Characterization of genetic resistance to Coffee Berry Disease (*Colletotrichum kahawae* Waller and Bridge) in Arabica coffee (*Coffea arabica* L.) that is introgressed from *Coffea canephora* Pierre.

BY

GICHURU, Elijah Kathurima
BSc (Agriculture, UoN), MSc (Plant Pathology, UoN)

THIS THESIS IS SUBMITTED IN FULL FULFILMENT OF THE
REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY
IN
PLANT PATHOLOGY

FACULTY OF AGRICULTURE
COLLEGE OF AGRICULTURE AND VETERINARY SCIENCES
UNIVERSITY OF NAIROBI.

2007

DECLARATION BY THE CANDIDATE

I declare that this is my original work and it has not been submitted in any other University for award of a degree.

GICHURU, Elijah Kathurima .... Date..30/7/07
     Department of Plant Science and Crop Protection
     Faculty of Agriculture
     College of Agriculture and Veterinary Sciences
     University of Nairobi
     P O Box 29053
     Nairobi

This thesis is submitted for examination with our approval as university supervisors

1.   Signed .... Date ..30/07/07
     Prof. Eunice W. MUTITU
     Associate Professor (Plant Pathology)
     Department of plant Science and Crop Protection
     University of Nairobi
     PO Box 29053
     Nairobi

2.   Signed .... Date..30/7/07
     Dr. Eliud C. K. NGUGI
     Senior Lecturer (Molecular Genetics)
     Department of plant Science and Crop Protection
     University of Nairobi
     P O Box 29053
     Nairobi

3.   Signed ..P. Lashermes.. Date ..30.07.07
     Prof. Philippe LASHERMES
     UMR RPB (Résistance des Plantes aux Bioagresseurs)
     Equipe DIVersité et Amélioration (DIVA)
     GeneTrop,
     Institut de Recherche pour le Développement (IRD),
     BP 64501
     F-34394, Montpellier Cedex 5
     France,

DEDICATION

I sincerely dedicate this thesis to my dear family:
Cecilia, Karean and Kelvin Wesley

ACKNOWLEDGEMENTS

Karean and Kelvin Wesley, thanks for your kindness, understanding and prayers during my absence and stressing periods. I wish to thank my relatives and in-laws for their support to me and my family during the trying period of this study. I passionately remember our late grandma, *Cucu* Wairimu wa Kamotho, who despite her enviable age of about a dozen decades, always preserved a sweet banana in her farm to await me after every trip from France. What a wonderful ritual trip I always made to see her and rediscover true tropical taste after a winter in Montpellier!!

I kindly wish to extend my thanks to the examiners appointed by the University for their useful suggestions and comments that helped to enrich this thesis

Finally but most significantly, I thank Almighty God who is "*a master-planner*". We were all His instruments in this work to deliver what, when, where and how He wished. I pray that He will never forsake us as individuals, institutions and industry.

LIST OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF APPENDICES

LIST OF MAJOR ABBREVIATIONS

AFLP	Amplified Fragment Length Polymorphism
BAC	Bacterial Artificial Chromosome
BBC	Bacterial Blight of Coffee
BC_i	i^{th} Back Cross
bp	base pairs
CRF	Coffee Research Foundation
CBD	Coffee Berry Disease
CIFC	Centro de Investigação das Ferrugens do Cafeeiro (translation into English: Coffee Rusts Research Centre, Portugal)
CLR	Coffee Leaf Rust
cM	centimorgan
cv(s)	cultivar(s)
DH(s)	Doubled haploid(s)
DNA	Decoy-ribonucleic acid
dNTPs	deoxyNucleotide Triphosphates. This refers to the four DNA building nucleotides: dATP: deoxyadenosine triphosphate; dCTP: deoxycytodine triphosphate; dGTP: deoxyguanosine Triphosphate and dTTP: deoxythymidine triphosphate
F_i	i^{th} Filial generation
g	gram
hr	hour
HDT	Hibrido de Timor
IRD	Institut de Recherche pour le Développement
kb	kilobase pairs
MAS	Marker assisted selection
Mb	Mega (million) base pairs
mg	milligram
ml	millilitre
mM	millimolar
ng	nanogram
PCR	Polymerase Chain Reaction
pg	picogram
QTL	Quantitative Trait Loci
RAPD	Randomly Amplified Polymorphic DNA
RFLP	Restriction fragment Length Polymorphism
SCAR	Sequence Characterised Amplified Region
SSRs	Short Sequence Repeats
V	Volts
W	Watts
µg	microgram
µl	microlitre
µM	micromolar
°C	degrees centigrade/celcius

ABSTRACT

Coffee Berry Disease (CBD) is an anthracnose of young berries of Arabica coffee (*Coffea arabica* L.) that is caused by the fungus *Colletotrichum kahawae*. It is a major limitation to economic production of the crop in Africa. Various sources of resistance to the disease have been identified and are used in breeding resistant cultivars. One such source of resistance is Hibrido de Timor (HDT), which is a natural hybrid between *C. arabica* and *C. canephora*. In Kenya, accessions of HDT progenies and its derivatives (cv Catimor) are used as donors of resistance to both CBD and CLR. The objective of this study was to decipher the genetic basis of CBD resistance derived from Hibrido de Timor and to identify molecular markers associated with it, which can be used for selection purposes.

Potential Amplified Fragment Length Polymorphism (AFLP) and microsatellite markers for the resistance were identified by characterisation of HDT derived polymorphism in resistant lines of cv Catimor. The accessions analysed included two lines of cv Catimor), eight resistant accessions of $BC_1 F_1$ progenies (Catimor x (Catimor x SL28)), up to 76 plants from three $BC_1 F_2$ populations and two accessions of the susceptible cv SL28. A Sarchimor line (T5296) and accessions of its F_2 progeny derived from its cross with a wild *C. arabica* collected from Ethiopia (ET6), which was used to map introgressed *C. canephora* chromosomal fragments, were included in some of the experiments. Three mapped *C. canephora* chromosomal fragments (T2, T3 and T4) were found to be present in the cultivars Catimor and Sarchimor and were therefore considered to be candidate carriers for CBD resistance. However fragment T4 was considered to be a weaker candidate because it was absent in one resistant $BC_1 F_1$ plant. Some AFLP markers of the introgressed fragments were cloned and converted into sequence characterised amplified regions (SCARs), and then assessed for polymorphism in a doubled haploid (DH) population so as to identify their linkage to coffee chromosomes. The SCARS

displayed very low polymorphism and it was possible to identify chromosome linkage for only one SCAR (J3), derived from the *C. canephora* chromosomal fragment T1. This SCAR was duplicated in chromosomes 2 and 8 of coffee genome.

Two F_2 populations (D and E) were raised by from crosses between two lines of cv Catimor (lines 127 and 88 respectively) and cv SL28. Phenotypic segregation for CBD resistance was verified by inoculation of half of each seed lot on the sixth week after germination by hypocotyls inoculation method. Resistant seedlings obtained from these tests were established in a nursery as Group 1 sub-populations and were used as checks in subsequent molecular studies. The other halves of the seed lots were transferred directly to the nursery without inoculation as Group 2 sub-populations representing unaltered F_2 populations for later studies. Segregation of candidate molecular markers of the resistance was verified using three microsatellites (Sat 11, Sat 32 and Sat 207) that are mapped onto the introgressed *C. canephora* chromosomal fragments T3, T1 and T2 respectively.

All the seedlings (both Groups 1 and 2 sub-populations) were screened for CBD resistance after one year by young seedlings inoculation method developed in this study. The method achieved a degree of success that was considered to be sufficient for identification of DNA markers of the resistance, despite of an expectation of some phenotypic misclassifications. Misclassification was expected due to the observation that plants with low vigour (stunted and/or thin) exhibited exceptionally high susceptibility including plants from Group 1 (resistant sub-populations) and some plants of cv Caturra failed to be infected.

Fifty-seven (57) microsatellites were screened for polymorphism amongst accessions of cvs Catimor, T5296, SL28 and the two F_2 populations (D and E). Twenty three (23) microsatellites

were variously polymorphic within or between lineages. Seven microsatellites had alleles that were common in the HDT derivatives, polymorphic in the two F_2 populations and absent in cv SL28. These were considered to be candidate markers of resistance to CBD. The seven microsatellites were then analysed in 95 Group 2 plants from Population E for segregation fitness and possible linkage to CBD resistance. Six of the microsatellites displayed segregation ratios that fitted Mendelian inheritance but one microsatellite (Sat 11) had distorted segregation in favour of the introgressed allele. It was further observed that Sat 207 and Sat 235 had marker alleles that were linked to CBD resistance. The same plants were analysed for an AFLP marker of the T4 fragment and it was observed to be present in 70.23% of the plants which suggested that it followed random Mendelian inheritance and it did not co-segregate with CBD resistance.

Further confirmation that the markers were linked to CBD resistance, the seven potential candidate microsatellites were amplified in fifty-six (56) Group 1 plants consisting of 29 and 27 individuals from Populations D and E respectively. These plants were also analysed with selected AFLP markers of the introgressed fragments T2, T3 and T4. The fragment T2 was confirmed to be linked to CBD resistance and further studies focussed it. Analysis was done with AFLP markers spread on the T2 fragment in plants selected from the two F_2 populations to cover the two screening methods, resistant and susceptible phenotypes. Sat 235 that was observed to be linked to CBD resistance was mapped using the same samples which had originally been used to map the introgressed *C. canephora* fragments. The established limits of the location of the gene confined it to a 26.9 cM segment, with high possibility of the gene to be within or near the limits of a 10.6 cM segment. The segregation of Sat 207 and Sat 235 in 47 resistant and 18 susceptible plants included in the 95 plants of Group 2 amplified earlier was re-examined with the mapping information. It was observed that two resistant plants had the introgressed Sat 207 allele but not the introgressed Sat 235 allele, while one susceptible plant without the introgressed Sat 207

allele had the introgressed Sat 235 allele. This prompted the assumption that the two markers maybe located on the opposite sides of the gene. If this is proved to be true, then the gene is located within a 13.2 cM chromosomal segment. No prominent skew in favour of homozygous introgressed genotypes compared to the heterozygous ones was observed in the resistant category of plants, indicating that the gene is of major action. It is therefore concluded that the locus carries a major resistance gene that was designated Ck-1 and is likely to be synonymous to T gene described earlier by other researchers.

Four out of five AFLP markers of the introgressed *C. canephora* chromosomal fragment T2 were successfully cloned, sequenced and specific primers designed. One primer pair amplified a monomorphic band whose intensity in agarose gel was related to the presence and absence of the parent AFLP marker at the theoretical optimum annealing temperature of 60 °C. At a higher annealing temperature of 62 °C, it amplified a dominant marker (AGC-CTG-c_{AA4}). The SCAR marker was analysed against Sat 207 and Sat 235 and it amplified as expected except in two plants that were assumed to be recombinant

RAPD markers for CBD resistance identified earlier by other researchers could not be reproduced, but specific primers designed from their sequences were tested in the F_2 populations by radioactive PCR and separated in denaturing polyacrylamide gels. One amplified a monomorphic band in all accessions while the other amplified two polymorphic bands, one of which was derived from HDT and it was linked to the T2 fragment. A survey of the microsatellite markers for CBD resistance was carried out in twenty-two (22) accessions bred from different accessions of HDT and agreement with earlier results was demonstrated.

Ninety one (91) accessions of *Coffea* species consisting of *C. arabica*, its putative parents namely *C. canephora* (and its close relative *C. congensis*) and *C. eugenioides* (and its close relative *C. anthonyi*) were analysed with eighteen (18) microsatellite markers of *C. canephora* chromosomal fragments introgressed into *C. arabica* and seven (7) SCARs developed from AFLP markers of some of the introgressed fragments. Different amplification characteristics of the microsatellites and SCARs were observed in the different *Coffea* species. Un-introgressed *C. arabica* accessions exhibited low variability. In cases where two microsatellite alleles per accession were amplified in *C. arabica*, there was amplification in all the species analysed with or without distinction between the canephoroid species (*C. canephora* and *C. congensis*) and eugenioid species (*C. eugenioides* and *C. anthonyi*). In cases where the un-introgressed *C. arabica* had one allele per accession, there was no amplification in all or most of the eugenioid species (*C. eugenioides* and *C. anthonyi*). Species specificity was also observed regarding some SCAR alleles, but no null alleles observed in amplifications in this system. In all cases there was an allele in canephoroid species (*C. canephora* and *C. congensis*) that was similar to the introgressed allele in HDT derivatives in regard to both microsatellites and SCARs. Sat 235 had no alleles shared between any of the un-introgressed *C. arabica* accessions and the accessions of the canephoroid group.

The maximum number of microsatellite alleles observed was seventeen and the minimum was three alleles, while the maximum number of SCAR alleles was five and the minimum was one. *C. canephora* had the highest number of alleles and the least polymorphic was the eugenioid group (*C. eugenioides and C. anthonyi*). The un-introgressed *C. arabica* accessions as a group had more alleles than the introgressed ones despite the introgressed accessions having extra alleles due to the introgression. In some cases, alleles similar to the marker alleles for introgression were observed in some accessions of the un-introgressed accessions of *C. arabica*.

In all cases, the genotypes of the HDT derivatives could be constituted by a combination of alleles observed in *C. arabica* and the canephoroid group. The alleles of HDT that were shared with the eugenioid group (*C. eugenioides* and *C. anthonyi*) were all observed in the un-introgressed *C. arabica* accessions. In HDT derivatives, only one of their alleles was replaced by the introgressed allele, even where there was more than one allele per accession of the un-introgressed *C. arabica*.

Microsatellites with potential for use as breeding tools for CBD and CLR resistance from the donor varieties Rume Sudan (resistant) and K7 (tolerant) were identified by their polymorphism between these varieties and the susceptible cultivars SL28 and Caturra. However it was noted that this potential would be attained by high performance techniques like LICOR fluorescence system that was used in this phase of study.

Key words: Coffee Berry Disease, *Colletotrichum kahawae*, *Coffea arabica*, *Coffea canephora*, Hibrido de Timor, introgression, resistance, chromosomal fragment, AFLP, Microsatellite, marker, allele

CHAPTER 1.   INTRODUCTION

Coffee is an important export crop and a major foreign currency earner for many countries located in the tropics of Africa, Asia and Latin America. It provides the livelihood for over 120 million people worldwide (Pare, 2002; Osorio, 2002). Arabica coffee (*Coffea arabica* L.) accounts for about 75% of the total world coffee production and the rest is mainly Robusta coffee (*Coffea canephora* Pierre). Major constraints to coffee production include pests and disease epidemics with various extents of impact in different regions, countries and continents.

1.1   Coffee production and its constraints in Kenya

Coffee is among the top three agricultural exports in Kenyan and it contributes up to 12% of the total export revenue (International Trade Centre, 2002). However, coffee productivity in Kenya is low with a national average of 400kg of clean coffee per hectare (International Trade Centre, 2002). Smallholders produce an average of 2.8 kg of cherry per tree while large estate growers realise an average of 5.6 kg per tree. This is very low compared to yields of 18.4 kg per tree, which are practically achieved in some estates (Karanja, 1996). Between 1989 and 1999, the national coffee production fell from 126,000 metric tons of clean coffee to 56,000 tons amounting to a loss of US$870 million. Although there is an interplay of factors whose individual level of contribution to the decline in production is difficult to isolate, one of the factors is poor disease management. This is partly due to high costs of pesticides that currently constitute the main control method. The strategy involves intensive pesticides spray programmes that accounts for up to 30% of the total cost of production. Disease management is a major limitation to economic coffee production especially to the smallholders, who find the use of pesticides beyond their financial and technical capabilities (Griffiths *et al.*, 1971; Walyaro *et al.*, 1984; Wrigley, 1988; Masaba and Waller, 1992). The major coffee diseases in Kenya are Coffee Berry Disease (CBD) caused by *Colletotrichum kahawae*, Coffee Leaf Rust (CLR) caused by

1

Hemileia vastatrix and Bacterial Blight of Coffee (BBC), caused by *Pseudomonas syringae pv garcae* (Kairu, 1998). *Fusarium* bark disease (FBD) and Fusarium root disease (FRD) that are caused by *Fusarium stilboides* and *F. solani* respectively, are becoming increasingly important in certain areas especially in lower altitude coffee growing areas ($\leq$ 1500m above sea level). Minor coffee diseases include those caused by *Cercospora coffeicola, Botrytis cinerea, Armilaria mellea* and nematodes. Coffee is mainly produced by developing countries and it is here where the impact of crop diseases is particularly acute (McDowell and Woffenden, 2003). An attractive alternative strategy for disease management is the development of resistant varieties. This strategy involves introduction of disease resistance genes from other varieties followed by backcrossing to the commercial cultivars to restore desirable traits especially yields and quality.

Conventional breeding methods take a long time due to the long generation interval of coffee (5 years) (Agwanda *et al.*, 1997). Furthermore, it would take at least 25-30 years after an inter-specific cross to eliminated undesirable traits and restore genetic makeup of the recipient coffee cultivar using conventional breeding methods (Anthony and Lashermes, 2005). The seedling hypocotyls inoculation method developed by Van der Vossen *et al.* (1976) shortened the period required to detect resistance to CBD. However it is limited when the programme requires procedures such as back crossing. The time required for breeding by traditional method can be shortened by use of DNA based marker assisted selection (MAS). The markers help in detecting a targeted genomic fragment and therefore selects for a desirable trait that is linked to it such as disease resistance, and this can be done in the early stages of plant growth. Selection by use of molecular markers results in a gain of about two generations of backcrossing and this gain can be higher if the objective is to reduce linkage drag (Riesenbierg *et al.*, 2000). Development of

2

modern breeding methods, whereby the genotypes of a progeny in a breeding cycle can be accurately detected early, is therefore of high priority.

1.2   Coffee Berry Disease (CBD)

As the name highlights, the main tissue infected is the berry. This is also the infection of highest economical importance, especially on green immature fruits, a stage in which it can cause up to 80% crop loss if not controlled and conditions are favourable (Griffiths *et al.*, 1971; Masaba and Waller, 1992).

1.2.1   The pathogen

The disease is caused by the species *Colletotrichum kahawae*, which belongs to the Genus: *Colletotrichum*; Family: *Phyllachoraceae*; Order: Phyllachorales; Class: Sardariomycetes; Phylum: Ascomycota; Kingdom: Fungi (Kirk *et al.*, 2001; Online site: http://www.Indexfungo rum.org/Names/fundic.asp). Like many other members of the species *Colletotrichum, C. kahawae* is considered to be an anamorph of the genus *Glomerella*. Until 1993 when it was renamed, the fungus was referred to as *Colletotrichum coffeanum* (Waller *et al.*, 1993). This was composite species taxon that included *C. gloeosporioides* and *C. acutatum* strains isolated from coffee, although the CBD pathogen displayed specific differentiating features like virulence on immature green coffee berries and colouration (whitish grey to dark grey). There are various differences in isolates of the pathogen including their aggressiveness (Rodrigues *et al.*, 1991; Rodrigues, *et al.*, 1992; Omondi *et al.*, 2000, 2001), but no conclusive evidence on the existence of its races has been demonstrated. Moreover, the isolates of the pathogen in Kenya belong to one vegetative compatibility group (Gichuru *et al.*, 2000) and are therefore of a clonal nature. However the possibility of appearance of races of the pathogen cannot be ruled out, especially due to the continued planting of resistant varieties in the field.

