	Type	CLR and CBD Resistance phenotype
Jackson 2/1257	DRC	Cultivar	susceptible
Matinho	Angola	Semi wild	resistant
Catimor	Costa Rica	Breeding line	resistant
BM 71	DRC	Cultivar	susceptible
Selection 5A	India	Breeding line	resistant
Harar	Ethiopia	Cultivar	susceptible
CIFC 8224	Portugal	Breeding line	resistant
Pop 3303/21	DRC	Cultivar	susceptible
Mibilizi	DRC	Cultivar	susceptible
SL 28	Kenya	Cultivar	susceptible
Rume Sudan	Sudan	Semi wild	resistant
Hibrido de Timor	Kenya	Wild	resistant
Selection 6	India	Breeding line	resistant
BM 139	DRC	Cultivar	CBD resistant but susceptible to CLR

2.2. DNA Extraction

Genomic DNA was extracted from fresh leaves following the method described by Diniz et al. [12] with minor modifications. Five hundred milligrams of fresh leaf material without mid-veins were frozen in liquid nitrogen and ground to powder and 0.35 – 0.4 g of the powder was collected in eppendorf tubes and 800 µl of extraction buffer (400 µl of lysis and 400 µl of extraction buffer) added. The mixture was incubated for 30 min at 62^{o} C and 1 ml of chloroform and isoamyl alcohol (24:1 v/v) was added and centrifuged at 13 000 rpr for 5 min and the supernatant was discarded.

The pellet was then washed with 250 µl of 70 % ethyl alcohol and the mixture was homogenized with gentle inversion and the supernatant was discarded. The pellet was air dried and dissolved in 50 µl of TE (10 mM Tris-HCl, 1 mM EDTA pH 8) and left at 4^{o} C over night to dissolve. The resulting aqueous fraction was incubated with 20 µl of RNAse (10 mg/ml) at 37^{o} C for 30 min and kept at – 20^{o} C for later use. The DNA quality was checked by electrophoresis in 1 % agarose gel at 50 W for 45 min. The DNA was visualized and photographed in UV trans-illuminator chamber after staining in Ethidium bromide (2.5 mg/l) for 20 minutes. The DNA quantity was estimated by visually comparing the obtained bands for different varieties with standardized Lambda DNA ladders.

2.3. Determination of Genetic Diversity by RAPD-PCR Analysis

Fifteen primers were screened on all samples to identify the ones which could detect polymorphism among varieties. Nine primers which showed variations were selected for further analyses (Table 2). The DNA amplification was performed on 25 µl reaction mix containing 12 µl of double distilled water, 2.5 µl of the buffer (75 mM Tris – HCl, pH 8.8, 20 mM $(NH_4)_2SO_4$, 0.01% (v/v) Tween 20), 2.5 µl of $MgCl_2$ (25mM), 1 µl of the primer (10 mM), 0.2 units of Taq polymerase, 3.8 µl of dNTPs (500 µM) (100 µM each of dATP, dCTP, dGTTP and dTTP) and 1 ng per µl of sample DNA. A master mix was prepared for each primer to minimize measurement deviations.

Amplification reactions were carried out in a

Techne-thermocycler with one cycle of initial strand separation at 94° C for 3 minutes followed by 45 cycles of 1 minute at 94° C, 1 minute at 37° C, and 2 minutes at 72° C. The last cycle was followed by an additional extension at 72° C for 10 minutes. The PCR amplification products were separated on 2.3% agarose gel and run in 1 x TE buffer (40 mM Tris acetate pH 8.0, 1 mM EDTA) at 100 volts for 4 hours. Staining was done using ethidium bromide (2.5 mg/l) for 30 minutes and the DNA was visualized on a UV transilluminator chamber and photographed using polaroid film. The gel photographs were evaluated as the presence (1) or absence (0) of the amplified bands.

