International Journal of Biotechnology and Food Science
Vol. 2(8), pp. 156-166, December 2014
ISSN: 2384-7344
Research Paper

Analysis of simple sequence repeat markers linked to coffee berry disease resistance genes in a segregating population of arabica coffee (*Coffea arabica* L.)

Kiguongo A.P.K.[1]* • Omondi C. O.[1] • Gichuru E. K.[1] • Kasili R. W.[2]

[1]Coffee Research Institute, P.O. Box 4 – 00232, Ruiru, Kenya.
[2]Jomo Kenyatta University of Agriculture and Technology, P. O. Box 62000 - 00200, Nairobi, Kenya.

*Corresponding author. E-mail: allankiguongo@gmail.com.

Accepted 3rd November 2014

Abstract. Arabica coffee (*Coffea arabica*) production in Africa is majorly constrained by coffee berry disease (CBD) caused by *Colletotrichum kahawae*. Transfer of desired genes from related wild diploid *Coffea* species into the cultivated allotetraploid *C. arabica* has been known to confer better traits such as pest/disease resistance. Thus, this study was aimed at establishing microsatellite markers that co-segregate with resistance to CBD in an F_2 population. Among the 12 simple sequence repeats (SSR) markers tested, six markers were polymorphic, but only two discriminated between the parents, F_1 and the F_2 population derived from a cross between resistant variety (Rume Sudan) and susceptible cultivar (SL 28). Phenotypic studies using *Colletotrichum kahawae* inoculum to screen F_2 population was carried out in order to understand the segregation of CBD resistance as well as their association with SSR markers. Only two SSR markers, M 24 and Sat 227 showed the expected Mendelian segregation ratio (1:2:1) for single gene effect (d.f = 1.0, $P < 0.05$) in the chi-square (x^2) analyses. In the phenotypic data analysis, the F_2 population segregated in a 3:1 for a major gene action (R:S) ratio for resistant and susceptible plants, respectively. Therefore, there was a correlation between phenotypic data and molecular data with regard to resistance to *Colletotrichum kahawae* infection. The genotypes from F_2 population that showed resistance to *C. kahawae* were putatively linked to two alleles/loci of SSR markers, M 24 (~210bp) and Sat 227 (~200bp). These diagnostic markers could be used in breeding programs where Rume Sudan is used as the donor parent to develop CBD resistant coffee varieties.

Keywords: Coffee berry disease, *Colletotrichum kahawae*, disease resistance, simple sequence repeats markers, segregating population.

INTRODUCTION

There are numerous production constraints in coffee; however, the most serious one is diseases, mainly coffee berry disease (CBD) caused by *Colletotrichum kahawae*, Waller and Bridge (Waller et al., 1993). Coffee berry disease is responsible for reduced productivity and increased cost of production, thereby reducing the competitiveness of Africa's coffee. The disease, which is confined to the African continent, attacks all stages of the developing crop including flowers and occasionally leaves (Gichimu et al., 2014). Coffee berry disease is an anthracnose of the green and ripening berries. Under cold and wet conditions, the fungus sporulates forming a mass of pink conidia and penetrates the interior of the berries destroying the beans (Mouen et al., 2008). Maximum production losses occur when infection takes place in expanding green berries, leading to their premature dropping and mummification (Andreia et al., 2013). The disease severity is higher on coffee trees exposed to sunlight than on those that are located under the shade (Mouen et al., 2008). Van der Vossen and Walyaro (1980) carried out studies in Kenya and concluded that host resistance to CBD appears to be controlled by major genes on three different loci. The highly resistant variety Rume Sudan carries the dominant

R- and the recessive k-genes. The R-gene has two alleles with R_1R_1 in Rume Sudan and R_2R_2 in Pretoria. The variety is believed to have originated from the Boma plateau in Sudan (Walyaro, 1983). Hibrido de Timor which is a spontaneous hybrid between *C. arabica* and *C. canephora* carries a resistant gene on the T-locus. The partially resistant cultivar, K7 carries the recessive k-gene. Silva et al. (2006) also described high levels of resistance in Rume Sudan and some progenies of Hibrido de Timor (HDT). Host resistance to CBD is of a quantitative nature, but it can be complete in some Arabica coffee genotypes. Though, there is no consensus on the genetics of CBD resistance, other authors describe oligogenes (1 to 3 major genes) and other polygenes that determine CBD resistance (Van der Vossen and Walyaro, 2009). Agwanda et al., (1997) reported the existence of susceptible and resistant Rume Sudan varieties which originated from different geographic origins.

Microsatellites have been used in coffee research to characterize *C. arabica*, *C. canephora* and related species (Combes et al., 2000). Conventional breeding methods involving hybridization and selection take longer especially when the programme requires technical procedures such as backcrossing (Anthony and Lashermes, 2005). The time required for breeding by traditional methods can be shortened by use of DNA based marker assisted selection (MAS) (Riesenberg et al., 2000). These markers assist in detecting a targeted genomic fragment, hence selecting for a desirable trait such as disease resistance (Gichuru et al., 2008). Gichuru et al. (2008) was able to identify a microsatellite marker Sat 235 which was linked to CBD resistance and mapped it onto an introgressed *C. canephora* fragment which harbors the responsible Ck-1 gene using F_2 plants from a cross between *cv.* Catimor × *cv.* SL28 that were resistant and susceptible to CBD respectively. Gichimu et al. (2014) used the same SSR marker, Sat 235 in the study of occurrence of Ck-1 gene conferring resistance to Coffee berry disease in *Coffea arabica cv.* Ruiru 11 and its parental genotypes, where the Sat 235 cannot be applied to Rume Sudan, hence this study. In view of the long time duration it takes to develop resistant varieties, this study was formulated with the sole objective of analyzing SSR markers that co-segregate with CBD resistance genes in an F_2 population derived from a cross between Rume Sudan (resistant variety) and SL 28 (susceptible cultivar) for possible use in Marker Assisted Selection (MAS) that would considerably shorten the breeding process.