3

Apart from genetic factors, the susceptibility of coffee berries to CBD depends on their age and they are most susceptible when they are expanding between 4 and 16 weeks after flowering, and also when they are ripening (Mulinge, 1970). The infection agents are conidia whose optimum germination temperature is 22°C in water but it is higher in presence of leachates from coffee berries (Nutman and Roberts, 1960). After germination and infection, success of subsequent disease progress requires cool and humid weather conditions which are usually encountered on higher altitudes and in particular months depending on location. Chemical control aims at protecting the berries when they are at the susceptible stage especially if it coincides with favourable conditions (Griffiths *et al.*, 1971).

1.2.2 Symptoms of the disease

The CBD pathogen is able to infect several coffee tissues either naturally or by artificial inoculation that result into variable symptoms.

1.2.2.1 Berries

Infection of green expanding berries is the major and the most economically important natural occurrence of the disease. The first symptoms of infection on green immature are dark-brown slightly sunken spots. Under suitable environmental conditions, the spots enlarge to cover the whole berry and masses of conidia maybe visible (Plate 1). The lesions may reach the beans that become black and shrivelled. Finally the berries become brown or black and if desiccation occurs, they are mummified (Plate 1). The stalks of the berries are also attacked and destroyed and the berries are shed or they remain on the tree in mummified form. Infection on ripe berries is seen as dark sunken patches that spread rapidly and may cover whole berries resulting in symptoms referred to as brown blight. The disease may occur in another form where buff-coloured scab lesions develop, with scattered dark-coloured stromata during the hard berry stage

(Wrigley, 1988). Few spores if any are produced on these lesions. The fungus may die out in these areas and the infected tissue may be sloughed off. Scabs are frequently formed on resistant plants or in susceptible plants if the environmental conditions are unfavourable for the disease (Masaba and van der Vossen, 1982). In dry weather conditions, progress of the disease is halted and the lesions take on an ash-grey colour except where it is ringed by a dark brown edge. The mycelia under a scab may penetrate deeper and destroy the beans. During berry ripening, the scab-lesions may become active if weather conditions are ideal.
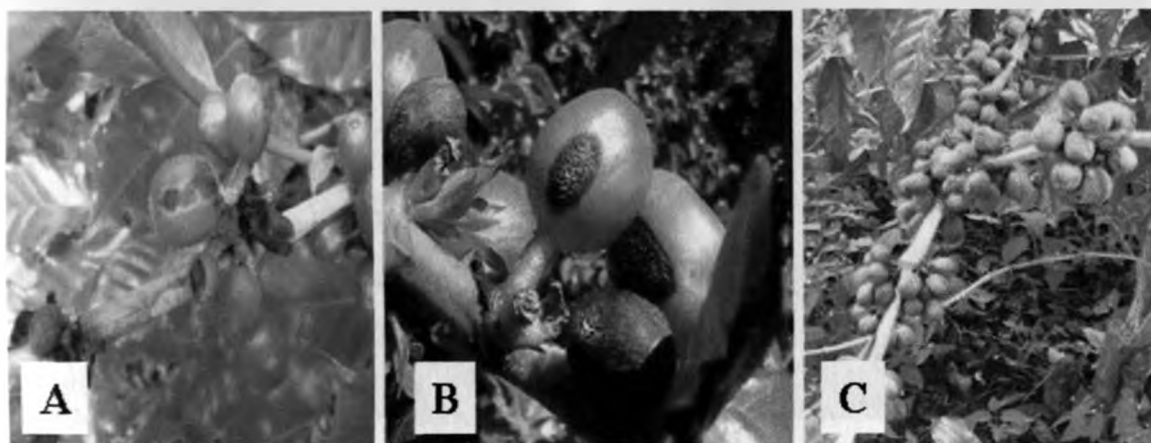
Plate 1. Symptoms of infection by *C. kahawae* on susceptible green coffee berries in the field (A) Active CBD lesions and dried young berries on susceptible cv SL28, (B) infection of cv SL28 showing conidial masses on green berries, blackened berries and stalks from which infected berries have detached (C) infection on cv Caturra showing dry brown mummified berries

1.2.2.2 Seedling hypocotyls and shoot tips

Infection of seedling hypocotyls and shoots is largely induced by artificial inoculation under controlled conditions, and symptoms largely depend on the degree of resistance of the seedlings that determine the degree of progress of the disease. In the most resistant cultivars, the symptoms do not develop beyond small scabs or brownish superficial lesions (van der Vossen *et al.*, 1976). In the moderately resistant cultivars, the symptoms develop into deeper black lesions that either become larger or increase in number as susceptibility increases. In the most susceptible

seedlings, the lesions coalesce and the hypocotyls stem or shoot tips become completely girdled, shrivelled, blackened and are finally killed. These symptoms are also occasionally observed in the field, especially on hypocotyls and shoots growing under infected trees when the whether is favourable for the disease (Plate 2).
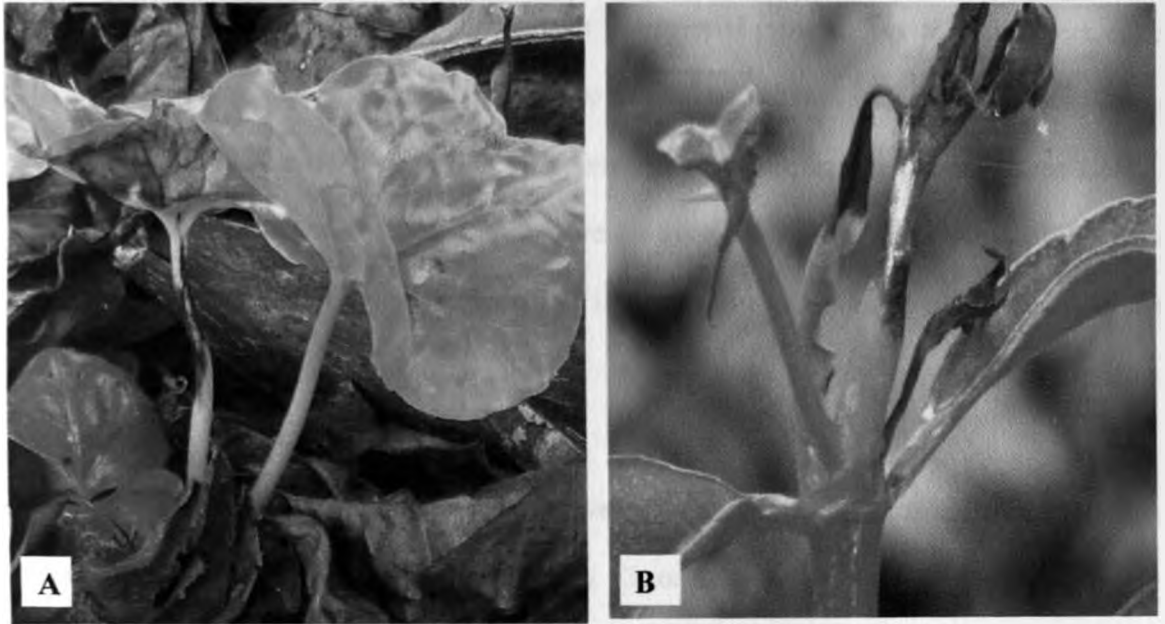
Plate 2. Infection of *C. kahawae* on coffee hypocotyls and shoot tips growing under a canopy of a cv SL28 tree in the field: (A) symptoms on a seedling hypocotyl; (B) symptoms on a young shoot growing on the stump of the tree.

1.2.2.3 Flowers

Infected flowers develop dark brown blotches or streaks on the white tissue that then turns black and are destroyed.

1.2.2.4 Leaves

Sometimes, the CBD fungus attack leaves. Leaf infection is seen as brown to black spots or elongated lesions mainly on the margins. This infection is relatively rare and not important in Kenya.

1.3 Sources and breeding for CBD resistance

There are numerous sources of different degrees of resistance to CBD in accessions of *C. arabica*. This was recognised quite early in the history of CBD whereby bronze tipped trees were observed to be more resistant than green tipped ones under field conditions (Rayner, 1952). However, systematic breeding for resistance to the disease started much later. Varieties such as Blue Mountain and K7 were recommended for commercial growing due to their tolerance to CBD and CLR that allowed acceptable yields to be realised without spraying. Van der Vossen and Walyaro (1980, van der Vossen, 2006) reported four CBD resistance genes in three loci i.e. R_1 and R_2 (in variety Rume Sudan and Pretoria respectively but in the same locus), T (in variety Hibrido de Timor or Timor hybrid) and k (in K7). The authors described R_1 as dominant, R_2 and T as intermediate and k as recessive.

Hibrido de Timor (HDT) is a natural cross between *C. canephora* and *C. arabica* that was first observed in 1927 in ex-Portuguese Timor; now Timor Lorosae (Bettencourt, 1973). A single plant without symptoms of CLR was observed and seeds from it were used to establish small coffee plantations. These plants exhibited vigour but yields were low and seeds from the best of these plants were selected. From 1956, large coffee plantations were established with the selected plants in all regions of Timor and they exhibited heterogeneity in morphology, interspecific origin and yields. The seeds of this hybrid were first sent to Centro de Investigação das Ferrugens do Cafeeiro (CIFC) (translation into English: Coffee Rusts Research Centre) in Portugal in 1957. Different introductions (such as HDT accession numbers 832/1, 832/2, 1343 and 2570) were done at different times. HDT is a heterogeneous population and out of the various introductions that were made to CIFC, only those that were resistant to all known races of CLR (i.e. of physiological group A) were used as resistant parents. Subsequently, this hybrid was distributed free of charge to almost all coffee research centres in the world, either as straight

progenies or as crosses with the best Arabica cultivars. Progenies of HDT have therefore been used in breeding programmes all over the world as sources of resistance, especially against CLR, CBD and nematodes. The hybrid acquired the resistance from genomic material from *C. canephora*.

The main hybrids produced at CIFC with HDT include HW26 (Caturra Vermelho x HDT 832/1), H46 (Caturra Vermelho x HDT 832/2), H361 (Villa Sarchi x HDT 832/2), H528 (Catuai Amarelo x HW26/13) and H529 (Caturra Amarelo x H361/3). In the pedigree selections, F_3 and F_4 generations of HW26 and H46 received the designation of "Catimor" by the Universidade Federal de Viçosa (UFV), Brazil. The hybrids H361, H528 and H529 were introduced in the American Continent in 1970, and their F_3 and further generations received the designations of Sarchimor, Cavimor and Cachimor (Bettencourt, 1983). Catimor and Sarchimor are the most advanced selections and have been widely distributed in the coffee-growing countries, not only in Latin America but also in Africa (Malawi), Asia (India) and Oceania (Papua New Guinea). After local selection for several years, Catimor received regional designations such as Oeiras, Tupi, Obata, Iapar59 (Brazil), Catrenic (Nicaragua), Costa Rica 95 (Costa Rica), Ihcafe-90 and Lempira (Honduras), Catisic (el Salvador) and Mida 96 (Panama). In Colombia, HDT 1343 was crossed with Caturra to produce the original hybrid from which variety "Colombia" is derived. More details on HDT and its utilization in breeding can be obtained in Bettencourt (1983), Rodrigues Jr. *et al.* (2000) Varzea and Marques (2005), Pereira *et al.* (2005) and Silva *et al.* (2006). There are still introductions of HDT derivatives that are being introduction in more countries or into the same countries under different names or of lineages. This has contributed to confusion about the derivatives of HDT and wrongful use of the word "Catimor" to refer to any kind of HDT derivative, a situation that can lead to use of wrong genotypes.

In Kenya, straight progenies of HDT of accession number HDT 1349/269 (Omondi *et al.*, 2001) were introduced in 1960 from CIFC (Portugal). Later in 1975 and 1977, F_3 and F_4 progenies of cv Catimor, from a cross between HDT (CIFC accession number 1343) and *C. arabica* cv Caturra, were received from Colombia (Van der Vossen and Walyaro, 1981; Walyaro, 1983). These cv Catimor lines are homozygous for compact growth and are resistant to CLR. In Kenya they were screened for resistance to CBD and CLR. When these Catimors were introduced into Kenya, they were seeds from single trees and each seed lot had a number that signified its lineage. The numbers were retained by CRF's Coffee Breeding Unit and denoted as *"Progeny"* numbers hence designations like Catimor 88, Catimor 90, Catimor 127 etc. In this thesis, the term *"line"* is used to refer to the coding of *"Progeny"*. The introduced lines of cv Catimor established in Kenya as they were received or were advanced by selfing. This means that the Catimors presently in Kenya are either F_3, F_4 or F_5 progenies. These Catimors and do not have adequate cup quality for direct commercial planting when compared to the major commercial cultivars in Kenya (Van der Vossen and Walyaro, 1981, Omondi *et al.*, 2001). However, they are used as donors of disease resistance in breeding programmes up to date. In fact the cv Catimor lines are the maternal parents of the hybrid cultivar Ruiru 11 bred in Kenya. More HDT derivatives are still being introduced into the country.

Due to filial advancement of the original HDT accessions and their use in different breeding programmes, there are different genomic fragments derived from the initial *C. canephora* genome that occur in different derivatives of the hybrid across the world. Such coffee progenies individually contain 9-29% of the *C. canephora* genome, while in combination they contain an estimate of 51% of the *C. canephora* genome (Lashermes *et al.*, 2000a), and are of great value in *C. arabica* breeding especially for resistance to pests and diseases. Molecular analysis can help in characterising desirable and undesirable *C. canephora* genomic fragments present in the HDT

derivatives and consequently select elite lines for breeding programmes or commercial cultivation.

A coffee-breeding programme was started at CRF in 1971 with a total of 35 coffee varieties as progenitors and it resulted in the release of cv Ruiru 11, which is a composite of 60 hybrids (van der Vossen and Walyaro, 1981, Omondi *et al.*, 2001). Several of the progenitors were included as donors of CBD resistance such Rume Sudan, HDT, Blue Mountain, K7, Pretoria and Geisha 10. Several multi-cross lineages were developed and backcrossed to the commercial cvs SL28 and SL34 to restore quality and yield while selecting for resistance to CBD and CLR. This resulted in different lines that are used as males to make the final hybrid cross with cv Catimor lines as maternal parents.

Individual Ruiru 11 hybrids are realised by pollinating trees of specific Catimor lines with pollen bulked from specific males of the same lineage. The males may posses different assortment of the resistance genes and most likely in heterozygous state. The guaranteed genetic resistance in the Ruiru 11 hybrids is therefore that present in cv Catimor. Although phenotypically they are homogeneous (due to dominant compact growth habit of cv Catimor), Ruiru 11 hybrids are genetically heterogeneous which theoretically buffers them against pathogen and environmental variations. However, this heterogeneity leaves room for genetic improvement and selection for environmental adaptation and possibly other traits. Another source of resistance to CBD is that observed in collections of wild *C. arabica* from Ethiopia (van der Graaff, 1978; van der Vossen and Walyaro, 1981). However this is yet to be exploited in commercial coffee production.

1.4 Selection for CBD resistance

Apart from natural CBD infection in the field, several artificial inoculation methods have been developed to screen coffee plants for resistance to the disease. The major ones are inoculation of detached green berries; seedling hypocotyls and seedling shoot tips (van der Vossen *et al*, 1976). The last one is less used partly due to the time it would require to raise the seedlings and their bulky nature. In the hypocotyls inoculation method, seedlings are germinated in sterile sand and their hypocotyls are double inoculated on the 6th week after germination. Typically, the seedlings have unopened cotyledons and the inoculation is done twice at 48-hour interval by spraying with a *C. kahawae* spore suspension of 2×10^6 conidia/ml. After an initial incubation at room temperature for 96 hours after the first inoculation, the seedlings are incubated in a temperature controlled room at $18 \pm 2°C$ for 2 weeks. They are then transferred back to room temperature and scored one to two weeks later. The most resistant seedlings with no infection signs are scored into class 1 and thereafter progressively in upper classes as the symptoms increase from small specks, to brown superficial lesions, to deep larger black lesions and finally to girdling and seedling death in class 12, which is the most susceptible (Plate 3, Appendix 1). Despite different opinions especially on data interpretation, this method is very valuable especially in screening populations to obtain resistant plants and/or using the averaged results to classify the CBD phenotype of the mother plant (Van der Vossen, *et al*., 1976; van der Graaff, 1978, 1982; Dancer, 1986; Owour and Agwanda, 1990).

In shoot tips inoculation, one-year-old seedlings with 1-2 cm long young shoots are inoculated once with the same inoculum conditions as for hypocotyls and incubated for 48 hours in a moist chamber. The seedlings are then left in the nursery for symptom development and scored as for the hypocotyls but from Class 0 to 11. It is imperative that for the symptoms to develop well, the ambient temperatures have to be favourable and the seedlings have to be selected so as to have

young shooting tips of 1-2 cm length. This limits random or total population screening since they cannot all be in the right stage at the same time.