2.4. Determination of Genetic Diversity by Microsatellite Analysis

Fifteen SSR primer pairs were screened for PCR amplification and only six primers that generated polymorphism were used for SSR analysis. Reaction master mix consisted of 25 µl containing 5 µl of 10 ng/ µl genomic DNA, 2.5 µl of buffer (10X, Promega), 2.5 µl of the $MgCl_2$ (25 mM, Promega), 7.5 µl of SSR dNTPs (dNTPs stock with a little dATPs), 2.5 µl each of right and left primers, 0.2 units of Taq DNA polymerase and 2.3 µl of double distilled water. The PCR programme consisted of an initial denaturation at 94° C for 2 min, followed by 5 cycles of 45 seconds of denaturation at 94° C, 1 minute primer annealing at 60° C reducing by 1° C every cycle, elongation for 1 minute at 72° C and 30 cycles of 45 seconds of denaturation at 90° C for 45 seconds, primer annealing at 55° C for 1 minute and elongation at 72° C for 1 minute 30 seconds and final extension of 8 minutes at 72° C.

Denaturing polyacrylamide (6%) gel was prepared in 33 cm x 39 cm casting plates separated by 0.35 mm spacers. A plane mould was inserted at the top and the gels were left overnight for use the next day. The gels were assembled in vertical electrophoresis and 1X TE buffer for running was placed into the top reservoirs to cover the inner smaller plates. After ascertaining that there was no leakage, the plane moulds were removed and the gels rinsed with the running buffer. More running buffer was put into the bottom reservoir and pre-runs were made at 55 W per gel for about 20 min before the samples were loaded. Similar to RAPD analysis, the polyacrylamide gels were interpreted for the presence (1) or absence (0) of the bands. Each of the bands was treated as an independent character.

2.5. Statistical Analysis

Genetic distances (GD) between genotypes were estimated using the method of Van der Peer & De Wachter [13] as follows:

$GDxy = (Nx + Ny)/(Nx + Ny + Nxy)$

Where,

Nx : Number of bands in line X and not in line Y

Ny : Number of bands in line Y and not in line X

Nxy: Number of bands in line Y and in line X

The similarity matrices computed for each pair of genotypes were subjected to cluster analysis using the Unweighted Pair – Group Method with Arithmetic Averages (UPGMA) and dendrograms were generated using SPSS (2007) to visualize the genetic similarity between varieties. Data generated using microsatellites and RAPD analyses were also analyzed for correlation using Microsoft Excel 2007.

3. Results

3.1. Polymorphic Information Generated by RAPD Analysis

The nine RAPD primers generated a total of 43 polymorphic bands across 14 coffee varieties. The number of bands per primer varied from two (N-18) to eight (I-7) with an average of 4.8 bands per primer, and the estimated molecular weight was in the range of 180 to 1000 base pairs (Table 2). Of the nine RAPD primers, 60.5 percent of the polymorphic bands were produced by 4 primers (X-20, M-4, I-7 and Y-15) whose frequencies ranged from 0.14 to 0.19.

Table 2. Primers Used in the RAPD Analysis Along with Their Nucleotide Sequences, Number of Polymorphic Bands and Estimated Molecular Size Bands

Primer	Sequences 5' to 3'	Number of polymorphic bands	Molecular size range
X-20	CCCAGCTAGA	6	300 to 700 bp
Y-10	CAAACGTGGG	4	200 to 800 bp
M-4	GGCGGTTGTC	6	200 to 700 bp
L-18	ACCACCCACC	3	320 to 600 bp
I-7	CAGCGACAAG	8	180 to 1100 bp
N-18	GGTGAGGTCA	2	350 to 900 bp
J-19	GGACACCACT	5	400 to 950 bp
X-16	CTCTGTTCGG	3	220 to 500 bp
Y-15	AGTCGCCCTT	6	350 to 800 bp
Total		**43**	
Mean		**4.8**	
Range		**2 to 8**	**180 to 1100 bp**

Table 3. Microsatellite Primers Used, the Number of Alleles Detected Per Primer and Their Estimated Allele Sizes