MATERIALS AND METHODS

Plant materials

Parental genotypes; Rume Sudan and SL 28 cultivars, one F_1 tree and 49 F_2 population (Table 1) were selected for analysis with 12 SSR markers to determine if CBD resistance co-segregated with the markers. The F_2 were obtained after selfing F_1 trees. Rume Sudan is a resistant variety that originated from the Boma plateau in Sudan and is currently maintained at the Kenya Agricultural and Livestock Research Organization - Coffee Research Institute (KALRO-CRI) in *ex-situ* gene bank. The variety carries the dominant R- and the recessive k-gene. The R-locus has two alleles with R_1R_1 in Rume Sudan and R_2R_2 in Pretoria (Van der Vossen and Walyaro, 1980). SL 28 coffee cultivar was selected at the former Scott Laboratories on a single tree basis from the Tanganyika Drought Resistant variety selected in Northern Tanzania in 1931. It combines high yield, fine beverage quality and suited for medium to high altitudes. It is susceptible to coffee berry disease (CBD), coffee leaf rust (CLR) and bacterial blight of coffee (BBC) (Lashermes et al., 1999).

Screening for resistance to CBD

Laboratory disease evaluation in F_2 genotypes

To determine segregation patterns for coffee berry disease resistance, parental genotypes, one- F_1 and 49 F_2 genotypes were evaluated by inoculation with freshly prepared inoculum of *C. kahawae*. Expanding berries of 15 weeks of age were collected during the mid-week of March 2014. This stage of maturation is the most susceptible stage for CBD infection before the hardening stage, when berries exhibit a higher level of resistance (Muller, 1980). All berries originated from single mature trees of the resistant Rume Sudan, susceptible *cv.* SL 28, and F_1 and F_2 plants. Clean plastic boxes were partially filled with water and a grid supporting absorbent paper was placed inside to bear the berries and create a humid atmosphere. The berries were cleaned with liquid soap (0.01%), rinsed and dried. The wounded stalk end of the berries was removed with a sterile scalpel to avoid contamination and to limit the development of saprophytic fungi. A total of 10 berries per genotype were placed in three rows in each box (Figure 1) across three replicas which were arranged in a completely randomized block design. Each replication had 19 plastic boxes and the berries were inoculated with a freshly prepared CBD inoculum 2×10^6 spores ml^{-1} from a standard CBD pathogen isolate on PDA in the laboratory. Control treatments of Rume Sudan and SL 28 were inoculated with sterile water. The sterile inoculation room was maintained at 21°C. Inoculation by *Colletotrichum kahawae* was carried out as described by Bock, (1956) and Pinard et al. (2012). Disease assessment was regularly scored at 7 days post inoculation (dpi) for three weeks, a period during which the berries remained free of contamination other than CBD.

CBD scoring

CBD development was assessed using a visual scale from 0% to 100% of the total berry surface affected on a

Table 1. List of coffee genotypes evaluated for marker assisted selection.

Lab serial no	Genotypes		Source/plots
1	Rume Sudan	KALRO-CRI	Plot 4
2	SL 28	“	Plot 3
3	F1 49	“	Plot 14
4	F2 8	“	Plot 16
5	F2 9	“	Plot 16
6	F2 13	“	Plot 16
7	F2 14	“	Plot 16
8	F2 15	“	Plot 16
9	F2 16	“	Plot 16
10	F2 18	“	Plot 16
11	F2 20	“	Plot 16
12	F2 22	“	Plot 16
13	F2 25	“	Plot 16
14	F2 26	“	Plot 16
15	F2 27	“	Plot 16
16	F2 28	“	Plot 16
17	F2 29	“	Plot 16
18	F2 30	“	Plot 16
19	F2 33	“	Plot 16
20	F2 44	“	Plot 16
21	F2 46	“	Plot 16
22	F2 49	“	Plot 16
23	F2 72	“	Plot 16
24	F2 75	“	Plot 16
25	F2 76	“	Plot 16
26	F2 77	“	Plot 16
27	F2 78	“	Plot 16
28	F2 80	“	Plot 16
29	F2 82	“	Plot 16
30	F2 83	“	Plot 16
31	F2 86	“	Plot 16
32	F2 87	“	Plot 16
33	F2 92	“	Plot 16
34	F2 93	“	Plot 16
35	F2 97	“	Plot 16
36	F2 98	“	Plot 16
37	F2 99	“	Plot 16
38	F2 104	“	Plot 16
39	F2 105	“	Plot 16
40	F2 108	“	Plot 16
41	F2 110	“	Plot 16
42	F2 111	“	Plot 16
43	F2 115	“	Plot 16
44	F2 116	“	Plot 16
45	F2 117	“	Plot 16
46	F2 121	“	Plot 16
47	F2 122	“	Plot 16
48	F2 123	“	Plot 16
49	F2 124	“	Plot 16
50	F2 125	“	Plot 16

Table 1. Contd.

51	F2 454	"	Plot 16
52	F2 468	"	Plot 16

KALRO-CRI- Kenya Agricultural and Livestock Research Organization-Coffee Research Institute.