There are various possible mechanisms of resistance to infection by *C. kahawae* in coffee. They include pre-formed and induced antifungal compounds and structural barriers as reviewed by Gichuru (1997). Since then, more mechanisms have been reported which include rapid localised cell death, accumulation of callose, lignin-like and phenolic compounds (Gichuru, 1999, Rodrigues *et al.*, 1999; Silva *et al.*, 2006). Some of these mechanism are likely to be pathogen non-specific and could also be induced by mechanical injury. It may be possible to develop some of the observed biochemical and structural changes into methods of screening for CBD resistance. Widespread MAS application of RAPD markers identified by Agwanda *et al.* (1997) is hampered by their lack of reproducibility in different laboratories and over time. They could be improved by developing them into SCAR markers. Mapping them in relation to the CBD resistance gene(s) would help in judging their genetic reliability. *In vitro* selection methods reported by Nyange *et al.* (1995, 1997) maybe technically demanding and therefore limit their routine and large-scale use. The methods would also require further studies for adaptation to explants obtained from other plant tissues rather than hypocotyls, which involves destruction of the individual seedlings sampled.
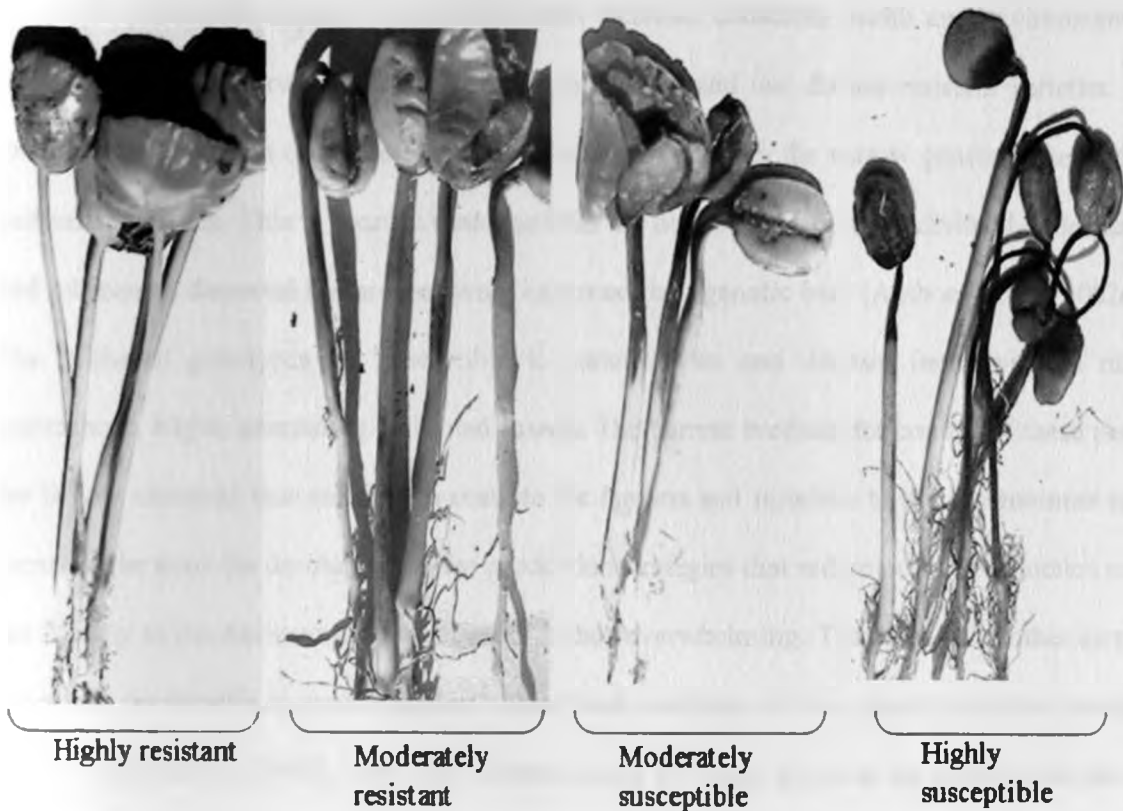
Plate 3. Photographs of hypocotyls of coffee seedlings showing symptoms of infection by *C. kahawae* at scoring time on the fifth week after inoculation. The phenotypic categories comprise of highly resistant seedlings (Classes 1-4), moderately resistant (Classes 5-7), moderately susceptible (Classes 8-10) and highly susceptible (Classes 11-12) as categorised in this study.

CHAPTER 2.   JUSTIFCATION

As coffee production expands, production costs increase, consumer health and environmental issues become of priority, it becomes crucial to develop and use disease resistant varieties. In totality, the production of coffee is extremely vulnerable due to the narrow genetic base of the cultivated varieties. This is because these varieties are derived from a few individual collections and subsequent dispersal has progressively narrowed their genetic base (Anthony *et al.*, 2002a). The cultivated genotypes are susceptible to various pests and diseases including leaf rust, anthracnose, blight, nematodes, wilts and insects. The current methods for control of these pests are largely chemical that are of high costs to the farmers and injurious to the environment and humans. The need for developing coffee production strategies that reduce cost of production and are friendly to the environment and humans is thus overwhelming. Therefore as in other crops, the search for durable resistance against coffee pests continues to be a priority objective though elusive (Michelmore, 2003). Host plant resistance may be singly adequate for commercial use or be incorporated into integrated disease management programmes.

As a contribution to this objective, Coffee Research Foundation (CRF), Kenya, released an Arabica coffee cultivar (cv Ruiru 11) in 1985 (Nyoro and Sprey, 1986). This cultivar combines resistance to CBD and CLR with high yields, fine cup quality and compact growth habit. However some isolates of the pathogens are isolated from this variety raising the concern of the durability of its resistance. Inheritance studies for CBD resistance revealed three genes on separate loci: R and T (dominant/intermediate) and k (recessive) (van der Vossen and Walyaro, 1980, van der Vossen, 2006). Cultivar Ruiru 11 is a composite of about 60 hybrids, each derived from a cross between a specific female and male population (Omondi *et al.*, 2001). Further more, each hybrid may express only the T gene or both T and R genes but not the recessive k gene. The population is therefore not genetically uniform, raising a need to conduct detailed studies to

establish the genomics of its resistance to facilitate tracing of the genes. However, it is difficult to study the individual genes in a complex product like Ruiru 11 without previously developing molecular markers and other basic knowledge for each gene. This study addresses this need by focusing on resistance gene(s) introgressed into *C. arabica* from *C. canephora* via HDT, which as noted earlier (Section 1.3) is of great importance in breeding for CBD resistance in Kenya.

The *C. canephora* chromosome fragments introgressed into *C. arabica* through HDT are important in breeding for pest and disease resistance, (Orozco-Castillo *et al.*, 1994, Lashermes *et al.*, 2000a), though some of them may lead to reduction of cup quality compared to pure Arabica varieties (Bertrand *et al.*, 2003). This study was formulated with the general objective of generating DNA based information on these *C. canephora* introgressed genomic fragments with emphasis on resistance to CBD. Development of DNA markers for the introgressed *C. canephora* fragments will hasten selection for the desired ones in future and against undesired ones. Selection would best be for the smallest fragments carrying the desired gene(s). Identification of markers linked to the resistance is possible by analysing the segregation of polymorphic bands and CBD resistant phenotype in an F_2 generation between a donor variety (such as cv Catimor) and a recipient variety (such as cv SL28). The markers can then be mapped and used as selection tools in the development of resistant varieties as well as refining the current varieties. The mapping population(s) can also be a source of different genetic assortments which can be of great value as elite breeding parents, be developed into pure line cultivars or used for gene mining. This study took into account all these aspects and plants of the developed F_2 populations were established in the field for later uses.

Although traditional breeding methods are considered difficult, they are essential in the development of new varieties and in verification of molecular markers. Suitable CBD screening

method(s) had to be selected from documented procedures and modified if necessary to ensure maximum reliability of identified markers. One disadvantage of the hypocotyls inoculation method (Van der Vossen *et al.*, 1976) is that susceptible seedlings are killed very early and they cannot be used for later studies in living form. It is also very difficult to obtain enough DNA from these seedlings (e.g. from roots) before the tissues are colonised by the fungus or die. Another disadvantage is that the results of inoculations of seedlings even from the same source may give different results in different repeats overtime thus creating inconsistency. The other screening method developed by the same authors is the inoculation of young seedlings with young shoots. As reported by the authors, there was no control of temperature and this would limit the tests to periods with favourable conditions. Furthermore, the need of selecting seedlings at the right growth stage would not allow the whole population to be screened at the same time. The option of raising the plants to maturity, for field evaluation or laboratory tests on the berries or seeds that they produce, is time consuming because it requires a whole generation interval. There was thus a need to develop a method that addresses the above limitations. The method would have to give a high value to the individual seedling disease reaction, allow extraction of DNA from the entire population and enhance survival chances for susceptible plants. In this study, a modification of the shoot tip inoculation method was assessed and used. The modifications aimed at enhancing infection and disease progress, and also developing a scoring scale with reduced intermediate classes.

There is little work done on the genomics of disease resistance in Arabica coffee, and more so in regard to CBD. Previous work includes the classical gene identification through inheritance studies by Van der Vossen and Walyaro (1980), search for isozyme markers for CBD resistance by Gichuru (1993) and identification of RAPD markers for CBD resistance by Agwanda *et al.* (1997). Molecular work by Noir *et al.* (2001, 2003) focussed on Resistance Gene Analogs

16

(RGAs) and nematode resistance while that of Prakash *et al.* (2004) focussed on resistance to CLR. There is therefore need to widen the knowledge of the genomics of disease resistance in coffee and develop appropriate markers. Development of easy to use and/or highly informative DNA marker(s) for disease resistance is of major priority. This was the major objective of this study in relation to CBD using microsatellites and the versatile AFLP methodology coupled with development of SCARs to improve reproducibility.

HDT derivatives are increasingly becoming more important in production of *C. arabica* especially as donors of resistance to various pests and diseases. Molecular studies have been carried out on these materials in relation to genetic diversity, relatedness to diploid relatives, cultivar identification, effects of the introgression on traits like beverage quality, and mapping of pest resistance (Lashermes *et al.*, 2000a; Steiger, *et al.*, 2002; Anthony *et al.*, 2002b; Bertrand *et al.*, 2003; Moncada and McCouch, 2004). Some anticipated studies in the future include identification of the functions of the introgressed fragments in the *C. arabica*, walking on *Coffea* genome in attempt to isolate and clone various genes of interest, use of transferable markers to search and transfer homologous genes from different *Coffea* species/genotypes into elite *C. arabica* cultivars, and subsequently fingerprinting the developed varieties. This can be done with highly reproducible markers like microsatellites and SCARs, but these may be complicated depending on degree of their diversity or repetition in the *Coffea* genome. This study explored the practical application potential of these marker systems by analysing their diversity in *C. arabica* and its progenitors with emphasis on markers of *C. canephora* chromosomal fragments introgressed into *C. arabica*.

CHPTER 3. OBJECTIVES

The general objective of this study was to decipher the genomics of *C. canephora* chromosomal fragments that are introgressed into *C. arabica* genome via Hibrido de Timor (HDT) and subsequently identify the one(s) that confers resistance to CBD.

The specific objectives were:

1. to identify and characterise introgressed *C. canephora* chromosomal fragments in cv Catimor lines that are used as donors of resistance to CBD and CLR in Kenya.

2. to develop mapping F_2 populations from crosses between cvs SL28 x Catimor

3. to develop a suitable method for early screening the of F_2 populations for CBD resistance while preserving susceptible seedlings

4. to identify and map DNA markers of resistance to CBD

5. to assess the diversity of microsatellite and AFLP-derived SCAR markers of introgression from *C. canephora* into *C. arabica* in *C. arabica* and its putative parents

CHAPTER 4.   LITERATURE REVIEW

4.1   Molecular markers

Each gene or DNA sequence occupies a particular place on a chromosome called "locus". Stansfield (1986) stated that the term marker usually refers to "locus marker". Due to mutations, genes can be modified into several mutually exclusive forms called "alleles" or allelic forms, and all allelic forms of a gene occur at the same locus on homologous chromosomes. All "molecular markers" are loci markers related to DNA and they can also be biochemical or morphological. Allozymes (or isozymes) are different forms of the same enzyme, coded for by alleles of the same gene and can be separated by electrophoresis, which enables their use as molecular markers. DNA based markers are better markers for close relatives and can be detected at all stages of development, unlike allozymes that may be age or environment dependent. DNA markers are also more numerous than allozymes. Over time, various methodologies for generating DNA markers have been developed and used in various plants, including coffee, with diverse objectives such as mapping traits of interest, evolutionary studies and biosystematics. Characteristics of good markers include:

1. Mendelian inheritance: transmitted from one generation to another in a predictable manner

2. Polymorphic: present several alleles at the locus investigated (multi-allelic)

3. Co-dominant: allow the discrimination between homozygotes and heterozygotes

4. Neutral: all alleles have the same fitness

5. Not epistatic: the genotype of a phenotype can be determined irrespective of the other loci

6. Independent of environment: no phenotypic plasticity

7. Frequent occurrence in the genome

8. Even distribution throughout the genome

9. Highly repeatable

10. Easy to generate and interpret

One type of molecular marker may not meet all the above qualities and generally none is suitable for all applications. Different marker systems vary in technical requirements, cost (development and running), speed (throughput), amount and quality of DNA needed, level of polymorphism revealed, precision of genetic distance estimates and statistical power. The thrust in developing new marker systems has been the need to increase the resolution of the different systems and to overcome the limitations of each one (Rafalski *et al.*, 1996). Allozymes and Restriction Fragment Length Polymorphisms (RFLP) were the earlier ones developed but are not numerous enough for high-density mapping. Since the development of Polymerase Chain Reaction (PCR), more maker systems have been developed and they include Randomly Amplified Polymorphic DNAs (RAPD), Simple Sequence Repeats or Simple Sequence Repeat polymorphisms (SSRs)[also referred to as Variable Number of Tandem Repeats (VNTR), Microsatellites. Short Tandem Repeats or Simple Tandem Repeats (STRs), or Simple Sequence Length Polymorphisms (SSLP)], Cleavable Amplified Polymorphic Sequences (CAPS), Amplified Fragment Length Polymorphism (AFLP) and Inter-SSR Amplification (ISA)[also referred to as Inter-SSR (ISSR)].

A marker that is randomly generated without prior knowledge of its sequence can be sequenced and specific primers be designed to amplify the region. The subsequent PCR-based marker is referred to as Sequence Characterised Amplified Region (SCAR, or Sequence Tagged Site, STS). PCR products can be analysed for conformational polymorphism by Single Strand Conformational Polymorphism (SSCP) whereby it is denatured and then electrophoresed in non-denaturing polyacrylamide gel (Orita *et al.*, 1989). The DNA strands fold onto themselves

resulting into different conformations of different mobility. The conformation is dependent on the sequences of the fragments and thus reveals sequence differences such as point mutations.

Typically the choice of which fingerprinting technique to use depends on (1) the application (e.g. DNA genotyping, genetic mapping, population genetics); (2) the organism under investigation and its state of knowledge (e.g., prokaryotes, plants, animals,); (3) the resources available (time, skills, money, equipments, availability and supply of chemicals), and (4) amount and quality of DNA (Vos *et al.*, 1995; Rafalski *et al.*, 1996; Robinson and Harris, 1999; Grivet and Noyer, 2003). The emergence of analysis kits and automation of procedures reduces the technological skills required, and increases the throughput of samples analysed. Collaboration between experts in the different types of marker technologies is a good way to realise objectives in an efficient and most cost effective way (Farooq and Azam, 2002). This is true not only for people starting the technologies, as in developing world, but also in developed world. This justifies the initiation of various international genomic networks including the International Coffee Genomics Network (ICGN). Below are brief descriptions of the various DNA markers, many of which were used in various stages of this study.

4.1.1   Restriction Fragment Length Polymorphism

Restriction Fragment Length Polymorphism (RFLP) involves digestion (restriction) of the DNA being analysed, agarose electrophoresis, southern blotting and probing with labelled sequence-specific probes (Botstein *et al.*, 1980). It is limited by requirement of large amounts of high quality DNA (1-10 μg per gel lane), but it has low start-up cost especially if probes are available (probes from one species may work in several other species) and involves simple techniques. RFLP markers are co-dominant and thus can analyse multiple alleles in a locus. Depending on the probe, coding and non-coding sequences of DNA can be analysed. This methodology was not used in this study.

4.1.2 Cleavable Amplified Polymorphic Sequences

Cleavable Amplified Polymorphic Sequences (CAPS) technique is related to RFLP in that polymorphism is revealed by restriction, but differs in that the substrate is a locus specific PCR product and revelation is by ethidium bromide (Konieczny and Ausubel, 1993). A segment of DNA is amplified with locus specific primers and the product is restricted using various enzymes. The restriction product is then analysed for polymorphism by electrophoresis in agarose gel and staining in ethidium bromide. Total polymorphism is by both the PCR (present/absent and size) and cleavage. This method is somehow less informative than RFLP because only the amplified region of the genome is analysed. Specific restriction enzymes may be used if the sequences of the PCR products are known and are polymorphic between individuals screened in a population. Otherwise several enzymes are tested at random and those that generate polymorphism are identified. This methodology was used to analyse PCR products from DH plants using primers specific to the sequences of RAPD markers identified by Agwanda *et al.* (1997). The products were first sequenced and potentially polymorphic cutting sites were identified (Section 5.1).

4.1.3 Randomly Amplified Polymorphic DNAs

Randomly Amplified Polymorphic DNAs (RAPD) is based on the fact that using short arbitrary primer sequences; they can by chance anneal on random sequences within the genome in close proximity and in opposite orientation to be amplified in a PCR programme (Williams *et al.*, 1990; Welsh and McClelland, 1990). The amplification products are then separated in agarose gel and revealed by ethidium bromide, but they can also be analysed in acrylamide gel. The technology is simple, low cost and the random primers are easily available. This method requires low amount of DNA, which can be of lower quality than for RFLP, but optimization of PCR conditions is needed to improve repeatability. The markers are scored as dominant. This

22

methodology was used to regenerate RAPD markers of CBD resistance that were identified by Agwanda *et al.* (1997).

4.1.4 Sequence Characterised Amplified Regions

Sequence Characterised Amplified Region (SCAR) technique involves sequencing of markers (DNA fragments) and designing locus specific primers can overcome some limitations of particular markers like sensitivity to PCR conditions. The subsequent sequence-specific PCR products may maintain the polymorphism of their parental markers, exhibit different polymorphism like co-dominance while the parent markers were dominant, or loose the polymorphism (Paran and Michelmore, 1993). New methodology for analysis of polymorphism may then be employed like identification of polymorphic restriction sites in different alleles followed by electrophoresis of the restriction products (CAPS), or by single strand conformational polymorphism (SSCP). In this study, SCARs were developed from AFLP markers to facilitate mapping of AFLP markers onto coffee chromosomes (Section 5.1), to make the AFLP markers more repeatable (Section 5.4) and for analysis of the diversity of the SCARs between *C. arabica* and its putative parents (Section 5.5).

4.1.5 Simple Sequence Repeats and Inter- Simple Sequence Repeats

Simple Sequence Repeats (SSRs) consist of variable stretches of repeated motifs of one to six nucleotides, which are abundantly and randomly found in eukaryotic genomes (Rafalski *et al.*, 1996). However, recent data suggest that their genomic distribution is non-random and are found mainly in non-coding DNA regions (Li *et al.*, 2002). First the genomic DNA is sequenced, SSRs are identified and PCR primers are designed from the two sides flanking the SSR motif. The primers are orientated such that they amplify the region carrying the SSR. Polymorphism is largely due to how many times the motif is repeated in different chromosomes, but can also be

due to the sequences between the primer annealing sites and the repeated motifs. If the primers are orientated such that they amplify the region between the repeated motifs, the product is referred to as Inter-Simple Sequence Repeats (ISSR) and polymorphism is due to size of these regions. The products are separated in acrylamide sequencing gels and can be revealed by radioactive/fluorescent labelling or silver nitrate staining. SSR markers are generally co-dominant, highly repeatable and transferable between laboratories, making them ideal for sharing. ISSRs are generally dominant but may sometimes be co-dominant. However, these marker systems are more costly to develop because the genomic sequence has to be established first. The most common steps in identification of SSR polymorphisms are:

1. Genomic library construction

2. Library screening by hybridization to enrich certain bases

3. DNA sequence determination of positive clones

4. Designing of locus specific primers

5. PCR analysis and identification of polymorphism

6. Mapping of polymorphic markers

High resolution electrophoretic separation is required to reveal allelic polymorphism that may differ by only two base pairs. Denaturing poly-acrylamide gels are the best, but they require radioactive, fluorescent labelling or silver staining to reveal the bands. However, electrophoresis in high concentration agarose gels followed by ethidium bromide staining can sometimes offer satisfactory results (Rafalski *et al.*, 1996). Such a procedure would make them relatively easy to use, especially in low technology laboratories. Although the expectation is the occurrence of one locus, multiple loci may occur due to duplication, or as in polyploids, due to homologous loci in the different genomes. Null alleles may also occur due to deletion or alteration of the priming sites. SSRs have a mutation rate ranging from 10^{-2} to 10^{-6} events per locus per generation, which

24

is very high compared to point mutations especially at coding gene loci (Li *et al.*, 2002). They have the highest information content compared to other markers but their very high cost of development may not be justified in many laboratories. This limitation might be reduced once more laboratories submit the SSR sequences they develop into public domain. Use of SSRs is also made easier by the fact that those developed from sequences of one taxon may work in other taxa. For example, SSRs that are developed from *C. arabica* or *C. canephora* work well in other *Coffea* species and even in the closely related genus *Psilanthus* (Combes *et al.*, 2000; Coulibaly *et al.*, 2003, Poncet *et al.*, 2004). Microsatellites from *Coffea* spp were tested for linkage to CBD resistance in Section 5.4 and for their diversity between *C. arabica* and its putative parents (Section 5.5).