Primer	Nucleotide sequence	Number of alleles	Estimated allele size
M24	GGCTCGAGATATCTGTTTAG (Forward)	5	450 - 800 bp
M24	TTTAATGGGCATAGGGTCC (Reverse)		
Sat 235	TCGTTCTGTCATTAAATCGTCAA (Forward)	4	320 - 750 bp
Sat 235	GCAAAATCATGAAAATAGTTGGTG (Reverse)		
Sat 172	ACGCAGGTGGTAGAAGAATG (Forward)	3	350 - 600 bp
Sat 172	TCAAAGCAGTAGTAGCGGATG (Reverse)		
Sat 227	TGCTTGGTATCCTCACATTCA (Forward)	2	220 -700 bp
Sat 227	ATCCAATGGAGTGTGTTGCT (Reverse)		
Sat 229	TTCTAAGTTGTTAAACGAGACGCTTA (Forward)	3	180 - 550 bp
Sat 229	TTCCTCCATGCCCATATTG (Reverse)		
Sat 254	ATGTTCTTCGCTTCGCTAAC (Forward)	2	250 - 650 bp
Sat 254	AAGTGTGGGAGTGTCTGCAT (Reverse)		
Total		**19**	
Mean		**3.2**	
Range		**2 to 5**	**180 - 800 bp**

The similarity matrix revealed values ranging from 0.12 to 0.67 with an average of 0.45 (Table 3). The highest genetic distances were obtained between BM 139 and HDT and between Harar and Catimor implying that these varieties were genetically different. However, the lowest values for genetic distance were recorded between BM 71 and BM 139 and between Matinho and Rume Sudan implying in turn that these varieties were genetically similar. The generated dendrogram for all analyzed varieties showed five clusters (*figure 1)*

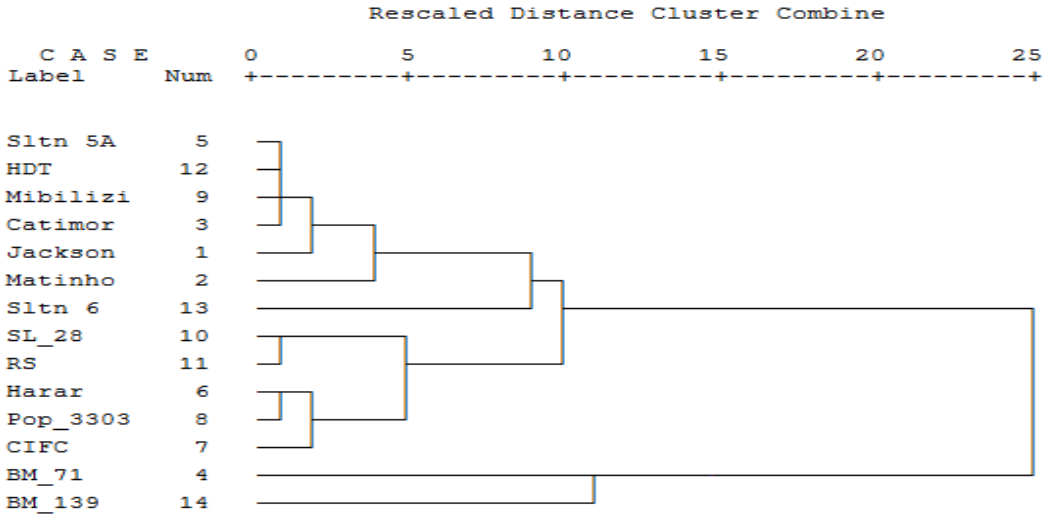

Figure 1. Relationships between 14 Arabica Genotypes Generated by Unweighted Pair Group Method Using Arithmetic Averages (UPGMA) Based on RAPD Markers

3.2. Polymorphism Obtained with SSR Markers

The analysis with six SSR markers revealed a total of 19 alleles across the fourteen varieties. The number of alleles per primer ranged from two (Sat 254 and Sat 227) to five (M24) with an average number of 3.2 alleles per used primer. The estimated allele size for all primers ranged from 180 bp to 800 bp (Table 3). Three primers (M24, Sat 235 and Sat 227) generated 57.9% of the total number of alleles with an overall frequency ranging from 0.11 to 0.26. The highest genetic distances were obtained between Mibilizi and Rume Sudan and between BM 139 and Hibrido de Timor indicating that these varieties were genetically distinct. The lowest values were obtained between varieties BM 71 and Mibilizi and BM

139 and BM 71, respectively indicating that these varieties were genetically similar. The similarity matrix showed values ranging from 0.13 to 0.78 with an average of 0.46 (Table 4). Unlike with the RAPD analysis, CLR and CBD resistant varieties were clustered separately from the commercial coffee cultivars (Figure 2).