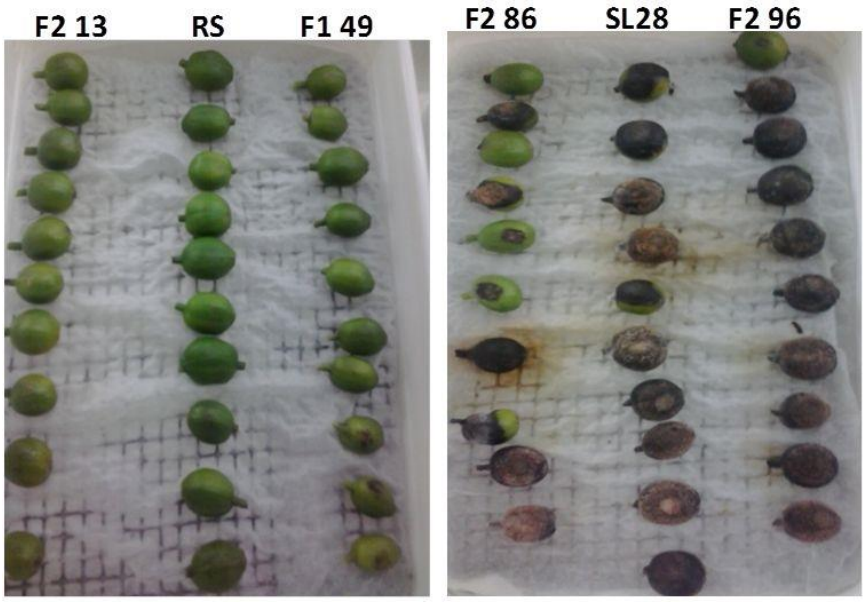

Figure 1. Coffee berries of Rume Sudan (RS) (resistant) and SL 28 (susceptible) inoculated with conidia of *Colletotrichum kahawae* after 21 days at 21°C.

scale of 1 to 5, where 0 to 5% = 1, 6 to 10% = 2, 11 to 25% = 3, 26 to 50% = 4 and 51 to 100% = 5. A score range of ≤ 2 was considered resistant while that of ≥3 was regarded to be susceptible. After scoring each coffee berry individually, average infection (AI) on each genotype across the replicas was calculated as follows:

$$AI = \Sigma [Ir1 + Ir2 + Ir3 + \cdots Irn]/N$$

Where, I is the sum of disease score; *n* is the number of replication; I*rn* is the sum of disease score in replication *n; N* is the total number of berries scored in the replications.

Data analysis

Scored data after 21 days was subjected to analysis of variance (ANOVA) using XLstat software 2014 version and effects declared significant at 5% level using Fisher (Least Significance Difference) method. The coffee genotypes showing a score that was not significantly different from Rume Sudan were considered to be resistant (R) while the rest were considered to be susceptible (S). Segregation data were analyzed by the chi-square (x^2) test. The chi-square analysis for the genotypic and phenotypic ratio was calculated using the formula, $x^2 = (O - E)^2/E$, where O is the observed value and E is the expected value. Each chi-square value was considered to be significant ($P \leq 0.05$) (3.84).

Screening for DNA markers linked to CBD resistance

Extraction of genomic DNA

Disease-free leaves were picked from first and second nodes from the growing tips of the coffee branches from the parental and progenies 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) instead of cetyltrimethylammonium bromide (CTAB).

Quantification of DNA

One per cent agarose gel in 0.5X Tris Boric Ethylenediaminetetraacetic acid (TBE) was prepared by weighing 0.7 g of Agarose in 70 ml 0.5X TBE. The solution was then heated in a microwave at short

Table 2. Simple sequence repeats (SSR) forward and reverse primer sequence used in the analysis.

Locus	Forward primer (5' > 3')	Reverse primer (5' > 3')
Sat11	ACCCGAAAGAAAGAACCAA	CCACACAACTCTCCTCATTC
Sat32	AACTCTCCATTCCCGCATTC	CTGGGTTTTCTGTGTTCTCG
Sat172	ACGCAGGTGGTAGAAGAATG	TCAAAGCAGTAGTAGCGGATG
Sat207	GAAGCCGTTTCAAGCC	CAATCTCTTTCCGATGCTCT
Sat227	TGCTTGGTATCCTCACATTCA	ATCCAATGGAGTGTGTTGCT
Sat229	TTCTAAGTTGTTAAACGAGACGCTTA	TTCCTCCATGCCCATATTG
Sat240	TGCACCCTTCAAGATACATTCA	GGTAAATCACCGAGCATCCA
Sat254	ATGTTCTTCGCTTCGCTAAC	AAGTGTGGGAGTGTCTGCAT
Sat255	AAAACCACACAACTCTCCTCA	GGGAAAGGGAGAAAAGCTC
Sat262	CTGCGAGGAGGAGTTAAAGATACCAC	GCCGGGAGTCTAGGGTTCTGTG
Sat283	GCACACACCCATACTCTCTCTT	GTGTGTGATTGTGTGTGAGAG
M 24	GGCTCGAGATATCTGTTTAG	TTTAATGGGCATAGGGTCC

Source: Lashermes et al., 2010.

intervals of 15 to 30 s with occasional shaking until it was clear. Due to evaporation that occurs during heating, the solution was weighed again, after which water was added to obtain the original volume and left to cool to about 55°C. The gel was then poured on the tray of the mini electrophoresis unit (MUPID) and any bubbles removed after which the combs were fixed and allowed to settle. After settling, the combs were removed and 0.5X TBE buffer added on the mini electrophoresis unit to cover the gel.

The standard DNA was then prepared (lambda DNA/EcoR1 +Hind111 marker 500 µg/ml).The lambda preparation mixture was heated at 65°C for 10 min and immediately chilled on ice for 5 min before use. After five minutes, 10 µl of lambda and 12 µl of sample DNA preparations were then loaded onto the one per cent agarose gel and run at 50 V for 45 min. The gel was stained in 1 mg/ml Ethidium Bromide (50 µl of 10 mg/ml Ethidium Bromide in 500 ml dH_2O) for 20 min and placed into the UV transilluminator and photographed. Lambda preparation table was used to estimate the quantity of DNA.