4.1.6 Amplified Fragment Length Polymorphism

Amplified Fragment Length Polymorphism (AFLP) has most characteristics of a good marker, except co-dominance and a degree of non-repeatability. It combines the random polymorphism of restriction sites and a few nucleotides (typically 6) adjacent to the restriction sites. It takes advantage of the polymerase chain reaction (PCR) and the specificity of restriction enzymes, to amplify a limited set of DNA fragments from a specific DNA sample. It analyses many loci over the entire genome in one reaction (Vos *et al.*, 1995; Rafalski *et al.*, 1996; Blears *et al.*, 1998; Robinson and Harris, 1999). Due to the high polymorphism that they can detect, AFLP markers are a priority as the most efficient markers (Mueller and Wolfenbarger, 1999). Garcia *et al.* (2004) found AFLP to be best suited for fingerprinting and assessing relationship in maize compared to other markers based on its practicability and precision of results. Although AFLP markers analyse a large part of the genome simultaneously, they occasionally exhibit clustered distribution especially in centromeric regions. This may be affected by the choice of enzymes

25

(methylation sensitive or insensitive) and this feature reduces the degree of whole genome coverage (Saliba-Colombani *et al.*, 2000; Wang *et al.*, 2005).

The AFLP methodology involves digestion of high quality genomic DNA (0.20-0.50µg) with restriction enzymes (usually a combination of a rare and a frequent cutter), ligation of double-stranded adaptors to the restriction ends, pre-selective and selective amplifications and then electrophoresis. During the amplifications, oligonucleotide primers complementary to the adaptors are used. One selective nucleotide is added to the 3' end of the primers during pre-selective stage, and two additional nucleotides in the final amplification stage. During final amplification, the primer matching the adaptor to the rare cutter is end-labelled by a radioactive or fluorescent label, and therefore only fragments with this primer are visualised after electrophoresis. Many laboratories use *Eco*RI and *Mse*I restriction enzymes, both of which have restriction sites distributed over the genome, are insensitive to methylation and can digest in the same buffer and at the same temperature (Rafalski *et al.*, 1996). These enzymes also permit restriction and ligation reactions to be done simultaneously under the same conditions.

Despite the high informative potential of AFLP, it is more sophisticated involving more steps and reagents, thus making it more laborious and requires higher technical skills. The method also requires relatively higher amount of high quality template genomic DNA to start a reaction. However, there is high multiplicity in down stream reactions making it possible to test many primer combinations without going back to the genomic DNA stock. The method is relatively expensive, much of the cost being the synthetic primers and labelling chemical (radioactive or fluorescent) (Rafalski *et al.*, 1996). Capital requirements vary with the electrophoresis and revelation methodologies used. AFLP polymorphism can be co-dominant but this relationship is not obvious and requires more inheritance or sequence analysis for verification. Co-dominance is

mainly due to differences in fragment sizes while dominance is mainly due to presence or absence of restriction sites or primer annealing sites. Pearl *et al.* (2004) observed eight AFLP bands to be co-dominant in *C. arabica*. In tomato, Saliba-Colombani *et al.* (2000) observed as many as twenty eight markers to be co-dominant, while Wang *et al.* (2005) observed two co-dominant AFLP bands that were amplified by different primer pairs in pawpaw (*Asimina triloba*). However, AFLPs are usually analysed as dominant. In this study, AFLPs were used to map the gene for resistance to CBD (Sections 5.1 and 5.4).

In most cases, no one fingerprinting technique is ideal for all applications. However, AFLPs are quickly becoming the tool of choice for many applications and organisms. Potential applications include screening DNA markers linked to genetic traits, parentage analysis, forensic genotyping, diagnostic markers for pathogen borne diseases, and population genetics. Since the AFLP technique can be applied to a wide variety of organisms with no prior sequence information, this technique has the potential to become a universal DNA fingerprinting tool. Universal reagents and kits for this methodology are also now commercially available. Molecular markers of choice therefore include amplified fragment length polymorphism (AFLP) for rapid genome wide analysis, and microsatellites and sequence characterised amplified regions (SCARs) for highly repeatable anchor markers.

4.2   *C. arabica* genome

The subjects of origin, evolution and diversity of *C. arabica* genome have been reviewed recently by Anthony and Lashermes (2005). There are about 100 species in the genus *Coffea* but *C. arabica* is the only tetraploid ($2n = 4x = 44$) (Charrier and Berthaud, 1985). It has for long been suggested that *C. arabica* is an allotetraploid with diploid meiotic behaviour, and has a centre of diversity outside the centre of origin of its parental diploid *Coffea* species (Carvalho,

1952). The various molecular markers developed in recent times have been used to study this subject. Genomic analysis using Restriction Fragment Length Polymorphism (RFLP) of chloroplast DNA (cpDNA), which is maternally inherited, demonstrated that *Coffea eugenioides* (or a closely related ecotype such as *Coffea anthonyi*: formerly referred to as *Coffea* sp moloundou (Anthony *et al.*, 2006)) donated the maternal genome (Lashermes *et al.*, 1995). On the other hand, analysis of ribosomal DNA (rDNA) demonstrated that *Coffea canephora* (or a closely related ecotype like *C. congensis*) donated the paternal genome (Lashermes *et al.*, 1995). The amphidiploid nature of the *C. arabica* genome was further confirmed almost simultaneously, by two independent teams using genomic *in situ* hybridisation (GISH) (Raina *et al.*, 1998; Lashermes *et al.*, 1999). Using the genomes of the two potential diploid progenitors as probes, the affinity of the two sets of chromosomes in *C. arabica* to those of the putative progenitors was demonstrated. Lashermes *et al.* (1999) further suggested the speciation of *C. arabica* took place not more than 1 million years ago, and the two constitutive genomes in the *C. arabica* (E^a and C^a referring to the chromosome sets from *C. eugenioides* and *C. canephora* respectively) have not differentiated much from their unique donor parents. The two sets of chromosomes in the *C. arabica* genome display a disomic inheritance pattern due to genetic control rather than structural differences of the chromosomes (Lashermes *et al.*, 2000b; Herrera *et al.*, 2002).

The nuclear DNA content of *C. arabica* is about 2.61pg with a dihaploid (2x) estimate of about 1300Mb (Cros *et al.*, 1995). The genetic size of *C. arabica* genome can only be deduced from that of the haploid genome of *C. canephora* which is about 1400 cM corresponding to about 570 kb per cM (Lashermes *et al.*, 2001), though the physical size is less than twice that of *C. canephora* (700 Mb). There possibly could be factors such as gene loss/silencing or differential expression that occur after polyploidization (Adams and Wendel, 2005). These factors might

28

also have occurred in the speciation of *C. arabica*. It is evident that *C. arabica* is more susceptible to diseases than its diploid progenitors and it has diverged in other traits such as quality. The functional status of the constitutive genomes in the *C. arabica* is therefore an interesting subject to investigate.

4.3   Genetic variability and introgression into *Coffea arabica*

Morphologically, there are distinctive characters observed in varieties of *C. arabica* such as growth vigour, canopy habit, leaf size, leaf colour, shape of leaves, angle of branching, berry shape, bean size and pest resistance. However at DNA level, *C. arabica* generally exhibits low variability that is attributed to its allotetraploid origin, selfing reproductive nature, and recent speciation (Lashermes *et al.*, 1999). Cultivated Arabica coffee varieties are of even lower genetic diversity compared to wild accessions, because they are derived from a few individual collections (Lashermes *et al.*, 1996; Anthony *et al.*, 2002a). This means that cultivated *C. arabica* is more vulnerable to pests and diseases than the wild population. This is typical due to domestication bottleneck, intensive breeding and pedigree selection that make genetic variability within gene pools of many crops to be at risk (Tanksley and McCouch, 1997; Schneider, 2005). Inter-specific crosses help to increase the size of the gene pool and the contribution of wild species in the form of introgression lines is therefore of high value, especially in respect to traits like disease resistance. From the discussions above, Arabica coffee is certainly one such example.

Although breeders have managed to exploit this low variability to develop improved coffee varieties, transfer of traits of agronomic importance from other *Coffea* species is desirable. One avenue of such transfer is by use of Hibrido de Timor (HDT) (Timor hybrid). This is a spontaneous inter-specific cross between *C. arabica* and *C. canephora* that was observed as an

atypical tree in a *C. arabica* field planted in 1927, in the island of Timor (Bettencourt, 1973). Details are given in section 1.3. Progenies of this hybrid (mainly three accessions (numbers HDT 832/1, HDT 832/2 and HDT 1343) have been and continue to be used worldwide as the main source of resistance to various pests including CBD, CLR and nematodes (*Meloidogyne* spp). Molecular genetic analysis of derivatives of these progenies have demonstrated that they variously contain an estimate of 9-29% of the *C. canephora* genome, and they constitute a considerable source of diversity for Arabica coffee improvement (Lashermes *et al.*, 2000a). Breeding programmes utilizing these progenies have given rise to introgressed cultivars like 'IAPAR59' in Brazil, 'Variedad Colombia' in Colombia 'IHCAFE 90' and 'Costa Rica 95' in Central America 'Ruiru 11' in Kenya and 'Sln 12' in India (Anthony *et al.*, 2002b). The continued use of the derivatives of HDT for Arabica coffee breeding emphasizes the importance of these materials and introduction of genes from diploid relatives of C. *arabica*. It should be however noted that introgression of some *C. canephora* genomic fragments into *C. arabica* varieties may affect their beverage quality (Bertrand *et al.*, 2003). Marker assisted breeding can help to select for desired fragments and against unwanted ones.

Various researchers have used different DNA marker systems to assess the genetic variability of *C. arabica* including HDT derivatives. Lashermes *et al.* (1993) did not observe any RAPD polymorphism between pure *C. arabica* accessions, but there was some difference between them and an HDT accession. Combes *et al.* (2000) observed a mean heterozygosity value of only 0.04 in *C. arabica* using SSRs compared to 0.47 in *C. canephora*. Similarly, Moncada and McCouch (2004) observed highest average number of SSR alleles/locus in diploid *Coffea* species (3.6), less in wild *C. arabica* tetraploids from Ethiopia (2.5) and the least in *C. arabica* cultivars (1.9). Aga *et al.* (2003) observed a mean diversity value of 0.30 between wild *C. arabica* populations from different regions of Ethiopia by RAPD. Using RAPD and ISSR respectively, Masumbuko *et al.*

(2003) and Masumbuko and Bryngelson (2005) observed clustering of *C. arabica* accessions from different regions of Tanzania but with low overall genetic diversity. Pearl *et al* (2004) observed extremely low polymorphism between two pure Arabica varieties with only two polymorphic bands from 24 AFLP primer combinations (0.083 polymorphic bands per primer pair). They however observed a slightly higher polymorphism of an average of 1.34 polymorphic bands per primer pair between a pure Arabica cultivar and cv Catimor (a derivative of HDT).

AFLP variation within and between *C. arabica* cultivars is similar but accessions within a cultivar tend to form clusters (Steiger *et al.*, 2002). Consequently, it is difficult to identify cultivar-specific markers especially in the absence of introgressed genetic material, but some DNA markers can distinguish accessions derived from the two basic populations of cultivated *C. arabica* i.e. 'Typica' and 'Bourbon' (Anthony *et al.*, 2002b). Although Crochemore *et al.* (2004) reported the ability of identifying seeds of different coffee cultivars using RAPD, it should be noted that they used both HDT derivatives and interspecific crosses (*C. arabica* x *C. canephora*). Low variability of cultivated varieties compared to wild relatives has also been reported in maize using molecular methods (Matsuoka *et al.*, 2002) and cassava (Olsen and Schaal, 2003), which is an aspect of domestication bottleneck that reduces the genetic variability of cultivated crops (Tanksley and McCouch, 1997; Schneider, 2005).

Another source of genetic introgression into *C. arabica* is that from *C. liberica* genome that includes a fragment that carries the S_H3 gene for resistance to CLR (Prakash *et al.*, 2002; 2004). Genetic introgression into *C. arabica* from its wild relative has thus played an important role in its production and this will continue even in future. It also can be anticipated that artificial introduction of desirable genes will be done to supplement natural events. Molecular and genetic engineering tools will be vital in this respect.

4.4 Molecular markers of disease resistance in Arabica coffee

Development of molecular markers of resistance to CBD in coffee has been of interest for a period and still continues to be. There are differences in isozyme patterns, peroxidase and proteolytic activities between resistant and susceptible coffee plants infected by *C. kahawae* with some potential to be developed for screening purposes (Gichuru, 1993, Gichuru *et al.*, 1996, Gichuru and King'ori, 1999). Agwanda *et al.* (1997) were able to identify RAPD markers of CBD resistance derived from HDT. However analysis by methodologies like AFLP, microsatellites and SCARs would improve DNA markers in versatility and reproducibility. Noir *et al.* (2003) identified 14 AFLP markers derived from *C. canephora* by introgression through HDT that are associated with a major gene for resistance to *Meloidogyne exigua,*. Prakash *et al* (2004) similarly identified 21 AFLP markers introgressed from *C. liberica* that are linked to S_H3 gene for resistance to CLR. It therefore appears possible to use the same methodology and develop markers for resistance to other diseases. Nine families of resistance gene analogs (RGAs) of NBS type have been identified (Noir *et al.*, 2001). It is anticipated that use of primers targeting more conserved motifs might reveal more RGAs. Extensive studies of the RGAs are required and they might ultimately lead to mapping the RGAs as resistance gene candidates (RGCs). More research is thus required to develop markers for resistance genes and facilitate marker-assisted breeding and finally isolate the genes.

The actual genetic map of *C. arabica* has not been developed yet. On the other hand, a genetic map of *C. canephora* that distinguishes eleven (11) linkage groups that potentially correspond to the 11 chromosomes of *C. canephora* genome was developed by Lashermes *et al.* (2001). This map permits the mapping of different markers onto *C. canephora* genome, which in turn corresponds to the basic haploid *Coffea* genome. Such a strategy for mapping markers of resistance would enhance the knowledge of their genomic organisation.

32

4.5   Diversity of Microsatellites and SCARs

Microsatellites have characteristic genomic distribution and motif dependent dispersion in the genome, with most of them being concentrated in centromeric chromosomal regions (Schmidt and Heslop-Harrison, 1996). Microsatellites and their flanking DNA sequences are rarely conserved in a whole genus leave alone other genera in the family, but some may be conserved even across the genus (Hale *et al.*, 2005). However, microsatellites developed from one *Coffea* species may be transferable to other species with a fair degree of success, and even to the related genus *Psilanthus* (Combes *et al.*, 2000; Coulibaly *et al.*, 2003; Poncet *et al.*, 2004). Poncet *et al.* (2004) reported that the transferability of 110 microsatellite primer pairs developed from *C. arabica* ranged from 72.7 to 86.4% in other *Coffea* species. Microsatellites vary in the number of alleles in different *Coffea* species. Moncada and McCouch (2004) observed that diploid *Coffea* species averaged 3.6 alleles per microsatellite locus, wild tetraploid *C. arabica* averaged 2.5 alleles per locus and cultivated *C. arabica* had only 1.9 alleles per locus. In addition, 55% of the alleles found in wild *C. arabica* accessions were not shared with the cultivated genotypes. They also observed that the accessions of HDT in their study resembled *C. arabica* cultivars more than *C. canephora* accessions. On the other hand, Anthony *et al* (2002b) identified four microsatellite alleles related to introgression in HDT and observed closer similarity between the introgressed lines and *C. canephora* from Central Africa than with a *C. canephora* accession from West Africa. Poncet *et al.* (2004) observed a maximum of 9 and 8 alleles per locus in *C. canephora* and *C. pseudozanguebariae* respectively. The two species shared a total of thirty polymorphic loci, which indicated microsatellite evolution with shared ancestry.

Samples showing only one microsatellite allele are usually considered to be homozygous and this omits occurrence of null alleles. In *C. canephora* and *C. pseudozanguebariae*, Poncet *et al.* (2004) observed more than 3 alleles per polymorphic locus and estimated null allele percentages

33

of -9% and -11% in the two species respectively. In maize, Matsuoka *et al.* (2002) observed modest rates of null phenotypes averaging less than 5% when analysing microsatellites derived from maize in diploid *Zea* species. Microsatellite loci may also be duplicated in a genome but the duplicated loci may or may not amplify depending on conservation of the primer binding sites. Coulibaly *et al.* (2003) observed two microsatellite loci which were duplicated in both *C. canephora* and *C. heterocalyx* and that unlike AFLPs, SSRs are not clustered and are randomly distributed in the genome. Matsuoka *et al.* (2002) also observed duplicated microsatellite alleles in *Zea* species i.e. more than two products per plant (1.8% for teosinte and 0.02% for maize landraces). This could have been due to duplicated alleles, contamination of PCR or some other types of error such as inter well leakage. Such results may be treated as missing data and therefore eliminate bias.

Between species, a given microsatellite may have different genomic location and therefore be subjected to different evolutionary forces (Poncet *et al.*, 2004). Diversity of microsatellites may also differ in relation to the focal species (from which they were developed). Hale *et al.* (2005) observed that there were generally more repeats in the focal species in the genus *Clusia* than in non-focal species. Although they tested only 3 microsatellites, Hale *et al.* (2005) reported that there is a relationship between polymorphism and the size of a microsatellite. The diversity of microsatellites may also be affected by factors other than the number of repeat units. By sequencing the amplification products, Matsuoka *et al.* (2002) observed that variability was not restricted to repetition of the motifs but also included insertions and deletions (indels) in the regions flanking the repeat motifs. They showed that 40 out of 46 microsatellites have allele distributions that do not strictly adhere to the simple model of allelic variation based on changes in the number of repeated motifs. This high level of occurrence of indels prompted the authors to suggest the term Indel-Rich Regions (IRRs) to describe the maize microsatellites. The

occurrence of indels may have been enhanced by the pre-screening methodology like polymorphism in agarose that requires large size differences to be noticeable. Hale *et al.* (2005) also reported stepwise motif mutations and indels in *Clusia* species. Microsatellite data may differ with the method of analysis due to factors such as sensitivity of detection or efficiency of resolution. For example, Poncet *et al.* (2004) reported a discrepancy between positive amplifications observed in agarose and by fluorescent analysis in acrylamide gel, such that some samples that had detectable products in agarose were negative in the acrylamide. This is an indication of analytical complications that may be purely technical.