Table 4. Matrix of Genetic Distance between 14 Genotypes Based on SSR Markers

	BM 139	Json	Mno	Cmor	BM 71	Sel 5A	Hrar	CIFC	Pop3303	Mzi	SL 28	RS	HDT
Json	0.37												
Mho	0.66	0.48											
Cmor	0.55	0.53	0.58										
BM 71	0.19	0.28	0.55	0.56									
Sel 5A	0.39	0.57	0.67	0.37	0.69								
Harar	0.24	0.34	0.39	0.58	0.33	0.56							
CIFC	0.51	0.43	0.2	0.23	0.53	0.21	0.53						
Pop 3303	0.2	0.26	0.51	0.51	0.17	0.68	0.2	0.57					
Mzi	0.27	0.18	0.65	0.56	0.13	0.69	0.23	0.65	0.35				
SL 28	0.25	0.26	0.37	0.63	0.24	0.66	0.21	0.52	0.38	0.33			
RS	0.6	0.44	0.34	0.23	0.58	0.25	0.23	0.35	0.6	0.78	0.68		
HDT	0.71	0.67	0.55	0.31	0.52	0.37	0.54	0.69	0.53	0.56	0.51	0.45	
Sel 6A	0.68	0.7	0.56	0.32	0.67	0.26	0.63	0.8	0.62	0.7	0.67	0.34	0.3

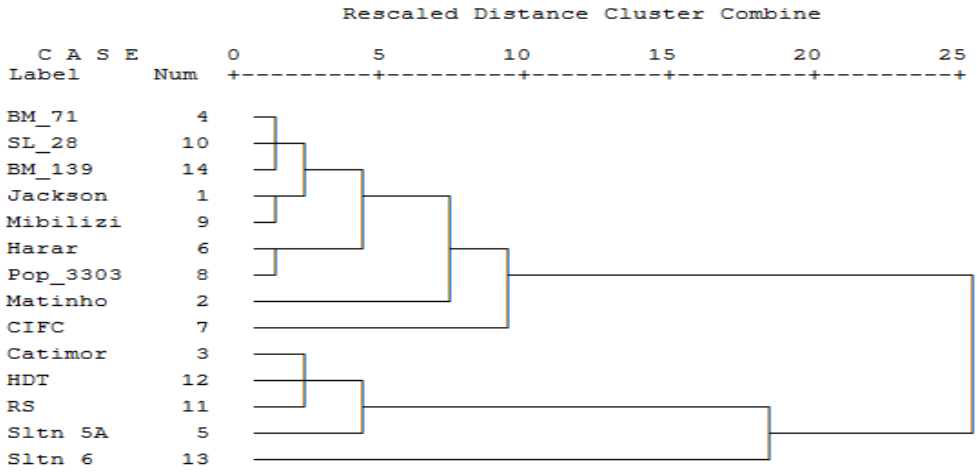

Figure 2. Relationship between 14 Arabica Genotypes Generated by Unweighted Pair Group Method Using Arithmetic Averages (UPGMA) Based on SSR Markers

3.3. Relationship between Genetic Distances Obtained with RAPD and SSR Markers

Estimates of correlation between genetic distances obtained by RAPD and SSR markers were moderate, with magnitudes of 55.52%, indicating that there is a relative pattern of association between results obtained using these two procedures *(Figure 3)*. The linear regression equation was found to be $Y = 0.8067 X + 0.1144$, where Y was the genetic distance generated based on RAPD markers and X the genetic distance with SSR markers. Both slope and intercept were highly significant ($p < 0.001$).