PCR amplification by SSR primers

PCR reactions were performed in a final volume of 25 µl containing 5.4 µl of double distilled water; 10 ng (10 ng/µl) of template genomic DNA, 2.5 µl of 10X PCR buffer (16 mM $MgCl_2$, Dongsheng), 1.0 µl of $MgCl_2$ (25 mM, Dongsheng), 3.75 µl of dNTPs (500 µM, Eurogentec), 1.0 µl each of forward and reverse Primer (10 µM, Eurogentec), 0.3 µl of Taq DNA polymerase (5U/µl, Dongsheng). Amplification was carried out in a Eurogene thermocycler (TECHNE, UK). The SSR amplification program started with one cycle of initial denaturation at 94°C for 5 min followed by 35 cycles of 45 s at 94°C (denaturation), 30 s at 55°C for primer annealing, and 90 s at 72°C for elongation. The final extension was done at 72°C for 10 min and final hold at 4°C. Twelve SSR primer pairs were used for DNA PCR amplification (Table 2). Selection of these primers was guided by the results of previous works done by Combes et al. (2000), Omondi et al. (2009) and Lashermes et al. (2010).

Genotyping for marker segregation

A total of 52 samples; consisting of two parental genotypes, one F_1 and 49 F_2 were genotyped for SSR marker alleles. Alleles at the SSR loci were detected on 2.3% agarose gels. Alleles were scored based on the parental and F_1 bands. A ladder was added with the first load to confirm the allele sizes observed in the parental and F_1 survey. The plants that showed a pattern similar to the resistant parent alleles were scored as (1) and those with a banding pattern similar to the susceptible parent alleles were scored as (0), and the heterozygous plants were scored as (2).

Using the SSR binary data, Agglomerative Hierarchical Clustering (AHC) method of Neighbor-Joining (NJ) analysis was used to construct a dendogram. Jaccard coefficient was used to check on the dissimilarity using Artemis 5.0 Software.

RESULTS

Phenotypic analysis of the F_2 population

Analysis of variance indicates that the effect of genotypes was highly significant ($p \leq 0.0001$) (Table 3). The effect of replication and interaction between replications and genotypes were non-significant ($p \leq 0.05$). There were 33 F_2 genotypes in the resistant class and 16 F_2 genotypes

Table 3. Analysis of variance table at P ≤ 0.0001.

Sources of variations	Degrees of freedom	Sum of squares	Mean squares	F	Pr > F
Coffee genotypes (G)	51	117.882	2.311	10.317	< 0.0001
Replications (R)	2	0.834	0.417	0.455	0.635
G X R	154	141.160	0.917	40.739	0.124

recorded in the susceptible class. Rume Sudan recorded an average infection score of 1.87, F_1 tree 1.80 and SL 28 an average infection score of 4.57 (Table 4). The control experiment of Rume Sudan and SL 28 berries remained symptomless across the replications. The F_2 progenies showed that resistance in this population segregated in a 3:1 ratio for major gene effect for plants with resistance and susceptibility (Table 5).

There were visual observations of the resistant F_2 genotypes having restriction scab lesions. The restricted scab lesions never progressed on the berry surfaces of these resistant F_2 genotypes during CBD development on the last date of data collection (21 days) as shown on Figure 2.

Marker segregation analysis

Among the 12 SSR markers used, six markers showed polymorphism among two parents, F_1 and F_2 populations. Two SSR markers, M 24 and Sat 227 showed polymorphism between susceptible and resistant parents and corresponding F_1 and F_2 genotypes upon a permutative test indicating their possible association with coffee berry disease resistance in the segregating population. Therefore, the F_2 population was genotyped with these two primers to study their possible association with CBD resistance.

Segregation study with marker M 24 recorded a resistant allele of ~210 bp amplified in 16 plants, whereas a susceptible allele of ~180 bp was amplified in 12 plants (Table 6, Figure 3). Twenty-one F_2 plants exhibited both the alleles (heterozygous) like the F_1 progeny. Genetic analysis with chi-square test indicated "goodness-of-fit" to the expected ratio of 1:2:1.

Segregation study with marker Sat 227 recorded a resistant allele of ~200 bp amplified in 11 plants, whereas a susceptible allele of ~220 bp was amplified in 17 plants (Table 7, Figure 4). Twenty-one F_2 plants exhibited both the alleles (heterozygous) like the F_1 progeny. Genetic analysis with chi-square test indicated goodness of fit to the expected ratio of 1:2:1 for single gene model indicating the possible association of Sat 227 with CBD resistance gene. The ratio 1:2:1 observed by the co-dominant SSR markers corresponds to the 3:1 ratio observed in the phenotypic data, thus indicating that the two markers were putatively linked to the resistance genes.

The dendrogram constructed using SSR binary data was used to determine genetic diversity of the F_2 genotypes. The F_2 genotypes separated into three main clusters. The F_2 genotypes clustered depending on the parental genotypes; Rume Sudan and SL 28 and the F_1 genotype banding patterns in regard to *C. kahawae* resistance. Cluster one consisted of F_2 genotypes that resembled F_1 banding pattern (colour green). Cluster two consisted of two sub-clusters that were closely related comprising of those F_2 genotypes that resemble SL 28 banding pattern (colour blue). Cluster three also consisted of two sub-clusters that were closely linked and comprised of those genotypes that resembled Rume Sudan banding pattern (colour red) as illustrated in Figure 5.