Microsatellites can be viewed as SCARs with highly variable segments in between the priming sites. Other types of SCARs such as those developed from AFLP bands lack such a segment and are expected, at least in theory, to be less polymorphic in size since this is entirely by indels. Poncet *et al.* (2005) tested the performance of 14 SCAR primers developed from AFLP markers specific to *C. pseudozanguebariae*, and they observed that the primers amplified only one band with a size similar to the parental band in other *Coffea* species. However there were some null allele phenotypes but the cross transferability was high with a minimum of 58%. Furthermore, the amplification pattern in *C. arabica* was a juxtaposition of the patterns of its putative diploid parent species, and the SCARs did not conserve the polymorphism of parental AFLP bands. These results not only confirm the ancestral parentage but may also help in analysing the behaviour of the sub-genomes in the tetraploid *C. arabica*, especially by analysing a large number of accessions of the different species.

The characteristics of a marker system including null alleles, low or lack of polymorphism, hyper-polymorphism and duplication of alleles in the genome present experimental challenges. In this study, the distribution of microsatellite and AFLP-derived SCARs was assessed in

relation to *C. canephora* chromosomal fragments introgressed into *C. arabica* via HDT. The aims were to evaluate the possibility of tagging or tracing these fragments and their homologs in *C. arabica* genome and its putative parents, seek to reveal genetic and evolutionary inter-relationships and evaluate challenges that are likely to be encountered in terms of practical utility of these marker systems in breeding.

4.6   Major commercial cultivars of *C. arabica* in Kenya

Two of the most widely grown Arabica coffee varieties in Kenya are SL28 and SL34. They belong to a series of single-tree selections done at Scott Laboratories between 1935 and 1939 in Kenya, thus the prefix as 'SL' (Jones, 1956). Both are of very fine cup quality and high yields but very susceptible to all major coffee diseases present in Kenya. Coffee breeding programmes in Kenya traditionally use these varieties as recurrent parents for agronomic traits, especially the fine cup quality. Another cultivar is K7, which is a progeny of one of two trees selected in 1936 from 'French Mission' for tolerance to CBD and CLR (Jones, 1956). The composite hybrid Ruiru 11 bred for compact growth and resistance to both CBD and CLR was released for commercial planting in 1985 (Nyoro and Sprey, 1986) and is still in high demand.

4.7   Coffee varieties used in this study

Apart from cvs SL28 and Catimor from Kenya that constituted the bulk of the study material, other *C. arabica* varieties and *Coffea* species were also used for comparison or to take advantage of knowledge previously generated using the varieties or their progenies. Cv SL28 is used as a recurrent parent in breeding programmes in Kenya and is a Bourbon type of *C. arabica*. Accessions of cv Catimor in Kenya were introduced from Colombia and have been shown to carry a resistance gene to CBD (Van der Vossen and Walyaro, 1980). All the Catimors currently present in Kenya were bred from the HDT accession number 1343 and the numbers after them

36

e.g. cvs Catimor 88 or 127, which were used a lot in this study, denotes single tree descent as received from Colombia. Before Catimor was introduced into Kenya, progenies of HDT (accession HDT 1349/269) had been introduced in 1960s and they were used in this study to survey for prevalence of markers for CBD resistance. *C. arabica* cvs Villasarchi and Caturra that are cultivated in Central and South America also belong to Bourbon type of *C. arabica* (Lashermes *et al.*, 2000a) and are susceptible to CBD, CLR and nematodes. They were crossed with derivatives of the original accessions of HDT to give rise to cvs Sarchimor and Catimor respectively. T5296 is a Sarchimor line derived from the HDT accession 832-2. ET 6 is a sub-spontaneous collection from Ethiopia whose F_2 progeny with T5296 was used to map some *C. canephora* chromosomal fragments present in HDT derivatives (Ansaldi, 2003). Analysis of markers of interest on the same DNA samples that she used would enable mapping them directly onto the same maps. Although there is intra-cultivar variation, hypocotyls inoculation tests have demonstrated that T5295 has resistance to CBD (Bertrand, Unpublished data). It was therefore expected that the fragment carrying the resistance would be common between T5296 and the resistant cv Catimor lines in Kenya.

The cultivar IAPAR59 is also a Sarchimor line that is cultivated in Brazil and was used by Noir *et al.* (2004) to construct a BAC library for *Coffea arabica*. These two varieties (T5295 and IAPAR59) represent highly introgressed derivatives of HDT. For chromosomal analysis of the coffee genome without complications due to heterozygosity or multiplicity expected in the tetraploid *C. arabica* genome, a *C. canephora* clone IF200 and a doubled haploid (DHs) mapping population derived from it were used (Lashermes *et al.*, 1994; Lashermes *et al.*, 2001). Since the same plants were used by Lashermes *et al.* (2001) to develop a genetic map, data generated in this study could be directly analysed alongside that of their study especially in mapping of linkage groups corresponding to chromosomes. *C. eugenioides* which is a putative

progenitor of the *C. arabica* genome was also included in some studies to generate information on alleles in *C. arabica* which could be of E^a sub-genome while the *C. canephora* clone IF200 played the counterpart role regarding the C^a sub-genome (Lashermes *et al.*, 1999). To assess the diversity of microsatellites and SCAR markers in *C. arabica* and its progenitor genomes, diverse accessions of *C. arabica* (wild, cultivated and introgressed), *C. canephora* and its close relative *C. congensis* plus *C. eugenioides* and its close relative *C. anthonyi* were used.

CHAPTER 5. SECTIONS ON SPECIFIC STUDY AREAS

SECTION 5.1. IDENTIFICATION OF *C. canephora* CHROMOSOMAL FRAGMENTS PRESENT IN LINES OF cv CATIMOR IN KENYA AND POTENTIAL MARKERS FOR CBD RESISTANCE

5.1.1 INTRODUCTION

Accessions of advanced generations (F_3 and F_4) of cv Catimor were first introduced in Kenya in 1970s and they were screened for resistance to CBD and CLR upon which susceptible ones were discarded (van der Vossen and Walyaro, 1981). The remaining accessions are homozygous for resistance to CBD and CLR and compact growth (van der Vossen and Walyaro, 1981) and they constitute very important donor genotypes. This selection is expected to have reduced the initial diversity. The disease resistance in these accessions is due to *C. canephora* chromosomal fragment(s) introgressed through the Hibrido de Timor (HDT) lineage. It is of interest therefore to identify the diversity of the introgressed fragments present in the current breeding populations because these are the candidate carriers for the resistance to the two diseases. This information would also facilitate identification of the presence of other useful fragments like those conferring resistance to nematodes, by comparison with results of other research work with similar genotypes. This information is also very useful in designing conservation and utilization strategies of accessions of HDT derivatives, such that accessions with fragments that are absent in cv Catimor can be given priority for conservation so as to widen the diversity.

It is also of interest to identify inbred lines that are unique in presence or absence of specific introgressed *C. canephora* fragments. Any unique phenotype of these plants can thus be attributed to the introgression genotype. Such plants can be obtained by analysis of F_2 of backcrosses or advanced selfings (Zamir, 2001; Jeuken and Lindhout, 2004; Von Korff *et al.*, 2004). In this study, analysis of accessions of cv Catimor, $BC_1 F_1$ and $BC_1 F_2$ progenies derived

from cultivars Catimor and SL28 was expected to widen the coverage of the polymorphism between and within the lineages of cv Catimor. It should be noted that these studies on the introgressed fragments in cv Catimor were done while at the same time two F_2 populations were being raised for mapping resistance to CBD and therefore advance identification of candidate markers for CBD resistance was advantageous.

Among coffee populations developed in various coffee breeding programmes in Kenya, there are different progenies of crosses between cvs Catimor and SL28 that are made with the aim of introducing disease resistance to the susceptible commercial cv SL28. It is possible to use these materials to identify markers or candidate markers for traits such as disease resistance depending on the appropriateness of the progeny, though some difficulties might be encountered. One problem is failure to identify individual parents because the crosses were not based on individual plants. The crosses were made using bulked pollen from several trees of a cv Catimor line to pollinate several trees of cv SL28 and the F_1 seed was bulked. In this study, individual parents of the F_1 and BC_1 F_1 plants were therefore not identified.

Five *C. canephora* chromosomal fragments that are introgressed into *C. arabica* through HDT have been mapped using an F_2 generation of a cross between a Sarchimor line (T5296) and a pure Arabica line (ET6) from Ethiopia (Ansaldi, 2003; Appendix 2). The fragments are serially designated as T1, T2, T3, T4 and T5. A genomic segment homologous to the introgressed fragment T4 has been identified in the genetic map of *C. canephora* corresponding to *Coffea* chromosomes (Lashermes *et al.*, 2001, Lashermes, Unpublished data), but the segments that are homologous to the other four fragments (T1, T2, T3 and T5) have not been identified. One way of identifying the location of these fragments is by sequencing their markers, designing sequence specific primers and then testing the PCR products for polymorphism in the *C. canephora*

mapping population (Lashermes *et al.*, 2001). This would enable chromosomal positioning of the fragments and reveal their organisation in the *Coffea* genome. In this study, the same double haploid (DH) population that was used by Lashermes *et al.* (2001) was used towards this endeavour. It is important to note that in different derivatives of HDT, the number and size of introgressed fragments of *C. canephora* genome may differ from those mapped by Ansaldi (2003). This would obviously have different implications in phenotypes of different introgressed lines/accessions. During this phase of the study, AFLP markers of the mapped *C. canephora* genomic fragments were used to detect the presence of these fragments in accessions of cv Catimor in Kenya. Alongside this observation, other AFLP bands that were polymorphic between the cvs Catimor and SL28 were identified. The identified fragments and polymorphic markers therefore constituted an inventory of candidates of disease resistance, for later investigation in F_2 populations and other subsequent studies. The genomic organisation of the fragments was then investigated as SCARs by sequencing their AFLP markers and designing specific primers.

Another possible way for identifying the candidate markers for CBD resistance was to establish any relationship between the polymorphic markers and any markers that are already developed. Agwanda *et al.* (1997) identified three RAPD markers of CBD resistance derived from HDT. However they did not map these markers though they recommended the analysis of the markers in a segregating F_2 population as a means of further confirmation. This could be achieved by regenerating the RAPD markers or SCARs developed from them in a segregating population. Furthermore, two of these markers (those amplified by RAPD primers N18 and M20) have been cloned, sequenced and SCAR primers designed (Lashermes, personal communication). However, apart from tests in agarose, in which there was no polymorphism, no further studies have been done with the SCARs. It was therefore of interest to try to map them onto the

introgressed fragments, an aspect which would indicate the priority candidate carrier(s) of CBD resistance. Additionally, this would improve the markers because SCAR markers developed from RAPD markers are more allele specific (non-random), can be amplified under higher stringency conditions and are less sensitive to PCR conditions (Paran and Michelmore, 1993; Zhang and Stommel, 2001). SCAR markers may not display the polymorphism of the parent bands and various approaches like redesigning of primers; optimization of PCR conditions and assessment by other methods like cleavage (CAPS) may help in recovery of polymorphism. All these options were differentially tried in this study with both the AFLP and RAPD derived SCARs. If polymorphic, the SCARs developed from the RAPD markers of resistance to CBD would make the linkage map denser and provide further proof of linkage to the resistance as identified from independent works. The SCARs would also be used to analyse their organisation in coffee genome using the DH population.

5.1.2 OBJECTIVE

The objective of this phase of study was to identify and characterise introgressed *C. canephora* fragments in lines of cv Catimor used as donors of resistance to CBD and CLR in Kenya, and subsequently establish an inventory of candidate markers for disease resistance.

5.1.3 MATERIALS AND METHODS

5.1.3.1 Plant genotypes

Mature coffee trees derived from crosses between different lines of cv Catimor that are resistant to CBD and CLR and the susceptible commercial cv SL28 were selected randomly in CRF field for this study. The sample constituted of two trees of different cv Catimor lines (line 127: P1 and line 88: P2), two trees of cv SL28 (P3 and P4), and eight $BC_1 F_1$ plants derived from different lines of cv Catimor (Catimor x (Catimor x SL28)). A total of 76 $BC_1 F_2$ seedlings were raised

and used in this study from selfed seeds of three different BC_1 F_1 plants to obtain three populations namely; population A and population B (both derived from cv Catimor line 127), and population C derived from cv Catimor line 129. It was not possible to trace the individual trees used in breeding of the BC_1 F_1 plants. DNA stock of an accession of Sarchimor line T5296 was also obtained at IRD for these studies. This accession was the HDT derived parent that was used to raise the mapping population used by Ansaldi (2003) to map the introgressed fragments.

5.1.3.2 Sampling and treatment of leaves

The details of how the plant materials were handled prior to DNA extraction at IRD in Montpellier, France was influenced by their stage of development.

5.1.3.2.1 Mature plants

The four representative parental trees of cvs Catimor and SL28 from Kenya (P1, P2, P3 and P4) and the BC_1 F_1 trees were mature trees growing in breeding fields at CRF, Ruiru, Kenya. Healthy disease free leaves were picked preferably from second and third nodes from the growing tips, wrapped in tissue papers and wetted slightly. Groups of the wrapped leaves were then packed in polythene bags, stapled to seal and then packed in paper envelopes. The parcels were then sent by express mail to IRD, Montpellier, France on the same day. Upon arrival at Montpellier which took 3-4 days after dispatch, the leaves were lyophilized immediately or dipped in liquid nitrogen and then stored at -80 °C until when they were lyophilized. Lyophilization was by dipping the leaves wrapped in paper towels into liquid nitrogen and then rapidly transferring them into a pre-cooled lyophilizer (Freeze mobile 5SL, Virtis Co. Inc. New York, 12525). After 72 hours of lyophilization, the leaves were stored in a cold room at 4°C awaiting DNA extraction.

5.1.3.2.2 Seedlings

Seeds obtained by selfing BC_1 F_1 plants were germinated in sterile sand at CRF until the cotyledons of the resultant BC_1 F_2 population were open. They were then transplanted into polythene bags and transferred into a nursery. When the seedlings developed two to three pairs of true leaves, the lowest pair was picked, wrapped in paper towels and dispatched to Montpellier for subsequently lyophilization as explained above for mature plants (Section 5.1.3.2.1).

5.1.3.3 Extraction of genomic DNA

Genomic DNA was extracted from the lyophilized leaves by the method of Diniz *et al.* (2005) with minor modifications. Fifty (50) to hundred (100) milligrams of lyophilized leaf material without mid-veins was placed into round bottomed 2 ml plastic tubes and two metal beads of 5 mm diameter were added into each tube. The tubes were put into horizontal mechanical shaker (Retch MM300) for 1min at a frequency of 30 cycles/s to obtain fine powder. In each tube, 400 µl each of extraction and lysis buffers respectively (Appendix 3) were added, vigorously shaken to mix and incubated at 62 °C in a water bath for 20-30 minutes with regular shaking. After incubation, 1 ml of chloroform/isoamyl-alcohol mixture, 24:1, was added to each tube, then mixed vigorously by shaking and centrifuged at 13000 rpm for 5 minutes in a desktop micro-centrifuge. The supernatants were carefully pipetted out into new 2 ml tubes. Though not of absolute necessity, a second centrifugation at this stage was generally helpful to eliminate any debris pipetted with the supernatant before proceeding to the next step. Twenty to thirty micro litres of RNase (10 mg/ml) was added to the supernatants and incubated at 37 °C in a water-bath for 30 minutes. A volume of isopropyl alcohol equal to the volume of each supernatant was added into each tube, and mixed gently by inverting the tubes several times to precipitate DNA. The suspended DNA was centrifuged at 13000 rpm for 5 min to obtain a DNA pellet and the

supernatant was carefully removed. The DNA pellets were then washed with 200µl of 70% ethanol and centrifuged at 13000 rpm for 3 minutes. The ethanol was drained by decanting or micro-pipetting, and the pellets dried in a vacuum centrifuge for 20 minutes. The pellets were dissolved overnight in 20-40 µl of TE (Tris-EDTA; Appendix 3) (depending on pellet size) at 4 °C. Estimation of DNA quantity was done by agarose gel electrophoresis. The stock solution and or their 10^{-1} dilution were electrophoresed in 1% agarose gel (QBiogene, France) and visually compared to standardized Lambda DNA ladders (Promega, Madison, WI, USA). This procedure also allowed assessment of the DNA quality attributes such as degradation and contamination that distort its migration. The gel was made in 0.5X TAE buffer (Tris/Acetic acid/EDTA; Appendix 3) and electrophoresis was done in the same buffer in a horizontal trough at 50 W for 1.5 hr. DNA was visualized and photographed in UV trans-illumination chamber after staining in Ethidium Bromide (2.5 mg/l) for 5 minutes.

5.1.3.4 AFLP analysis

The AFLP protocol used was basically as developed by Vos *et al.* (1995) and adopted by Lashermes *et al.* (2000a) for coffee but with some modifications. The stepwise procedure is presented below.

5.1.3.4.1 Digestion of DNA

The genomic DNA mother stocks were diluted to 50 ng/µl and 5 µl of this solution (a total of 250 ng of DNA) was pipetted into 0.5 ml tubes. The tubes contained 20 µl of digestion mixture comprising of 0.2 µl of *Eco*RI enzyme (10U/µl, Invitrogen, Life Technologies), 0.4 µl of *Mse*I enzyme (5 U/µl, Invitrogen, Life Technologies), 5.0 µl of T4 DNA Ligase buffer (5X, Gibco BRL) and 14.4 µl of water (Chromatography grade, Merck KGaA, Germany: hereafter referred to as PCR water). The mixture was vortexed slightly and incubated at 37 °C for 3 hr and the

45

digested DNA was visualized in 1% agarose gel. Samples that were difficult to digest were excluded from later procedures. The enzymes were not denatured at the end of this digestion step so as to allow any remnant undigested DNA to be digested during the ligation step below.

5.1.3.4.2 Ligation

To each digestion tube, 25 μl of ligation mixture containing; 1 μl each of *Eco*RI and *Mse*I adaptors (Appendix 3), 5 μl of T4 DNA Ligase buffer (5X, Invitrogen), 1μl of T4 DNA ligase (1 U/μl, Invitrogen) and 17 μl of PCR water was added. The ligase buffer was vortexed before pipetting the necessary volume to completely dissolve any precipitates. The components were mixed by gently turning the tube upside down several times because T4 ligase is fragile and needs gentle handling. The mix was incubated for 3 hr at 37 °C and the enzymes denatured at 60 °C for 10 min.