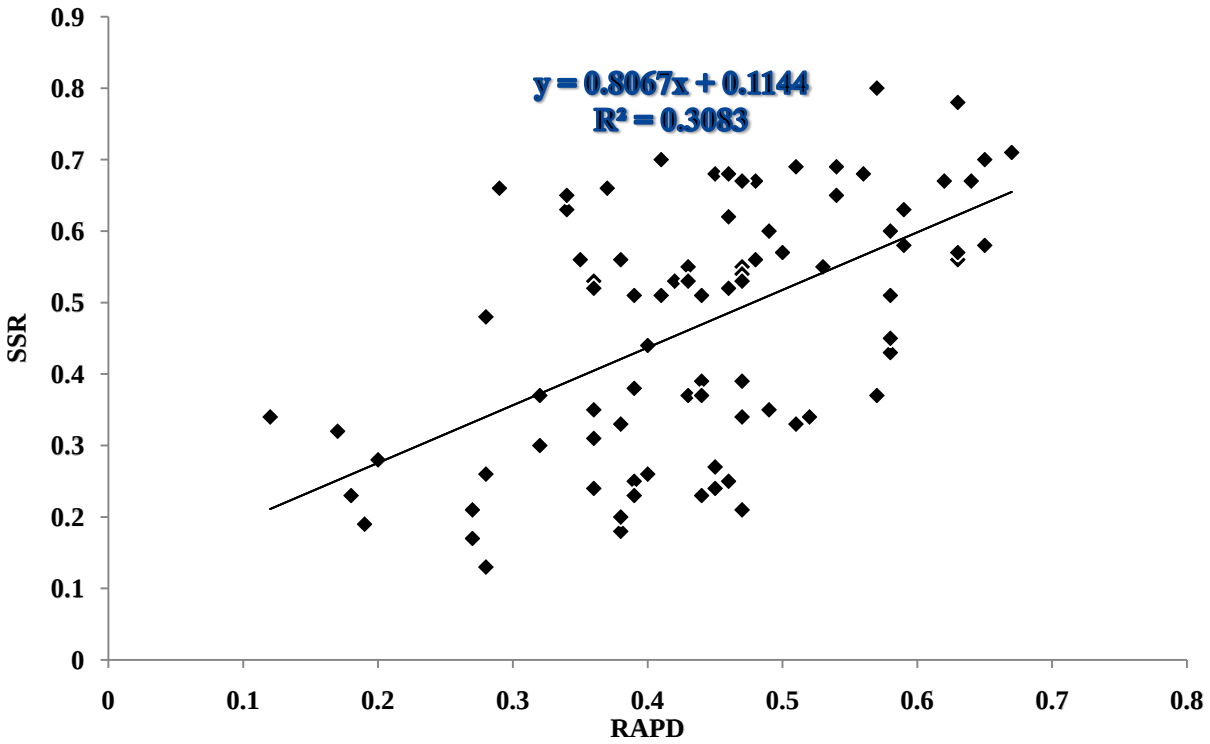

Figure 3. Relationship between Genetic Distances Generated by SSR and RAPD Markers

4. Discussion

The results of the study indicated that the highest values for genetic distance were obtained between Mibilizi and Rume Sudan and between BM 139 and Hibrido de Timor (HDT) for both markers. The genetic dissimilarity between BM 139 and HDT may be explained by their divergent background because BM 139 is a selection among Arabica coffee introductions in Mulungu while HDT is a natural interspecific hybrid between *Coffea arabica* L. and *Coffea canephora* Pierre found in East Timor [14]. While working on different coffee species, Lashermes et al. [8] also observed genetic differences between Arabica coffee and HDT. On the other hand, the genetic differences between Rume Sudan and Mibilizi may be attributed to their genetic genealogy because Mibilizi is a selection from Mulungu whereas Rume Sudan originated in the Boma plateau of Sudan, a region adjacent to the primary centre of genetic diversity situated in the highlands of south west Ethiopia. Leal et al. [7] working on lines with different origins also reported that varieties coming from different locations tended to be genetically different from lines of the same provenance.

While Mibilizi and Rume Sudan showed the highest genetic distance, BM 71 and Mibilizi and BM 139 and BM 71 displayed the lowest values. The genetic similarity between BM 71 and Mibilizi and BM 139 and BM 71 may be explained by their origins as they were selected among introductions from Mulungu. BM 139 and BM 71 were probably developed from the same parents by single tree selections. In addition, these are Arabica coffee varieties which are reported to have a low genetic diversity [6]. The narrow genetic base in Arabica coffee may also be explained by the high level of homozigosity as *C. arabica* is a self-pollinated species. Analysis by SSR markers grouped local cultivars and varieties that are resistant to CLR and CBD in separate clusters. Similar results are reported by Agwanda et al. [15] using RAPD markers; while Setotaw et al. [16] assessing the genetic diversity of Hibrido de Timor germplasm found that the trees constructed using SSR and RAPD systems were very similar.