DISCUSSION

Variation for resistance to *C. kahawae* among F_2 population of a cross between coffee cultivars, Rume Sudan (resistant) and SL 28 (susceptible) was mainly due to segregation. This conclusion was supported by the fact that the main effect of genotypes was significant (P ≤ 0.0001). There was uniformity in the inoculation conditions and therefore the effect of replication and interaction between replications and genotypes were non-significant (p ≤ 0.05). The uniform conditions were attained by having water inside the closed containers to increase humidity and maintaining temperature at 21°C in the cold room. Pinard et al. (2012) reported that presence of water (rain, mist or dew) on berry surfaces and favorable temperatures between 21 and 23°C are necessary conditions for infection and development of CBD epidemics. Uniformity was also achieved by using berries that were collected at the same growth stage, four months post flowering, at their soft stage which is most susceptible to *C. kahawae*. Mulinge (1970) reported that the first four weeks, the berry does not increase in size instead it remains at the "pinhead". This stage is resistant to CBD. The next 4 to 16 weeks after flowering, the expanding berry at this stage is the most susceptible which is unlike fully expanded green berries, which are resistant. Further uniformity was also attained by standardizing the incubation period to seven days and inoculum concentration to 2×10^6 spores per ml. During the course of the experiment, the controls remained symptomless, an indication of absence of latent infections which are frequent with anthracnose diseases of other fruit tree species and is suspected with CBD (Pinard et al., 2012).

Table 4. Variations in CBD infection among F_2 coffee genotypes.

Coffee genotypes	Mean score of CBD infection	Coffee genotypes	Mean score of CBD infection
Rume Sudan	1.867^{k-o}	F_2 78	3.100^{d-g}
SL 28	4.567^{a}	F_2 80	2.300^{h-m}
F_1 49	1.800^{k-p}	F_2 82	1.700^{k-p}
F_2 8	2.733^{f-j}	F_2 83	3.733^{b-d}
F_2 9	1.567^{m-p}	F_2 86	2.200^{h-n}
F_2 13	1.133^{o-p}	F_2 87	1.900^{k-n}
F_2 14	1.067^{p}	F_2 92	2.933^{e-h}
F_2 15	1.700^{k-p}	F_2 93	2.000^{j-n}
F_2 16	2.333^{h-l}	F_2 97	1.733^{k-p}
F_2 18	1.800^{k-p}	F_2 98	4.367^{a-b}
F_2 20	1.800^{k-p}	F_2 99	1.867^{k-o}
F_2 22	2.333^{h-l}	F_2 104	1.967^{k-n}
F_2 25	1.633^{l-p}	F_2 105	1.733^{k-p}
F_2 26	3.533^{c-e}	F_2 108	1.600^{l-p}
F_2 27	1.833^{k-o}	F_2 110	2.767^{f-i}
F_2 28	1.933^{k-n}	F_2 111	2.000^{j-n}
F_2 29	3.300^{c-f}	F_2 115	3.333^{c-f}
F_2 30	3.333^{c-f}	F_2 116	3.533^{c-e}
F_2 33	1.533^{n-p}	F_2 117	3.467^{c-f}
F_2 44	1.567^{m-p}	F_2 121	1.467^{n-p}
F_2 46	2.400^{g-k}	F_2 122	1.833^{k-o}
F_2 49	1.833^{k-o}	F_2 123	2.033^{i-n}
F_2 72	4.300^{a-b}	F_2 124	1.733^{k-p}
F_2 75	1.833^{k-o}	F_2 125	2.733^{f-j}
F_2 76	1.633^{l-p}	F_2 454	4.033^{a-c}
F_2 77	1.500^{n-p}	F_2 468	2.033^{i-n}

Means followed by the same letter(s) within the column are not significantly different at P ≤ 0.05 according to Fisher (LSD) method. Key: The hyphen (-) represents the alphabetical range between the letters.

Table 5. Observed and expected segregation ratios of resistant and susceptible plants in the F_2 generation from a cross between the Rume Sudan × SL 28 inoculated with *Colletotrichum kahawae.*

Generation	Category	Pathogenicity assay		χ2 (3:1)	P
		Observed number	Expected number		
F_2	Resistant	33	36.75	1.5307*	0.25
	Susceptible	16	12.25		
	Total	49	49		

d.f. = 1.0; χ2 (0.05, 1) = 3.84; significantly different (*).

Upon separations of means, the F_2 progenies segregated into two groups; 33 of them had disease scores that were statistically similar to the resistant parent, Rume Sudan and F_1 progeny. The rest exhibited scores that were similar to the susceptible parent, SL 28. It was however, not possible to categorize the resistant F_2 plants into homozygous and heterozygous types using the phenotypic data.

However, an analysis of the SSR data using primer M 24 and Sat 227 clearly delineated the F_2 plants into three categories. SSR markers are co-dominant in nature and therefore were able to distinguish homozygous resistant plants from heterozygotes and homozygous susceptible plants.

Visual observation showed that Rume Sudan had fewer CBD lesions compared to the susceptible SL 28. This was due to the antifungal compounds in the cuticular wax layer of the Rume Sudan berries. This is in agreement with studies reported by Steiner (1972) and Lampard and Carter (1973) on presence of antifungal compounds in