5.1.3.4.3 Pre-amplification

The ligation products were diluted ten times or less depending on the observed intensity of the digestion product in agarose (Section 5.1.3.4.1). Five micro-litres of this dilution was used for pre-amplification in a 50 μl mixture containing 0.5 μl of *Eco*RI pre-amplification primer (150 ng/μl), 0.5 μl of *Mse*I pre-amplification primer (150 ng/μl), 5μl of buffer (10X, Promega), 5 μl of MgCl$_2$ (25 mM), 0.2μl *Taq* DNA polymerase (5U/μl, Promega), 2 μl of dNTPs mix (5mM; Appendix 3) and 31.8 μl of PCR water. Only one *Eco*RI pre-amplification primer (E+A) and two *Mse*I preselection primers (M+A and M+C) were used. The letter 'E' refers to the universal *Eco*RI primer sequence 5'-GACTGCGTACCAATTC while 'M' refers to the universal *Mse*I primer sequence 5'-GATGAGTCCTGAGTAA. The letters after the 'E' or 'M' refer to additional selective nucleotides. Amplification consisted of 20 PCR cycles of denaturation at 94 °C for 30 seconds, primer annealing at 56 °C for 1 min and extension at 72 °C for 1 min in a

DNA Engine PTC-200 thermocycler (MJ Research Inc, Massachusetts). The amplification products were quantitatively assessed by their intensity in 1% agarose gel, using 5 µl of the product.

5.1.3.4.4 Labelling of *Eco*RI primers

For final amplifications, *Eco*RI primers were end labelled with a radioactive phosphate group (γP^{33} dATP). The labelling was in a 0.41 µl mixture containing 0.2 µl of the appropriate *Eco*RI (28 ng/µl, Eurogentec, Belgium), 0.04 µl of Kinase buffer 10X (QBiogene), 0.016 µl of T4 Kinase (10 U/µl, QBiogene) 0.06 µl of γP^{33} dATP (10 µCu/µl, Amersham Biosciences, UK) and 0.094 µl of PCR water. The Kinase buffer was left to thaw completely so as to have no precipitates. The labelling mixture was mixed by gentle tapping of the tubes because the T4 Kinase is fragile. The mix was incubated at 37 °C for 1hr followed by enzyme denaturation at 70 °C for 10 min.

5.1.3.4.5 Final amplification

The pre-amplification products were diluted by 1/30 or less (such as 1/10 or 1/20) if the intensity of the products observed in Section 5.1.3.4.3 was low, so as to achieved more uniform concentrations before final amplification. Five micro-litres of the diluted pre-amplification products were amplified in a 20µl reaction mixture containing 2µl of buffer (10X, Promega), 2 µl of $MgCl_2$ (25 mM, Promega), 0.8 µl of 5 mM dNTPs, 0.1 µl of *Taq* DNA polymerase (5 U/µl, Promega,), 0.33 µl of *Mse*I primer (100 ng/µl Eurogentec, Belgium), 0.41 µl of the labelled *Eco*RI primer mix (section 5.1.3.4.4) and 9.36 µl of PCR water. The pre-amplification and amplification primer combinations were matched such that if pre-amplification was by the primer pair EA x MA, the final amplification primer pairs had to be EAXX x MAXX; where the Xes refer to additional selective nucleotides. The amplification was done by a step down PCR

programme consisting of 12 cycles of 30 sec of denaturation at 94 °C, 30sec of primer annealing at 65 °C (reducing by 0.7 °C/sec) and 1min of elongation at 72 °C, followed by 33 (cycles with constant annealing temperature) consisting of 30sec of denaturation at 94 °C, 30 sec of primer annealing at 56 °C and 1min of elongation at 72 °C in a DNA Engine PTC-200 thermocycler (MJ Research Inc, Massachusetts, USA). After the amplification, 12.5 µl of loading stain (Formamide blue, Appendix 3) was added to each of the samples and stored at 4 °C until when required for electrophoresis.

5.1.3.4.6 Electrophoresis and revelation of radiographs

The ingredients for 6% denaturing polyacrylamide gel (Appendix 3) were mixed and carefully poured (avoiding trapping air bubbles) into 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 to set for at least 2 hr if used the same day or overnight for use the next day. The gels were assembled in vertical electrophoresis apparatus (Gibco, BRL sequencing system, Model S2, Life Technologies) and 1X TBE buffer for running (Appendix 3) 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 55W per gel for about 20 min before the samples were loaded. The samples were denatured by heating to 95 °C for 5 min and then immediately put into ice until when loaded. After pre-running, the gels were rinsed again and 62 well combs inserted at the top of the gels until the teeth slightly penetrated the gels. Aliquots of 4.5 µl of the samples were carefully loaded between the teeth of the combs. Electrophoresis was conducted at 55 W per gel for 2.5 hr and stopped when the slower moving xylene-cyanol dye was about two-thirds down the gel. After electrophoresis, the gels were fixed for 20min in a fixing solution (Acetic acid 1: Ethanol 2: Distilled water: 7), then removed and attached to blotting papers (3MM Whatman

paper) followed by drying at 80 °C for 1-2 hr in slab gel drier (Drygel Sr, Model SE 1160, Hoefer Scientific Instruments, San Francisco). Once the gels were well dried, they were put into cassettes with the sides attached to the gels up and Kodak Biomax X-ray films placed on top. After 4 to 7 days of incubation (depending on age of the radioactivity), the films were removed and developed to reveal the bands.

5.1.3.4.7 Primer combinations and samples analysed

Thirty one AFLP primers combinations (Table 1) were used to analyse the two Catimor trees (P1 and P2), two SL28 trees (P3 and P4), an accession of Sarchimor (T5296) and a selection of 6, 4 and 5 seedlings from BC_1 F_2 Populations A, B and C respectively (described in Section 5.1.3.1). The primers were chosen on the basis of their polymorphism between pure Arabica lines and HDT derivatives in the studies of Lashermes *et al.* (2000a), Noir *et al.* (2003) and Ansaldi (2003). Out of the tested primer pairs, 10 of them were selected, based on their polymorphism and amplification quality, for analysis in 61 additional plants of the BC_1 F_2 progeny, comprising of 25, 15 and 21 plants from the three populations A, B and C respectively, to confirm their segregation behaviour in the populations. Five primer pairs were further tested in the eight BC_1 F_1 plants of different cv Catimor lines.

5.1.3.4.8 Data scoring and identification of introgressed fragments

Bands that were polymorphic between the parental accessions, within the BC_1 F_1 or within BC_1 F_2 progenies were scored in binary form as "1" for presence and "0" for absence. Unclear bands were indicated by '?'. Bands that were monomorphic in all samples were not scored. If the primer combinations were the same as those used by either Noir *et al.* (2003) or Ansaldi (2003), the films were compared with theirs to identify the markers of introgressed *C. canephora*

fragments and consequently identify the mapped introgressed fragments T1, T2, T3, T4, and T5 (Ansaldi, 2003).

5.1.3.5 Identification of *C. canephora* linkage groups (chromosomes) associated with the introgression fragments.

This was anticipated by consideration of the possibility of transforming AFLP markers into SCARs, and then assessing their polymorphism in the DH mapping population of Lashermes *et al.* (2001). If any polymorphism was observed, the samples were amplified in a sample of 60 plants of the DH population and associated linkage group identified.

5.1.3.5.1 Extraction of DNA from AFLP bands

AFLP markers of four *C. canephora* chromosomal fragments introgressed into *C. arabica* (T1, T2, T3 and T5) were selected for this study based on size, (more than 100bp) and clear separation from other bands. The number of appropriate bands available and subsequently utilized was related to the number of markers mapped onto the fragments. DNA samples of seven HDT derivatives (4 from T5296 x ET6 and 3 from Catimor x SL28 populations) and a cv SL28 or cv Caturra sample (to confirm marker by absence) were used. However in a few tests, no accessions from the Kenyan populations were used because they had not been pre-amplified with the appropriate pre-selective primers in previous studies. For them to be used, it would have been necessary to pre-amplify just a few samples for these tests only. The samples were amplified with the appropriate final selective AFLP primers by the AFLP protocol outlined in Section 5.1.3.4. However instead of the normal 62-well comb for loading, one with alternate teeth removed to give double size wells was used, and 10 µl of each sample was loaded instead of 4.5 µl. After electrophoresis, the dried gels were stapled onto the films before placing them into the cassettes to avoid movements between the two, which could cause misalignment in later

steps. Before separating the gel from the film for development (after incubation), extra marks were made by cutting through the films and the Whatman papers to enhance correct realignment when extracting bands. To extract the DNA from the bands of interest, the developed films and the gels were re-matched with the aid of the cut and staple marks and held firmly together again by new staples. The films were then cut using sterile scalpels at the limits of bands of interest, such that the cuts extended through the films, gels and into the filter paper underneath. The delimited pieces of the gels were then detached from the Whatman papers, (sometimes with the top layer of the papers attached) with aid of clean forceps, and put into 0.5 ml tubes into which 50-100 µl of PCR water was added (depending on the size of the piece of gel). Two replicates of each band from different plants (preferably from the different populations) were cut for cloning as counter-checks, but when two adjacent samples from the same population had the band of interest, the band was extracted as one. The soaked gels were incubated at 4 °C for two days with occasional agitation to enhance diffusion of DNA into the water. The solution was then pipetted out into new 0.5 ml tubes and 2 µl used for amplification. The remaining solution was concentrated to 10-20 µl in a vacuum centrifuge and 2 µl taken for duplicate amplification as a precaution in case the first solution was too dilute.

Amplification was in 25 µl reaction mixtures containing 2 µl of the DNA solution, 2.5 µl of 10X buffer, 2.0 µl of $MgCl_2$ (25 mM), 0.5 µl of dNTPs (5 mM), 0.6 µl of each of the primers used to amplify the particular bands during AFLP (10µM), 0.1µl of *Taq* DNA polymerase and 16.7 µl of PCR water. The PCR programme consisted of an initial denaturation step of 5 minutes at 95 °C followed by 35 cycles of denaturation at 94 °C for 45 sec, primer annealing at 50 °C for 45 sec, elongation at 72 °C for 45 sec and a final extension step of 10 min at 72°C. Two microlitres of the amplification products were electrophoresed in 2% agarose gel and revealed in ethidium

bromide. Only samples with one clear band were judged to be good for cloning and the sample with more intense band between the two duplicates of the same AFLP band extract was used.

5.1.3.5.2 Cloning of the extracted DNA

The fresh PCR products were cloned using TOPO TA Cloning® kit with pCR® 2.1-TOPO® vector and chemically competent cells (Invitrogen, Life Technologies) according to the manufacturer's instructions (Appendix 4). Depending on the assessment of intensity in agarose of the extracted DNA in Section 5.1.3.5.1, 2-3 µl of the PCR products were used for ligation into the vector. After the transformation, 50µl of the *E. coli* cells were plated onto pre-selection plates of LB (Luria-Bertani) agar medium (25 g/L) containing 50 µg/ml ampicillin, onto which 40 µl of 40mg/ml X-gal in DMF (dimethylformamide) had been spread and dried. The inoculated plates were incubated at 37 °C for 14-16 hr (overnight) after which they were put into a refrigerator until when used for testing for positive clones. Ten white or light blue colonies were individually used for analysis to confirm that they had acquired the plasmid with the right inserts of DNA fragments. Blue colonies were disregarded as negative clones. The cultures were aseptically picked with sterile wooden toothpicks, which were then dropped into sterile plastic tubes containing 5 ml of LB liquid medium with ampicillin at a concentration of 50 µg/ml. The tubes with the inoculated media were incubated overnight in a rotary shaker (200 rpm) for 14-16 hr at 37 °C.

The cultures were then tested for inserts by PCR using 2 µl of the liquid culture and the same reaction mix as the one used to amplify the AFLP bands DNA before cloning. The PCR programme was also the same as during amplification of extracted AFLP DNA but the initial denaturation step was increased to 10 min, to ensure adequate rupturing of the bacterial cells to release the plasmids. To assess the inserts, 2 µl of the PCR products were electrophoresed in 2%

agarose gel and visualized under UV after staining with ethidium bromide. A sample of the PCR product used for cloning was included to ascertain that the size of the cloned fragments were the ones targeted. Clones with inserts of different sizes from the targeted fragments and those without inserts were discarded. A maximum of four and a minimum of two clones with the right size of insert per individual cloning reaction (depending on availability) were selected for extraction of plasmid DNA for sequencing. Before plasmid extraction, a glycerol stock of each of the selected transformed cultures was prepared for long-term storage (at -80°C). The stocks were made by adding 500 µl of the culture broths to 500 µl of LB/Glycerol mix (1:2) in 2 ml vials labelled with the details of the culture. Plasmid DNA was extracted from two of the selected cultures, hence four replicates of the same AFLP band for sequencing. The other cultures which were not extracted were centrifuged to precipitate the cells and then stored at -20 °C as a contingency measure, in case something went wrong with the first extractions or if the initial sequence results needed further confirmation by comparison with extra samples. Extraction of the plasmid DNA was done with QIAprep® spin kit (QIAGEN Sciences, Maryland, USA) according to manufacturers' manual (Micro-centrifuge option) (Appendix 5). The extracted DNA was estimated using spectrophotometer at 260 nm or in 1% agarose gel and diluted to 130 ng/µl before sending to Genome Express, France for sequencing.

5.1.3.5.3 Analysis of SCARs derived from the introgressed fragments

When the sequences were received, the actual plant DNA sequences were identified from the entire sequences to exclude AFLP primers and vector sequences. Replicate sequences of the same band were aligned using CLUSTAL W 1.82 programme (European Bioinformatics Institute, http://www.ebi.ac.uk/clustalw). Only sequences that were highly similar were considered as allelic. If the four sequences from the same band were highly divergent, the results were discarded and the corresponding culture stocks discarded from long-term storage.

Sequence specific primers were designed from one of the alleles of the same band using Primer3 programme (Whitehead Institute, USA, http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_w ww. cgi). The parameters considered in designing the primers targeted sizes between 18 and 22bp and optimum annealing temperature of 55 °C, so that they could all be analysed under the same PCR conditions. The primers (synthesised by Eurogentec, Belgium) were first tested for performance and possible polymorphism in 2% agarose gels. The samples used included two plants from which the AFLP bands were extracted, two un-introgressed accessions, the *C. canephora* clone IF200 and two DH accessions derived from it that are part of those used to construct *C. canephora* genetic linkage groups that correspond to the 11 chromosomes of *Coffea* sp (Lashermes *et al.*, 2001). Amplification was in 25 µl reaction mixtures containing 5 µl of genomic DNA (1 ng/µl), 2.5 µl of 10X buffer, 2.0 µl of $MgCl_2$ (25 mM), 0.8 µl of dNTPs (5 mM), 1.0 µl of each of the left and right primers (10 µM), 0.1 µl of *Taq* DNA polymerase (5 U/µl) and 13.7 µl of PCR water. The amplification regime consisted of an initial denaturation step of 5 min at 95 °C followed by 35 cycles of denaturation for 1min at 94°C, 45 sec of primer annealing at 55 °C (which was adjusted to improve the quality of product or when the optimum annealing temperature of the primers designed was different), 45sec of elongation at 72 °C and a terminal extension step of 10 min at 72 °C. The amplification was done either in a PTC-200 (DNA Engine, MJ Research Inc, Massachusetts, USA) or GeneAmp® PCR system 9700 (Applied Biosystems) thermocycler.

Where amplification was successful but without polymorphism in agarose, further amplification was done using a radioactive nucleotide ($\alpha dATP^{33}$) followed by electrophoresis in 6% denaturing acrylamide gel. In these tests, the samples consisted of *C canephora* clone IF200 and five DH plants derived from it, two or four F_2 plants, 2 un-introgressed Arabicas and an accession of *C. eugenioides*. Amplification was done in a 25 µl PCR mixture containing 5 µl of genomic DNA

(lng/µl), 2.5 µl of Buffer (10X), 2.0 µl of $MgCl_2$ (25 mM), 1.0 µl of SSR dNTPs (Appendix 3), 1.0 µl each of the right and left primers (10µM), 0.1µl of *Taq* DNA polymerase (Promega, 5 U/µl), 13.6 µl of PCR water and 0.08 µl of radioactive αdATP[33] (10 µCi/µl; Amersham Biosciences, UK). The amplification programme was the same as that used for agarose tests. Thereafter, electrophoresis and gel treatment was done as for AFLP (Section 5.1.3.4). However the exposure was either on film or on Amersham storage Phosphor screen. To reveal the results using the screen, the dried gels were incubated in cassettes with the screen and scanned after 24 to 48 hr with Typhoon scanner (9700 series, Amersham Biosciences) and TIFF digital images were obtained. Where polymorphism was observed within the DH plants, the total number of the DHs analysed was increased by 60 plants.

If no polymorphism was observed in the poly-acrylamide gel electrophoresis and the product was only one uniform band, genomic DNA of four DH plants was amplified with the same reagents and programme as for the agarose test. The PCR products were visualized in 1% agarose to estimate the quantity and then sent to Genome Express, France for sequencing. The sequences were analysed using RestrictionMapper programme version 3 (http://www.restriction mapper.org) to determine if there were polymorphic restriction sites that could be exploited to map the alleles in the DH plants by cleavable amplified polymorphisms (CAPs).

5.1.3.6 Analysis of RAPD markers of CBD resistance

Attempts were made to regenerate the RAPD markers amplified by primers N18 and M20 as described by Agwanda *et al.* (1997) using the accessions of cvs Catimor and SL28. Amplification was in a 25 µl mix consisting of 5 µl of genomic DNA (1ng/µl), 7.5 µl of dNTPs (500 µM; 1/10 dilution of the 5 mM dNTPs in Appendix 3), 2.5 µl of buffer (10X, Promega), 2.0 µl of MgCl2 (25 mM, Promega), 0.1µl of *Taq* DNA polymerase (Promega), 1 µl of primers (10

μM, Appligene) and 7.0 μl of PCR water. In attempt to optimise the amplifications, $MgCl_2$ was increased to 4 μl and water reduced appropriately. Taq DNA polymerase was increased to 0.2 μl and another DNA polymerase (HotGoldstar[TM], Eurogentec, Belgium) was tested. Amplification was done in a PTC-200 (DNA Engine, MJ Research Inc, Massachusetts, USA). Amplification programme consisted of initial denaturation at 95 °C for 5min followed by 45 cycles of denaturation at 95 °C for 1 min, annealing at 35 °C (or 37°C) for 1 min, elongation at 72°C for 2 min and final elongation at 72 °C for 7 minutes. The total PCR products were electrophoresed in 2% agarose gel in 1X TBE at 80 W in a 20 cm wide gel. Revelation was by UV after staining in ethidium bromide.