In this study, UPGMA analysis of the RAPD and SSR markers showed some similarities. For instance, varieties BM 71 and HDT were placed at the ends of dendrograms. However, some varieties such as Harar and Selection 5A occupied different positions. Maluf et al. [17] evaluating the genetic diversity of cultivated Arabica coffee inbred lines using RAPD, SSR and AFLP markers also observed that some genotypes were not similarly distributed in clusters produced with RAPD and SSR markers. The possible explanation to these dissimilarities may be the fact that molecular markers evaluate different components of DNA variations which can progress in diverse ways (Collard et al. [18]).

Moderate association was obtained between genetic distances based on RAPD and SSR markers, indicating some differences between the two molecular techniques. Similar observations are reported in other studies on maize by Souza et al. [19] and Leal et al. [7] who found correlations of 0.54 and 0.55, respectively, between RAPD and SSR markers.

The study showed genetic diversity between commercial coffee cultivars in Rwanda and CBD and CLR resistant varieties. The latter category included varieties such as Rume Sudan and Selection 6, Catimor and CIFC 8224. However, despite the poor yield and other inferior agronomic traits, variety Rume Sudan has good resistance to CBD and CLR and has been used along with Catimor in the breeding program for resistance to CBD and CLR in Kenya which led to the development of variety Ruiru 11 [14]. Similarly, concerning disease resistant variety CIFC 8224, Setotaw et al. [16] reported that it originated from natural interspecific hybridization between *C. arabica* and *C. canephora* and has also been used for the development of CBD and CLR resistant varieties all over the world.

The results obtained in this study may be useful in planning breeding procedures for the development of varieties that combine resistance to CLR and CBD and other desirable traits such as high yield and better quality. For example, crosses should be made between genetically distant susceptible and resistant varieties in order to derive hybrids that combine disease resistance with marked levels of heterosis particularly for yield.

5. Conclusions

The knowledge of the genetic diversity is important to identify parental combinations with distinct gene sets that can be used in crossing to obtain superior hybrids. Local cultivars such as BM 139, BM 71 and Mibilizi are genetically quite distinct from CBD and CLR resistant varieties like Rume Sudan, Selection 6 and HDT. Advantage should be taken of this situation to make crosses between these two groups of varieties in order to derive disease resistant hybrids that combine a marked level of heterosis for yield with improved adaptation and quality.

Acknowledgements

The authors would like to thank Common Fund for Commodities (CFC) for funding this study through a Coffee Leaf Rust Project. This project was executed by CABI and supervised by the International Coffee Organization. Authors are also grateful to Coffee Research Foundation (CRF), Ruiru, Kenya for providing the facilities for laboratory work. The views expressed are not necessarily those of CFC.

References

[1] Rani, V., Singh, K.P., Shiran, B., Nandy, S., Goel, S., Devarumath, R.M., … Raina, S.N. (2000). Evidence for new nuclear and mitochondrial genome organizations among high-frequency somatic embryogenesis derived plants of allotetraploid Coffea arabica L. (Rubiaceae). Plant Cell Reproductions, 19, 1013 -1020.

[2] National Bank of Rwanda, (2009). *Annual report*. Available at http: //www.bnr.rw/docs/publicnotices.

[3] Masaba, D.M. & Waller, J.M. (1992). Coffee berry disease: the current status. In: Bailey, J.A., & Jeger, M.J. (eds.), Colletotrichum: Biology, Pathology and Control (pp. 237-249). CAB International, Wallingford, UK.

[4] Van der Vossen, H.A.M. & Walyaro, D.J. (2009). Additional evidence for oligogenic inheritance of durable host resistance to coffee berry disease (Colletotrichum kahawae) in arabica coffee (Coffea arabica, L.). Euphtica, 165, 105 – 111.