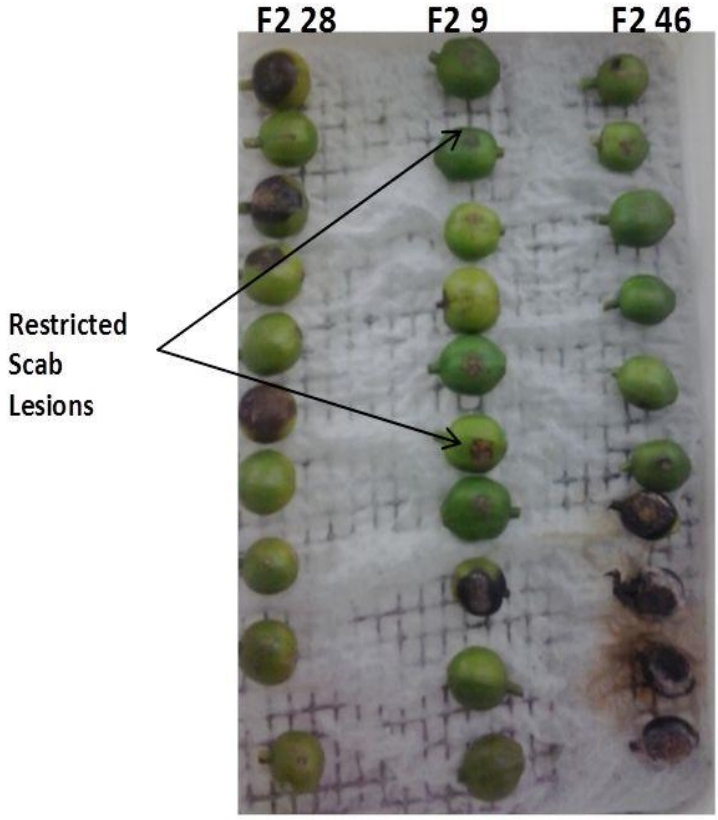

Figure 2. Presence of CBD restricted scab lesions on a resistant F_2 genotype inoculated with conidia of *Colletotrichum kahawae* after 21 days at 21°C.

Table 6. Evaluation of the F_2 population with SSR marker M 24.

Category	Observed genotype	Expected genotype	χ2(1:2:1)	P
Resistant	16	12.25	1.6684*	0.10
Heterozygote	21	24.50		
Susceptible	12	12.25		
Total	49	49		

Significantly different (*).

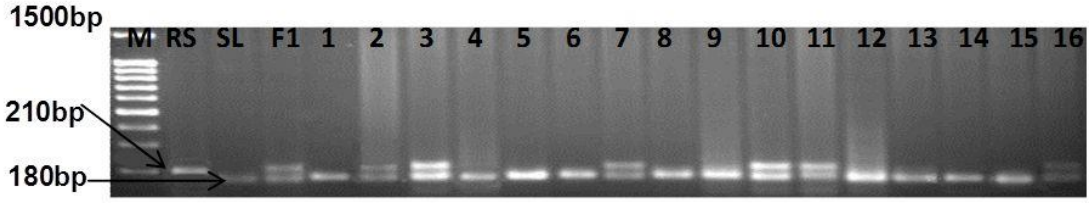

Figure 3. DNA banding patterns in an F_2 population of coffee derived from a cross between Rume Sudan (RS) × SL 28 (SL) for SSR marker M 24. M = 100-bp ladder; RS= Rume Sudan; SL= SL 28; F_1 and lanes 1-16 = F_2 progenies.

the cuticular wax layer in green berries from resistant cultivars such as Rume Sudan which significantly decreased the level of conidia germination. Resistance reaction in the F_2 genotypes to *C. kahawae* was presented

Table 7. Evaluation of the F_2 population with SSR marker Sat 227.

Category	Observed genotype	Expected genotype	χ2(1:2:1)	P
Resistant	11	12.25	2.4694*	0.10
Heterozygote	21	24.50		
Susceptible	17	12.25		
Total	49	49		

Significantly different (*)

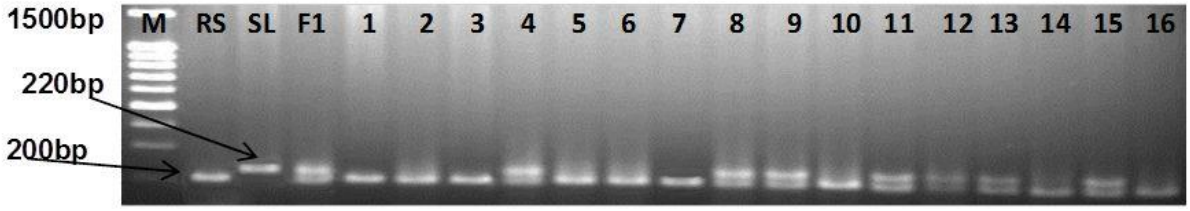

Figure 4. DNA banding patterns in an F_2 population of coffee derived from a cross between Rume Sudan (RS) × SL 28 (SL) for SSR marker Sat 227. M = 100-bp ladder; RS = Rume Sudan; SL= SL 28; F_1 and lanes 1-16 = F_2 progenies.

as restricted scab lesions. Restricted scab lesions hinder penetration of the CBD pathogen inside the coffee beans. These results agree with the findings of Gichuru (1997) that scab lesions through formation of cork barriers are the common macroscopic expression of resistance to CBD. This resistance to CBD are preformed and induced, and they operate at distinct stages of pathogenesis (Gichuru, 1997). Pinard et al. (2012) also came to a deduction that berry resistance could be separated into two types; one against the pathogen penetration and the other against its growth in berries through scab lesion formation. These were the possible explanation why there was phenotypic variation among the F_2 genotypes with regard to CBD resistance.

Chi-square analysis revealed a strong correlation between the phenotypic and the SSR data. A segregation ratio of 3:1 for resistant to susceptible plants was observed with phenotypic data while SSR data revealed a segregation ratio of 1:2:1 representing homozygous resistant, heterozygotes and homozygous susceptible respectively. Due to the co-dominance of SSR markers, the resistant genotypes scored by the phenotypic assay were further disaggregated into homozygous resistant and heterozygotes with SSRs, M 24 and Sat 227. The M 24 SSR locus that was putatively associated to CBD resistance in the study was also reported by Omondi et al. (2009) who concluded that one of the resistance genes from Rume Sudan was carried by the marker which could be used for assisted selection for resistance to CBD.