The primers designed from the sequences of RAPD markers identified by Agwanda *et al.* (1997) were then analysed in the same way as explained in section 5.1.3.5 for SCARS from AFLP bands. The SCAR products of the $M20_{830}$ consisted of two bands of which the introgressed one was identified. Subsequently, it was amplified in 60 of the same plant samples used by Ansaldi (2003) and the data generated was assessed in attempt to determine if it is linked to one of the mapped *C. canephora* fragments. The N18 SCAR which amplified one monomorphic band in all accessions was cloned and sequenced from four DH plants (one clone per plant) and analysed for restriction polymorphisms (CAPs) as explained in section 5.1.3.5. Potentially polymorphic restriction sites for enzymes *Mse*I and B*fa*I (an isoschizomer of *Mae*I and *Rma*I) were identified. The SCARs were then amplified and digested with *Mse*I (Invitrogen) and *Bfa*I (New England BioLabs Inc.) as per the manufacturers' instructions and separated in 2% agarose gel as explained for RAPD gels above. Digestion was in a 20 μl mixture consisting of 15 μl of the PCR product, 0.5 μl of the appropriate enzyme (5 U/μl), 2.5 μl of enzyme buffer and 2.5 μl of PCR water and incubated was for 3 hr at 37 °C. To check the efficiency of digestion without PCR contaminants, the PCR products were cleaned before the digestion. For the cleaning, entire PCR

products were transferred into 1.5 ml tubes into which 80 μl of 70% ethanol was added and vortexed slightly. The mix was left for 15 minutes at room temperature (precipitation stage) and then centrifuged for 20 min at maximum speed (13000 rpm) noting the orientation of the tubes. The supernatant was then carefully pipetted out. In some cases, the precipitate was not visible but consideration of the orientation of the tubes avoided its disruption. Two hundred microlitres of 70% ethanol was added, vortexed briefly and centrifuged for 10 min at 13000 rpm. The ethanol was carefully pipetted out and the tubes were dried in a vacuum centrifuge for 10 min. The DNA was then dissolved overnight at 4 °C in PCR water ready for digestion.

5.1.4 RESULTS

5.1.4.1 AFLP analysis

Thirty-one AFLP primer combinations were analysed for polymorphism between cv Catimor accessions (P1 and P2), cv SL28 (P3 and P4) and an accession of Sarchimor line T5296. They generated between 1 and 9 polymorphic bands (Table 1). The polymorphism was either within or between the two categories i.e. HDT derivatives (cvs Catimor and Sarchimor accessions) versus cv SL28. The two cv Catimor accessions had some polymorphism between them but the two SL28 accessions always exhibited the same banding pattern. A total of 100 polymorphic bands were observed (Table 1). Some polymorphisms were observed in the BC_1 F_2 progenies which were not observed in the parental representatives such that 2 bands were present and 2 others were absent in all the parental representatives but were polymorphic in the BC_1 F_2 progenies. Forty four (44) bands were present in both cv Catimor accessions but absent in cv SL28. Sixteen (16) of these "Catimor" bands were also shared with the cv Sarchimor line T5296. Fifteen (15) bands were present in cv SL28 but absent in the HDT derivatives. Eighteen (18) bands were present in at least one of the HDT derivatives but absent in SL28. Nineteen (19) bands were present in at least one of the HDT derivatives and present in cv SL28. Two (2) bands were

present in all the parental accessions and 2 bands were absent in all the parental accessions but they were polymorphic in the BC_1 F_2 plants. Polymorphic bands which were present in at least one of the HDT derivatives but absent in cv SL28 were classified as HDT bands (Table 1).

Table1. Summary of AFLP primer combinations tested on accessions of *C. arabica* cvs Catimor, Sarchimor and SL28 and BC_1 F_2 populations derived from the two cultivars and characteristics of polymorphic bands generated.

Primer combination	Number of polymorphic bands	HDT bands*	SL28 bands (P3 and P4)	Present in at least one HDT derivative and present in both P3 and P4	Monomorphic in both parental accessions
1. EAAC-MCAC#	2	1	-	1	-
2. EAAC-MCTG	4	3	1	-	-
3. EAAC-MCTT	1	1	-	-	-
4. EAAG-MAAG	2	1	1	-	-
5. EAAG-MACA	3	2	1	-	-
6. EAAG-MACT	1	1	-	-	-
7. EAAG-MCAA	9	7	1	1	-
8. EAAG-MCTA	2	-	-	2	-
9. EAAG-MCTC#	5	5	-	-	-
10. EAAG-MCTT	2	1	-	1	-
11. EACA-MCAA	1	1	-	-	-
12. EACA-MCAC	4	1	-	1	1**, 1***
13. EACC-MAAG#	2	1	1	-	-
14. EACC-MCAA	3	2	-	1	-
15. EACG-MCAA	3	2	1	-	-
16. EACG-MCAT	3	2	1	-	-
17. EACG-MCTA	7	2	2	3	-
18. EACT-MAAC#	8	5	1	1	1**
19. EACT-MAAG#	3	2	-	1	-
20. EACT-MACT#	3	2	-	1	-
21. EACT-MAGC#	1	1	-	-	-
22. EACT-MAGT#	4	3	-	1	-
23. EACT-MCAA	1	1	-	-	-
24. EAGA-MAAC	2	1	-	1	-
25. EAGA-MACA	2	1	1	-	-
26. EAGA-MCGA	1	1	-	-	-
27. EAGC-MCTG	4	2	2	-	-
28. EAGG-MCTC	3	2	-	1	-
29. EATC-MAAC#	4	2	-	1	1***
30. EATC-MAAT#	3	2	1	-	-
31. EATC-MACT#	7	4	1	2	-
Total	100	62	15	19	4

Notes

#	primers that were also tested on the enlarged BC_1 F_2 populations
*	bands that were present in at least one HDT derivatives but absent in SL28
**	bands that were absent in all parental accessions but polymorphic in the BC_1 F_2 progeny
***	bands that were present in all parental accessions but polymorphic in the BC_1 F_2 progeny

Ten primer combinations were tested on a larger number of BC_1 F_2 seedlings by analysing 60 additional plants from populations A, B and C in addition to the 15 used in pre-screening stage so as to confirm their behaviour in the populations. These primers generated 37 polymorphic bands (Table 2). Twenty three (23) of these bands were attributed to cv Catimor (absent in cv SL28) and 14 bands were attributed to cv SL28 (absent in cv Catimor). Out of the 23 bands attributed to cv Catimor, 15 were not segregating (always present in all individuals of the 3 populations), 5 were segregating in the Mendelian ratio of 3:1 only in populations A and B and were always present in population C. Three (3) bands had distorted segregations whereby one appeared overrepresented and another underrepresented in all populations. The third band was overrepresented in populations A and C but absent in population B.

During this phase of the study, some AFLP markers of *C. canephora* chromosomal fragments introgressed into *C. arabica* that revealed the presence of fragments T2, T3 and T4 in the cvs Catimor and Sarchimor accessions and the BC_1 F_1 and BC_1 F_2 breeding progenies were generated. No markers of *C. canephora* fragments T1 and T5 were observed in the cv Catimor accessions and derivatives. Most of the polymorphic markers in the BC_1 F_2 populations co-segregated with markers of *C. canephora* introgressed fragment T4. Some of the ever-present bands were identified as markers of the *C. canephora* fragments T2 and T3. Seven plants lacking the introgression fragment T4 were identified from the BC_1 F_2 seedlings and preserved as a resource for future studies alongside plants with other introgressed fragments. Preservation of plants with unique recombination in subsequent studies was envisaged.

Table 2. Scores of polymorphic AFLP bands between two trees of cv Catimor (lines 88 and127), two trees of cv SL28 and three BC1 F2 populations of the two cvs ((SL28 x Catimor) x Catimor)).

AFLP Primers	Band No.	Cat 127 (P1)	Cat 88 (P2)	SL28 (P3)	SL28 (P4)	Population A	Population B	Population C
EAAG/MCTC	i	1	1	0	0	m	m	m
	ii	1	1	0	0	m	m	m
	iii	1	1	0	0	p	m	m
EACC/MAAG	i	0	0	1	1	p	p	m-
	ii	0	0	1	1	m	p	m-
	iii	1	1	0	0	m	m	m
EACT/MAAC	i	0	0	1	1	p	p	p
	ii	1	1	0	0	m	m	m
	iii	0	1	0	0	m	m	m
	iv	1	1	0	0	m	m	m
	v	0	1	1	1	m-	p	p
	vi	1	1	0	0	p	p	m
	Vii*	1	1	1	1	p	p	p
EACT/MAAG	i	1	1	0	0	m	m	m
	ii	0	1	1	1	p	p	p
	iii	1	1	0	0	m	m	m
EACT/MACT	i	1	0	1	1	p	m	p
	ii	1	1	0	0	m	m	m
	iii	1	1	0	0	m	m	m
EACT/MAGC	i	1	1	0	0	m	m	m
EACT/MAGT	i	0	0	1	1	m-	p	p
	ii	1	1	0	0	m	m	m
	iii	0	1	1	1	p	p	p
	iv	1	1	0	0	m	m	m
EATC/MAAC	i	1	1	0	0	p	p	p
	ii	0	0	1	1	p	p	p
	iii	0	0	1	1	m-	p	p
EATC/MAAT	i	1	1	0	0	m	m	m
	ii	0	0	1	1	m-	p	m-
	iii	1	1	0	0	p	p	m
EATC/MACT	i	0	0	1	1	p	p	p
	ii	1	0	0	0	p	p	p
	iii	1	0	1	1	p	p	p
	iv	0	1	0	0	p	m	p
	v	1	1	0	0	m	m	m
	vi	1	1	0	0	p	p	m
	vii	1	1	0	0	p	p	m

Key:
m: monomorphic band that was present in all individuals of BC_1 F_2 populations
m-: monomorphic band that was absent in all individuals of BC_1 F_2 populations
p: a band that was polymorphic in the BC_1 F_2 populations
* a band that was present in all parental accessions but polymorphic in the BC_1 F_2 progenies

Five AFLP primer combinations were analysed in 8 accessions of $BC_1 F_1$ plants alongside the accessions of cvs Catimor and SL28 (P1-P2) and twenty-three 23 bands which were polymorphic between the parental accessions were generated (Table 3). Eight (8) of these bands were present in the accessions of cv Catimor and all the $BC_1 F_1$ plants while one was absent in both Catimor accessions and all the $BC_1 F_1$ samples. The rest displayed varied polymorphism between the parental accessions and the $BC_1 F_1$ plants. Some of the bands present in both accessions of cv Catimor were not present in all the $BC_1 F_1$ samples as would be expected for fixed dominant markers and this demonstrated further the diversity between the lines of cv Catimor. One lineage of cv Catimor line 88 appeared to be particularly low in bands derived from cv Catimor including fragment T4.

Table 3. Scores of polymorphic AFLP bands in two trees of cv Catimor (lines 88 and127), two trees of cv SL28 and 8 trees of $BC_1 F_1$ progenies involving different cv Catimor lines ((SL28 x Catimor) x Catimor)).

AFLP Primers Tested	Band *	Pure cultivar accessions				$BC_1 F_1$ plants bred from the following lines of cv Catimor and cv SL28							
		Cat. 88 (P1)	Cat. 127 (P2)	SL28 (P3)	SL28 (P4)	127	127	129	88	88	119	90	127
EACT/MACT	i	0	1	1	1	0	1	0?	0	1	1	0	1
	ii	1	1	0	0	1	1	1	1	1	1	1	1
	iii	1	1	0	0	1	1	1	1	1	1	1	1
EACT/MAAC	i	0	0	1	1	1	0	0	1	1	1	0	1
	ii (T2)	1	1	0	0	1	1	1	1	1	1	1	1
	iii	1	0	0	0	1	1	1	0	0	0	1	1
	iv	1	1	0	0	1	1	1	1	1	1	1	1
	v	1	0	1	1	0	0	0	0	1	1	1	1
	vi (T4)	1	1	0	0	1	1	1	0	1	1	1	1
EATC/MACT	i	0	0	1	1	0	0	0	1	0	0	0	0
	ii	1	1	0	0	1	1	1	1	1	1	1	1
	iii	0	1	1	1	0	1	1?	0	1	0	0	1
	iv	1	0	0	0	1	1	1	1	1	1	1	0
	v	1	1	0	0	1	1	1	1	1	1	1	1?
	vi (T4)	1	1	0	0	1	1	1	0	1	1	1	1
	vii (T4)	1	1	0	0	1	1	1	0	1	1	1	1
EACT/MAGT	i	0	0	1	1	0	0	0	0	0	0	0	0
	ii	1	1	0	0	1	1	1	1	1	1	0?	1
	iii	1	0	1	1	1	1	1?	1	0	1	1	1
	iv	1	1	0	0	1	1	1	1	0	1	0	1
EACT/AAG	i	1	1	0	0	1	1	1	1	1	1	1	1
	ii	1	0	1	1	1	1	1	1	1	1	1	1
	iii	1	1	0	0	1	1	1	1	1	1	1	1

Key: Cat.   Catimor

Notes

i. Polymorphic bands were serially numbered from the largest in size to the lowest.

ii. Where the markers identified *C. canephora* fragments, the fragments are indicated in brackets as mapped by Ansaldi (2003) (Appendix 2).

5.1.4.2 Mapping of the *C. canephora* chromosomal fragments introgressed into C. *arabica* genome

AFLP bands that are markers of different *C. canephora* fragments introgressed into C. *arabica* were cloned and sequenced from accessions of an F_2 progeny between Sarchimor line T5296 x ET6 and accessions of BC_1 F_2 progenies of (cv Catimor x (Catimor x SL28)). A total of ten (10) AFLP markers were successfully cloned and sequenced (some after repetition), two of which were markers of T1, four of T2, three of T3 and one of T5 (Table 4). One T2 marker band (AFLP-38) repeatedly amplified a weak unspecific band that was judged poor for cloning. Although the target was to obtain four similar sequences for primer design, in two cases there were only two sequences that were similar and in three cases, three sequences were similar. This was due to shortage of colonies with the right size of inserts, dissimilar sequences or poor sequencing due to clones that contained more than one sequence. In cases where the size of inserts differed from those targeted, the contaminants were mostly smaller. Mixed sequences could have been due to mixed cultures or, though less likely, due to a bacterium picking more than one plasmid.

The resultant sequences were indistinguishable and differences observed between the sequences were similar both within and between the lineages of HDT derivatives (Figure 1). The differences included possible substitution and additions/deletions as observed in Figure 1. Such observations from these results strengthened the decision that there was no need of making extra efforts to include samples from the Kenyan populations if they were not already pre-amplified. Most of the primers designed amplified clear SCAR products without further optimisation of the PCR conditions. However, one primer (A2) amplified a smear while another one (W3) did not amplify at 55°C and the temperature was reduced to 52°C to obtain amplification. No primer displayed distinct polymorphism in agarose gel.

Table 4. A Summary of characteristics of SCARs developed from AFLP markers of *C. canephora* chromosomal fragments introgressed into *C. arabica*.

AFLP primers combination	*C. canephora* fragment	Cloning code	Remarks on amplified SCARs
1. EACT-MAAC	T2 (AFLP-16)	A, B	SCAR A2 amplified unspecific products as a trail of DNA on agarose
2. EACT-MCAA	T3 (AFLP-29)	C, D	SCAR D4 was a monomorphic band in all test accessions whose sequence did not exhibit differential cleavable site in the DHs
3. EAAC-MCTT	T1 (AFLP-24)	E, F	SCAR F1 was monomorphic in the DHs but Arabicas had 2 alleles E^a and C^a. Sequences from the DHs lacked polymorphic cleavage sites
4. EAAC-MCTT	T5 (AFLP-25)	G, H	SCAR G3 was monomorphic in all accessions tested
5. ECAC-MCCA	T1 (AFLP-M8)	J, K	SCAR J3 had 2 loci in DHs which were mapped onto the *C. canephora* map
6. ECAC-MCTA	T2 (AFLP-93)	N, P	SCARs N2-R and N2-2R were four bands of which one was E^a, one C^a and two were common in all accessions. There was no polymorphism in the DHs.
7. ECAC-MCAT	T3 (AFLP-12)	S, T	SCAR S3 had 3 alleles of almost the same size which were monomorphic in all accessions
8. ECAC-MCAT	T3 (AFLP-12)	U, V	This was a band that perfectly co-segregated with S/T. SCAR U2 amplified 3 bands but not polymorphic in DH. One allele was E^a, another C^a and the third was present only in *C. canephora*
9. EACG-MCAT	T2 (AFLP-36)	W, X	SCAR W3 was polymorphic in DH but was not highly repeatable for exploitation. In Arabica accessions, one band was observed
10. EAGC-MCTG	T2 (AFLP-33)	AA4, AB5	SCAR AA4 was a monomorphic band in all accessions

Notes

   i. The number in parenthesis is the marker identity as mapped by Ansaldi (2003).
   ii. E^a and C^a refer to the two constitutive genomes in *C. arabica* from *C. eugenioides* and *C. canephora* respectively.

(a)   **Sequences an AFLP marker (AFLP-29) of the *C. canephora* chromosomal fragment T3 introgressed in *C. arabica* via HDT**

```
D4    CAACTAAATCGTTCACATAACACTCAATATTTTTGTGCAGCATGTCGTCAAAGATATTTT 60
D5    CAACTAAATCGTTCACATAACACTCAATATTTTTGTGCAGCATGTCGTCAAAGATATTTT 60
C1    CAACTAAATCGTTCACATAACACTCAATATTTTTGTGCAGCATGTCGTCAAAGATATTTT 60
C4    CAACTAAATCGTTCCCATAACACTCAATATTTTTGTGCAGCATGTCGTCAAAGATATTTT 60
      *************  *********************************************

D4    GCATTGCTCTTTGATACTTGGCTCCAGCATTTTTGAGATCAAAGGGCATTACTTTATAGT 120
D5    GCATTGCTCTTTGATACTTGGCTCCAGCATTTTTGAGATCAAAGGGCATTACTTTATAGT 120
C1    GCATTGCTCTTTGATACTTGGCTCCAGCATTTTTGAGATCAAAGGGCATTACTTTATAGT 120
C4    GCATTGCTCTTTGATACTTGGCTCCAGCATTTTTGAGATCAAAGGGCATTACTTTATAGT 120
      ************************************************************

D4    AACAAATACCCTTAGGAGTACGAAATGCAGT 151
D5    AACAAATACCCTTAGGAGTACGAAATGCAGT 151
C1    AACAAATACCCTTAGGAGTACGAAATGCAGT 151
C4    AACAAATACCCTTAGGAGTACGAAATGCAGT 151
      *******************************
```

(b)   **Sequences of AFLP marker (AFLP-36) of the *C. canephora* chromosomal fragment T2 introgressed in *C. arabica* via HDT**

```
W3    ACGGTGAATGGTTACAATTTGACAGGGCTGTTGCTTGGTGCTTGAGCCAAACAGACATTT 60
X3    ACGGTGAATGGTTACAATTTGACAGGGCTGTTGCTTGGTGCTTGAGCCAAACAGACATTT 60
X1    ACGGTGAATGGTTACAACTTGACAGGGCTGTTGCTTGGTGCTTGAGCCAAACAGACATTT 60
      *****************  ****************************************

W3    ATTTTTATGTGCCACGTCAGCCACAAGAATAAGTGGAACTACGTAGTTTTGTGTTGGACA 120
X3    ATTTTTATGTGCCACGTCAGCCACAAGAATAAGTGGAACTACGTAGTTTTGTGTTGGACA 120
X1    ATTT--ATGTGCCACGTCAGCCACAAGAATAAGTGGAACTACGTAGTTTTGTGTTGGACA 118
      ****  ****************************************************

W3    GGAAATTGGACACAGAAAATTGGACAGAGGATCCCGTCTGGTGTTGTGCATCCTTGCAGA 180
X3    GGAAATTGGACACAGAAAATTGGACAGAGGATCCCGTCTGATGTTGTGCATCCTTGCAGA 180
X1    GGAAATTGGACACAGAAAATTGGACAGAGGATCCCGTCTGATGTTGTGCATCCTTGCAGA 178
      ****************************************  ******************

W3    TTCTGTGCATGTATCCATGTTATTATTGATTTGAGTTAGTTCCATGGACGATTTGTAGAA 240
X3    TTCTGTGCATGTATCCATGTTATTATTGATTTGAGTTAGTTCCATGGACGATTTGTAGAA 240
X1    TTCTGTGCATGTATCCATGTTATTATTGATTTGAGTTAGTTCCATGGACGATTTGTAGAA 238
      ***********************************************************

W3    CAACCAGGCTGAATGCAGTTATTCATGTACAGAGCATG 278
X3    CAACCAGGCTGAATGCAGTCATTCATGTACAGAGCATG 278
X1    CAACCAGGCTGAATGCAGTTATTCATGTACAGAGCATG 276
      ******************* ******************
```

Figure 1. Alignment of sequences of AFLP bands aligned using CLUSTAL W (1.82) multiple sequence alignment programme. Sequences D4, D5and W3 are from AFLP bands of samples from F_2 generation of the T5296 x ET6 while sequences C1, C4, X1 and X3 were from samples of BC_1 F_2 ((Catimor x (Catimor x SL28)).