[5] Gatarahiya, C.M, Mushimiyimana, S., Bigirimana, J. & Phiri, N. (2010) Current status and management of coffee leaf rust disease in Rwanda. Proceedings of the 23rd ASIC Conference, Bali, Indonesia.

[6] Silvestrini, M., Maluf, M.P., Silvarolla, M.B., Filho, O.G., Filho, H.P.M., Vanini, M.M.T., … Fazuoli, L.C. (2008). Genetic diversity of a coffee germplasm collection assessed by RAPD markers. Genetic resources and crop evolutions, 55, 901 – 910.

[7] Leal, A.A., Mangolin, C.A., do Amaral, A.T.J, Gonçalves, L.S.A., Scapim, C.A., Mott, A.S. Eloi, J.BO. & Silva, M.F.P. (2010). Efficiency of RAPD versus SSR markers for determining genetic diversity among popcorn lines. Genetics and Molecular Research, 9 (1), 9 – 18.

[8] Lashermes, P., Cros, J., Marmey, P. & Charrier, A. (1993). Use of random amplified DNA to analyze genetic variability and relationships of Coffea species. Genetic resources and crop evolution, 40, 91 - 94.

[9] Aga, E., Bryngelsson, T., Bekele, E. & Salomon, B. (2003). Genetic diversity of forest arabica coffee (Coffea arabica L.) in Ethiopia as revealed by random amplified polymorphic DNA (RAPD) analysis. Hereditas, 138, 36 – 46.

[10] Masumbuko, L. I., Bryngelsson, T., Mneney, E. E. & Salomon, B. (2003). Genetic diversity in Tanzanian Arabica coffee using random amplified polymorphic DNA (RAPD) markers. Hereditas, 139, 56 – 63.

[11] Cubry, P., Musoli, P., Legnaté, H., Pot, D., Bellis, F., Poncet, V., … Leroy, T. (2008). Diversity in coffee assessed with SSR markers – structure of the genus Coffea and perpectives for breeding. Genome 51, 50 – 63.

[12] Diniz, L. E. C., Sakiyama, N. S. & Lashermes, P. (2005). Analysis of AFLP markers associated to the Mex-1 locus in Icatu progenies. Crop Breeding and Applied Biotechnology, 5, 387 - 393.

[13] Van der Peer, Y. & De Wachter, R. (1993). TREECON: a software package for the construction of evolutionary trees. Computer application bioscience, 9, 177 – 182.

[14] Rodrigues, C.J & Eskes, A.B. (2009). Resistance to coffee leaf rust and coffee berry disease. In: Wintigens, J. (eds.) *Sustainable Coffee Production and Processing* (pp 557 – 568), Montreal, Canada.

[15] Agwanda, C.O., Lashermes, P., Trouslot, P., Combes, M.C. & Charrier, A. (1997). Identification of RAPD markers for resistance to coffee berry disease, Colletotrichum kahawae, in Arabica coffee. Euphytica, 97, 241 – 248.

[16] Setotaw, T.A., Caixeta, E.T., Pena, G.F., Zambolim, E.M., Pereira, A.A. & Sakiyama, N.S. (2010). Breeding potential and genetic diversity of Hibrido do Timor coffee evaluated by molecular markers. Crop breeding and applied biotechnology, 10, 298 – 304.

[17] Maluf, M.P., Silvestrini, M., Ruggiero, L.M.C., Filho, O.G. and Colombo, C.A. (2005). Genetic diversity of cultivated *Coffea arabica* inbred lines assessed by RAPD, AFLP and SSR marker systems. Scientia Agricola 62 (4): 366 – 373.

[18] Collard, B.C.Y., Jahufer, M.Z.Z., Brouwer, J.B. and Pang, E.C.K. (2005). An introduction to markers, quantitative trait loci (QTL) mapping and marker-assisted selection for crop improvement-the basic concepts. Euphytica 142: 169-196.

[19] Souza, S.G.H., Carpentieri-P ́polo, V., Ruas, C.F. & Carvalho, V.P. (2008). Comparative analysis of genetic diversity among the maize inbred lines (Zea mays L.) obtained by RAPD and SSR markers. Brazilian Archive of Biological Technology, 51, 183 - 192.