The genetic variation in the F_2 population was observed in the dendogram. The first cluster composed of heterozygotes in F_2 genotypes similar to F_1. The second cluster had two sub-clusters which were closely linked since the confidence level was more than 50% and composed of homozygous susceptible F_2 genotypes similar to SL 28. The third cluster also had two sub-clusters with one cluster having a confidence level of 50%. One sub-cluster has Rume Sudan and three F_2 genotypes. It can be assumed that the three F_2 genotypes may be having the R- dominant and k-recessive genes while the rest of the F_2 genotypes in the other sub-cluster only the dominant R- gene alone. Both sub-clusters were composed of homozygous resistant F_2 genotypes similar to Rume Sudan.

CONCLUSION

It is therefore concluded that the SSR markers co-segregated with the resistance genes in Rume Sudan thus suggesting that there is a putative association of the two SSR loci and the resistance genes. A linkage analysis to determine the actual distances between the markers and the resistance genes requires to be done to establish the most suitable marker that can aid in selection for resistance to CBD. Since the more the marker is tightly linked to the gene, the better it is for Marker Assisted Selection (MAS). The findings of this study could be directly useful in molecular analysis of segregating generations, breeding lines and varieties which have Rume Sudan as one of the parents.

ACKNOWLEDGEMENTS

The authors are thankful to Dr. B.M. Gichimu, Mr. J.M. Ithiru, Mr. P.G. Njuguna, Mr. Cyrus Maina, Mr. D.O. Omari and Mr. B.N Maina as well as Coffee Breeding Section personnel for their technical support. This work

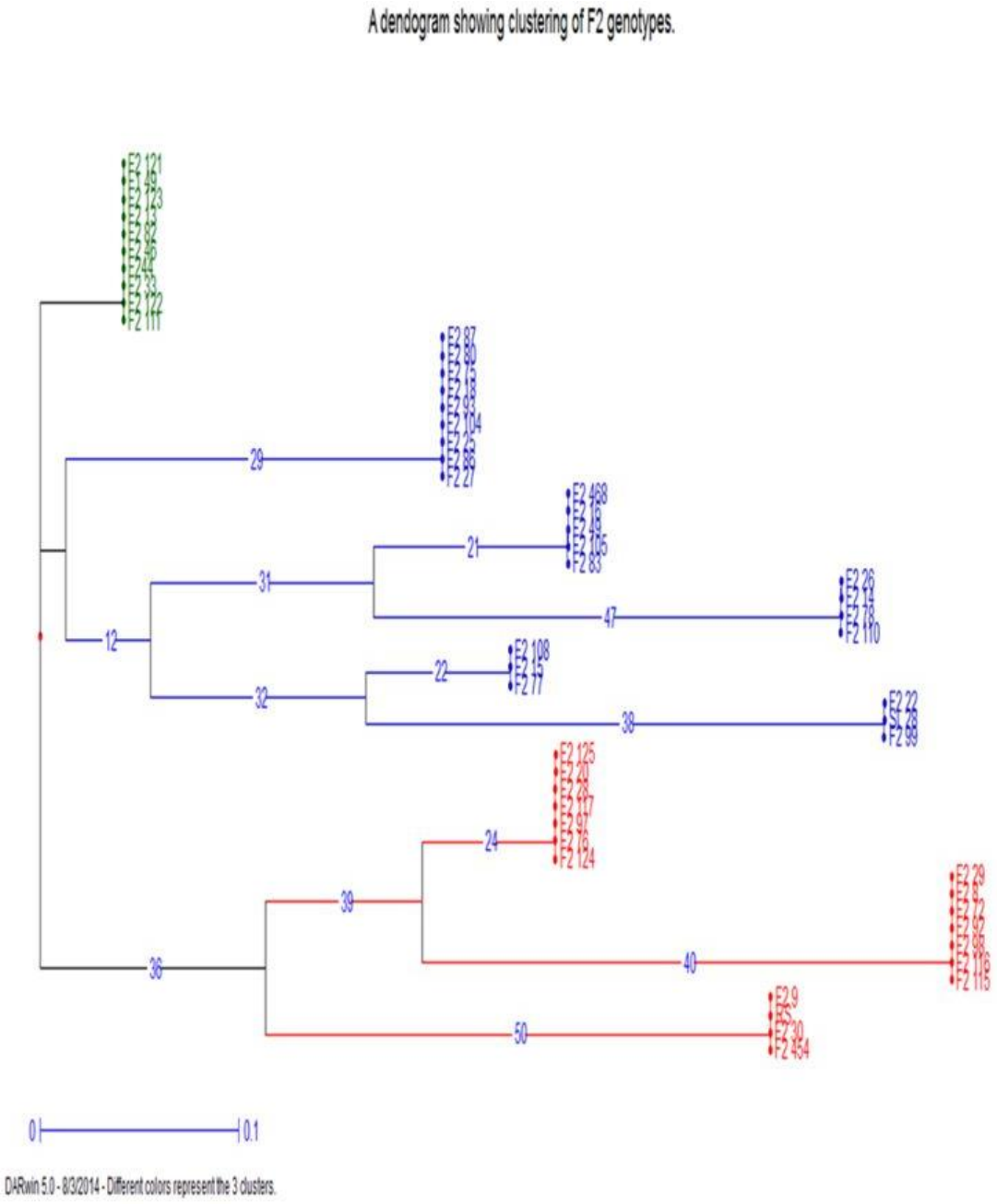

Figure 5. A dendogram showing clustering of the coffee genotypes generated by M 24 and Sat 227.

received the financial support from the Kenya Agricultural and Livestock Research Organisation-Coffee Research Institute (KALRO- CRI). This work is published with the permission of the Director of Institute, CRI, Kenya.

REFERENCES

Agwanda CO, Lashermes P, Pierre T, Combes MC, Charrier A (1997). Identification of RAPD markers for resistance to coffee berry disease, *Colletotrichum kahawae*, in Arabica coffee. Euphytica 97: 241-248.

Andreia F, Andreia L, Dora B, Filipa M, Vítor V, Maria SP, Gichuru EK, Maria Céu Silva (2013). Validation of reference genes for normalization of qPCR gene expression data from *Coffea* spp. hypocotyls inoculated with *Colletotrichum kahawae*. BMC Research Notes 6: 388.

Anthony F, Lashermes P (2005). Origin, evolution and diversity of the coffee (*Coffea arabica* L.) genome. In: Sharma, A. K. and Sharma, A. (Eds), Plant genome: Biodiversity and evolution Vol. I (B). Science Publisher, Inc. Plymouth, UK pp. 208-228.

Bock KR (1956). Investigations of coffee berry disease –laboratory studies. East African Agric. For. J. 22: 99-103.

Combes MC, Andrzejewski S, Anthony F, Bertrand B, Rovelli P, Graziosi G, Lashermes P (2000). Characterization of microsatellites loci in *Coffea arabica* and related coffee species. Mol. Ecol. 9: 1178-1190.

Diniz LFC, Ruas CF, Carvalho VP, Torres FM, Ruas EA (2005). Genetic Diversity Among Forty Coffee Varieties Assessed by RAPD Markers Associated with Restriction Digestion. Braz. Arch. Biol. Tech. 48(4): 511-521.

Gichuru EK (2007). Sensitive response and resistance to berry disease (*Colletotrichum kahawae*) of two coffee varieties (*Coffea arabica* and *C. canephora*): histological comparisons of interactions. Agronomie Africaine19: 233-240.

Gichuru EK, Agwanda CO, Combes MC, Mutitu EW, Ngugi ECK, Bertrand B, Lashermes P (2008). Identification of molecular markers linked to a gene conferring resistance to coffee berry disease (*Colletotrichum kahawae*) in *Coffea arabica*. Plant Pathol. 57: 1117-1124.

Gichimu BM, Gichuru EK, Mamati GE, Nyende AB (2014). Occurrence of *Ck-1* gene conferring resistance to Coffee Berry Disease in *Coffea arabica* cv. Ruiru 11 and its parental genotypes. J. Agric. Crop Res. 2(3): 51-61.

Lampard JF, Carter GA (1973) Chemical investigations on resistance to coffee berry disease in *Coffea arabica*. An antifungal compound in coffee cuticular wax. Ann. Appl. Biol. 73: 31-37.

Lashermes P, Combes MC, Robert J, Trouslot P, D'Hont A, Anthony F, et al. (**1999).** Molecular characterization and origin of the *Coffea arabica* L. genome. Mol. Gen. Genet. 261(2): 259-66.

Mouen Bedimo JA, Njiayouom I, Bieysse D, Ndoumbè Nkeng M, Cilas C, Nottéghem JL, (2008). Effect of shade on Arabica coffee berry disease development: towards an agroforestry system to reduce disease impact. Phytopathology 98: 1320-1325.

Mulinge SK (1970). Development of coffee berry disease in relation to the stage of berry growth. Ann. Appl. Biol. 65: 269-276.

Muller RA (1980). Contribution àla connaissance de la phytomycocénose constituée par *Coffea arabica, Colletotrichum coffeanum, Hemileia vastatrixet Hemileia coffeicola*. Bulletin Institut Français du Café et du Cacao 15: 174.

Omondi CO, Gichuru EK, Combes MC, Lashermes P (2009). SSR polymorphism in breeding populations of Arabica coffee with varying reactions to coffee berry disease. In: Plant and animal genomes XVII conference. San Diego, CA: Town & Country Convention Center pp. 44-48.

Pinard F, Omondi CO, Cilas C (2012). Detached berries inoculation for characterization of coffee resistance to coffee berry disease. J. Plant Pathol. 94(3):517-523.

Riesenberg LH, Baird SJE, Gardner KA (2000). Hybridization, introgression and linkage evolution. Plant Mol. Biol. 42: 205-224. Rieseberg

Silva MC, Várzea V, Guerra-Guimarães L, Azinheira HG, Fernandez D, Petitot AS, Bertrand B, Lashermes P, Nicole M (2006). Coffee resistance to the main diseases: leaf rust and coffee berry disease. Braz. J. Plant Physiol. 18(1): 119-147.

Steiner KG (1972). The influence of surface wax obtained from green berries of six selections of *Coffea arabica* on conidia of *Colletotrichum coffeanum*. Kenya Coffee 37: 107-108.

Van der Vossen HAM, Walyaro DJ (1980). Breeding for resistance to coffee berry disease caused by *Colletotrichum coffeanum* Noack (sensu Hindorf) in *Coffea Arabica* L. II. Inheritance for the resistance. Euphityca 29:777-791.

Van der Vossen HAM, Walyaro DJ (2009). Additional evidence for oligogenic inheritance of durable host resistance to Coffee berry disease (*Colletotrichum kahawae*) in Arabica coffee (*Coffea arabica* _L) Euphytica 165(1):105-111.

Waller JM, Bridge PD, Black R, Hakiza G (1993). Characterization of the coffee berry disease pathogen, *Colletotrichum kahawae* sp. nov. Mycol. Res. 97:989-994.

Walyaro DJ (1983). Considerations in breeding for improved yield and quality in arabica coffee (*Coffea arabica* L.). PhD Thesis, University of Wageningen, Netherlands.