Notes
  i.   * denotes the presence of identical nucleotides in all the sequences aligned.
  ii. Dashes (-) are introduced into the sequences to maximise similarity.

The primers were used for radioactive amplification on samples comprising of pure Arabicas, Arabicas with introgressed *C. canephora* genomic fragments, *C. canephora* and *C. eugenioides*. The amplification products were separated in denaturing acrylamide gel and various characteristic patterns were observed (Table 4). Some of the products amplified did not display any polymorphism in all the accessions, others amplified alleles specific to the sub-genomes in *C. arabica* i.e. E^a and C^a, but only one (J3) had polymorphism that could be mapped in the DH population (Plate 4). Analysis of the segregation pattern of the J3 SCAR derived from an AFLP marker of fragment T1 (Plate 4 B) revealed that alleles 'b' and 'c' segregated as alleles of the same locus (locus 2) while allele 'a' is a different locus (locus 1) possibly coupled by an allele slightly smaller than allele 'c'. Allele 'b' was observed in the un-introgressed *C. arabica* accessions and *C. eugenioides* though with a difference in intensity, which could have been due to more copies in *C. arabica*. Allele 'c' was observed in one plant of the F_2 population of T5296 x ET6 (Plate 4 B, sample 7) and was concluded to be the allele introgressed from *C. canephora* into *C. arabica*. The segregation pattern of the alleles in the DH population enabled mapping of locus 2 onto the linkage group corresponding to chromosome 8 while locus 1 tended to associate with linkage group/chromosome 2. Locus 1 could not be perfectly mapped due to some unexpected amplifications, for example in sample 4 (Plate 4 B) where it seemed not to have been amplified.

On general terms, primers designed from markers of fragments T3 and T5 gave monomorphic patterns in all the samples, except U2 that had polymorphism between the species (Plate 4 C, D, and E). In contrast, SCARs of fragment T2 generally had more bands with alleles that were monomorphic within the coffee species but polymorphic between the species. *C. arabica* exhibited alleles which were specific to the two sub-genomes shared with the other two species

(E^a and C^a). For example, the first primer pair from N2 sequence gave four clear bands of which one was specific to *C. canephora* and another specific to *C. eugenioides* genomes (Plate 4F).
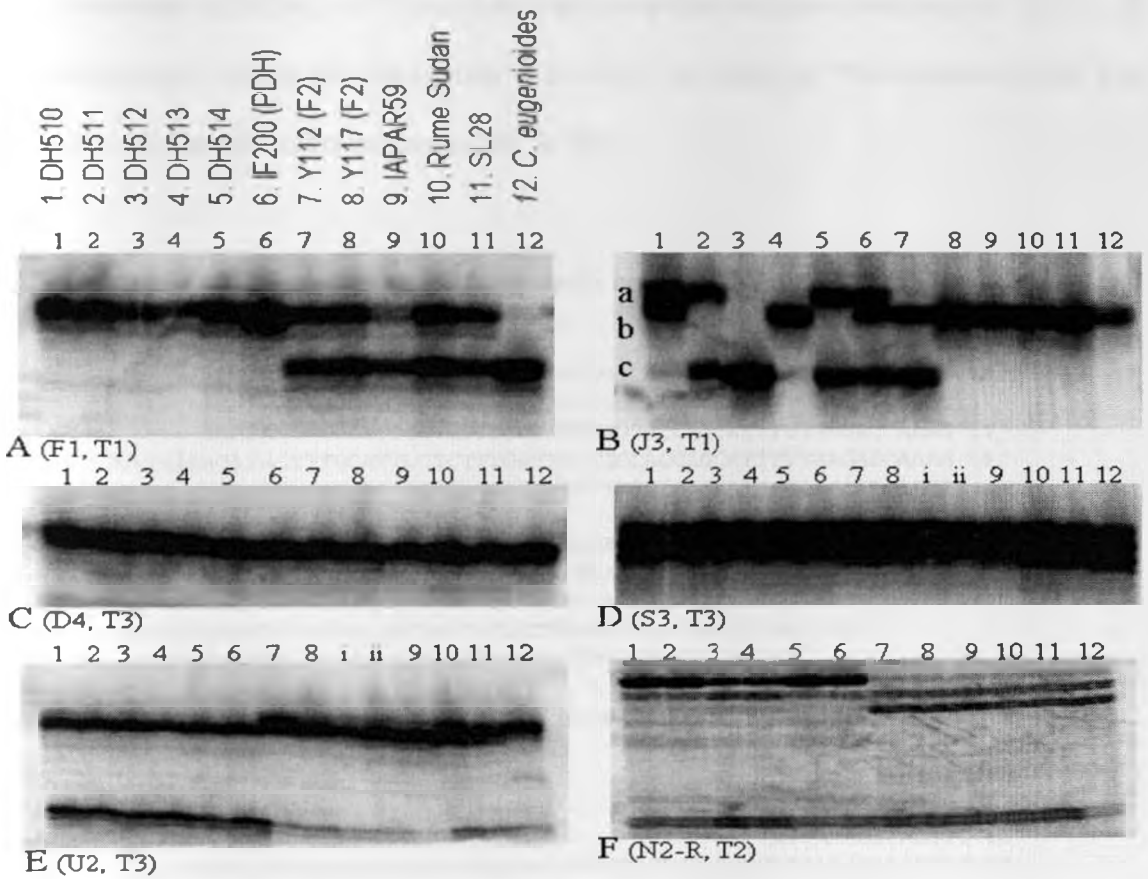
Plate 4. Radiographs of PCR products generated by SCAR primers designed from sequences of AFLP markers of the *C. canephora* chromosomal fragments introgressed into *C. arabica* genome.

Legend on samples

Samples 1 – 5 are Doubled Haploid (DH) plants accession numbers 510, 511, 512, 513 and 514 respectively; sample 6 is: IF200 (the *C. canephora* parent of the DHs); samples 7 and 8 are F_2 plants accession numbers 112 and 117 from a cross between T5296 x ET6; sample 9 is IAPAR 59 (a Sarchimor line); samples 10 and 11 are *C. arabica* varieties Rume Sudan and SL28 while sample 12 is an accession of *C. eugenioides*.

* Where included, i and ii refers to F_2 plant numbers 120 and 140 of the T5296 x ET6 cross.

Legend on panels of radiographs

Panel A represents SCAR F1 from AFLP marker of *C. canephora* fragment T1
Panel B represents SCAR J3 from AFLP marker of *C. canephora* fragment T1
Panel C represents SCAR D4 from AFLP marker of *C. canephora* fragment T3
Panel D represents SCAR S3 from AFLP marker of *C. canephora* fragment T3
Panel E represents SCAR U2 from AFLP marker of *C. canephora* fragment T3
Panel F represents SCAR N2-R from AFLP marker of *C. canephora* fragment T2

A second primer was designed on the right side of this sequence (N2-2R), which reduced the prominent bands to two but lacked the specificity to the two genomes. An exception in the general behaviour of SCARs of T2 was AA4 which amplified only one monomorphic band in all accessions tested. Unlike the observation with AFLP, no effect of PCR machine model was observed on the amplification patterns of the SCARs.

(a) **Alignment of sequences of PCR products amplified with the SCAR primer D4 from four DH plants (510, 511, 512 and 513).**

```
D4-512   --------GATACTTTGCATTGCTCTTTGATATGTGGCACCAGCATTTTTGAGACCAAAG 52
D4-513   ---ATCAAGATACTTTGCATTGCTCTTTGATATGTGGCACCAGCATTTTTGAGACCAAAG 57
D4-510   -------AGATACTTTGCATTGCTCTTTGATATGTGGCACCAGCATTTTTGAGACCAAAG 53
D4-511   TCATCAAAGATACTTTGCATTGCTCTTTGATATGTGGCACCAGCATTTTTGAGACCAAAG 60
                 ***********************************************************

D4-512   GG-CATCACTTT-ATAACAATAAATACCCTTAGGAGTACGAAATGCAG 98
D4-513   GG-CATCACTTT-ATAACAATAAATACCCTTAGGAGTACGAAATGCAG 103
D4-510   GG-CATCACTTT-ATAACAATAAATACCCTTAGGAGTACGAAATGCAG 99
D4-511   GGGCATCACTTTTATAACAATAAATACCCTTAGGAGTACGAAATGCAG 108
         **  ********* ************************************
```

(b) **Comparison of sequences of SCAR D4 in two DH plants and the sequence (D4) used to design the primers.**

```
D4-510   -------------------------------------------------------AGATACTTT 9
D4-511   ------------------------------------------------TCATCAAAGATACTTT 16
D4       CAACTAAATCGTTCACATAACACTCAATATTTTTGTGCAGCATGTCGTCAAAGATATTTT 60
                                                         *****  ***

D4-510   GCATTGCTCTTTGATATGTGGCACCAGCATTTTTGAGACCAAAGGG-CATCACTTT-ATA 67
D4-511   GCATTGCTCTTTGATATGTGGCACCAGCATTTTTGAGACCAAAGGGGCATCACTTTTATA 76
D4       GCATTGCTCTTTGATACTTGGCTCCAGCATTTTTGAGATCAAAGGG-CATTACTTT-ATA 118
         ***************  ****  *************** *******  ***** ***

D4-510   ACAATAAATACCCTTAGGAGTACGAAATGCAG- 99
D4-511   ACAATAAATACCCTTAGGAGTACGAAATGCAG- 108
D4       GTAACAAATACCCTTAGGAGTACGAAATGCAGT 151
              *****************************
```

Figure 2. Alignment of sequences obtained by direct sequencing (without cloning) of PCR products from *C. canephora* DH plants amplified with SCAR primer D4 designed from an AFLP maker of fragment T3: (a) CLUSTAL W (1.82) multiple sequence alignment of the sequences and (b) comparison of two of the sequences with the full length sequence of AFLP band cloned from *C. arabica* and used to design the primers.

Notes
  i. Dashes (-) are introduced in the sequences to maximise similarity.
  ii. The missing sequences from the DHs on the left ends are due to poor initial sequencing since the products were sequenced directly without cloning.
  iii. * indicates that the nucleotides in the three sequences are identical in the locus

Some products of the SCAR amplifications that consisted of a single band in the DHs were amplified and sequenced without cloning. The sequences from the DHs were highly similar to the ones obtained from the *C. canephora* fragments introgressed into Arabica coffee (Figure 2). The percentage of bases that were different between the individual sequences from the DHs and the sequence used to design the SCAR primers ranged from 1.73 to 8.08 %. No potentially polymorphic restriction sites were observed in the sequences.

5.1.4.3 Analysis of RAPD markers of CBD resistance

It was not possible to regenerate polymorphic bands with the RAPD primers N18 and M20 as reported by Agwanda *et al.* (1997). This was despite changes of the concentrations of Magnesium chloride and DNA polymerase in PCR reaction mixtures, source of DNA polymerase and PCR programme. Since SCAR primers had been previously designed from two of the RAPD markers but they do not exhibit polymorphism in agarose, (Lashermes, unpublished) they were tested by radioactive PCR and separation in polyacrylamide electrophoresis. The SCARs of the markers $N18_{250}$ and $M20_{830}$ consisted of one and two bands respectively in denaturing polyacrylamide gel (Plate 5). The smaller allele of $M20_{830}$ was clearly the one introgressed due to its presence in HDT derivatives and *C. canephora*. However, when amplified in a set of 60 samples of the F_2 population used by Ansaldi (2003) to map the introgressed fragments, the quality of amplification was not good despite several repetitions. It could therefore not be clearly mapped although it tended to be associated with markers of the introgressed fragment T2. There seemed to be competition between the two alleles especially in the heterozygous state in favour of the introgressed allele and also poor amplification in many samples.

The product amplified by primers designed from the sequence of the RAPD marker $N18_{250}$ was cloned from four of the DH plants to assess sequence polymorphism. Out of the four clones prepared for sequencing, three were successfully sequenced while the third had mixed sequences. The three sequences were highly similar to the sequence obtained from the *C. canephora* fragment present in HDT (Figure 3). Two potentially polymorphic restriction sites were observed but digestion of the PCR products with the two restriction enzymes, *Mse*I and *Bfa* I, yielded a mixture of bands that could be expected from a mixture of the sequences (Plate 6). Cleaning the samples after PCR before digestion and increasing the digestion time from three hours to twelve hours did not change the patterns. This was rather unexpected since no multiple bands were observed in the polyacrylamide gel.
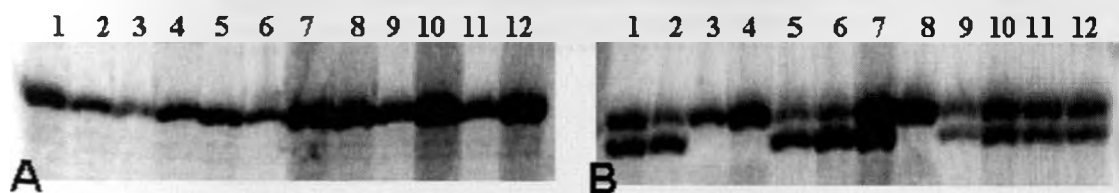
Plate 5. Radiographs of banding patterns of SCAR products amplified with primers designed from RAPD markers of CBD resistance identified by Agwanda *et al.* (1997) (A) $N18_{250}$ and (B) $M20_{830}$.

Samples:
 Samples 1 to 5 are F_2 plants of the cross between T5296 and ET6; sample 6 is a BC1 F_2 plant of SL28 x Catimor; sample 7 is a *C. canephora* clone (IF200); sample 8 is cv SL28 while samples 9 to 12 are DH plants

```
N18DH507-1    ------------------------------------CGATTGACCAATGGATAAAGTCATG 25
N18-Inverse   CGGTGAGGTCATGGTAGCCTGGTATAGAGCCCAGCCGATTGACCAATGGATAAAGTCATG 60
N18DH506-2    ------------------------------------CGATTGACCAATGGATAAAGTCATG 25
N18DH508-6    ------------------------------------CGATTGACCAATGGATAAAGTCATG 25
                                                  ************************


N18DH507-1    GTAGCCTGGTTATAAAGCCCAGCCGATTGACCAATTGAGAAAGTCATGGTAGCCTGGTAT 85
N18-Inverse   GTAGCCTGGT-ATAAAGCCCAACTGATTGACCAATTGAGAAAGTCATGGTAGCCTGGTAT 119
N18DH506-2    GTAGCCTGGT-ATAAAGCCCAACCGATTGACCAATTAAGAAAGTCATGGTAGCCTGGTAT 84
N18DH508-6    GTAGCCTGGT-ATAAAGCCCAGCCGATTGATCAATTGAGAAAGTCATGGTAGCCTGGTAT 84
              ********** ********** *  ****** *****  ********************


N18DH507-1    AAAGCCCAACCGAGTGACCCAAGAATGAAATGAGACCAATCGGTAGTCATGGAACTTATT 145
N18-Inverse   AAAGCCCAACCGAGTGACCCAAGAATGAAATGAGACCAATCGGTAGTCATGGAACTTATT 179
N18DH506-2    AATGCCCAGCCGAGCGACCCAAGAATGAAATGAGACCAATCGGTAGTCATGGAACTTATT 144
N18DH508-6    AAAGCCCAACCGATTGACCCAAGAATGAAATGAGACCAATCGGTAGTCACGAAACCTATT 144
              ** ***** ****  ******************************** * *** ****


N18DH507-1    GGTCGCCCACCTATAAGGGG-TAAAATCT███GCTGAGTACTCAGTAGCCGGTCATTGC 204
N18-Inverse   GGTCGCCCAC███AAGGGG-TAAAATCT██GCTGAGTACTCAGTAGCCGGTCATTGC 238
N18DH506-2    GGTCGCCCAC███AAGGGGGTAAAATCT██GCTGAGTACTCAGTAGCCGGTCATTAC 204
N18DH508-6    GGTCGCCCAC██TAGGGG-TTTAATCTCTA GCTGAGTACTCAGTAGCCGGCCATTAC 203
              ************* ***** *  ************************** **** *


N18DH507-1    ATGTA███TTATGAGCTGATTTGATGGAATGATTATGAACTGATTTGAAATGAGACTTC 264
N18-Inverse   ATGTA██TTATGAGCTGATTTGATGGAATGATTATGAACTGATTTGAAATGAGACTTC 298
N18DH506-2    ATGTACTTGTTATGAGCTGATTTGATGAAAGATTATGAACTGATTTGAAATGAGACTTC 264
N18DH508-6    ATGTACTTGTTATGAGCTGATTTGATGGAATGATTATGAACTGATTTGAAATGAGACTTC 263
              ******  ****************** ** ****************************


N18DH507-1    TGAACTGATATGAGTTGATTTACGAATGGAAATGAAGAGATTGGA--------------- 309
N18-Inverse   TGAACTGATATGAGTTGATTTACGAATGGAAATGAAGAGATTGGTAACTTGAGACGAATT 358
N18DH506-2    TGAACTGATATGAGTTGATTTACGAATGGAAATGAAGAGATTGGA--------------- 309
N18DH508-6    TGAACTGATATGAGTTGATTTACGAATGGAAATGAAGAGATTGGA-------------- 308
              ******************************************


N18DH507-1    -----------------
N18-Inverse   TAGTTATGACCTCACCA 375
N18DH506-2    -----------------
N18DH508-6    -----------------
```

Figure 3 Alignment (CLUSTAL W (1.82)) of sequences amplified from three *C. canephora* double haploid plants (accessions 506, 507and 508) and the original sequence (N18-Inverse) of the RAPD band (RAPD marker N18$_{250}$) that was used to design SCAR   primers

Notes
1. The notation 'inverse' refers to the fact that the sequence is in inversed complementary sense to the one actually sequenced.
2. The sequences in bold are the restriction sites for restriction enzyme *Mse*I while those shaded grey are restriction sites for the enzyme *Bfa* I.
3. Dashes are introduced in the sequences to maximise similarity.
4. * indicates that the nucleotides in the three sequences are identical in the locus
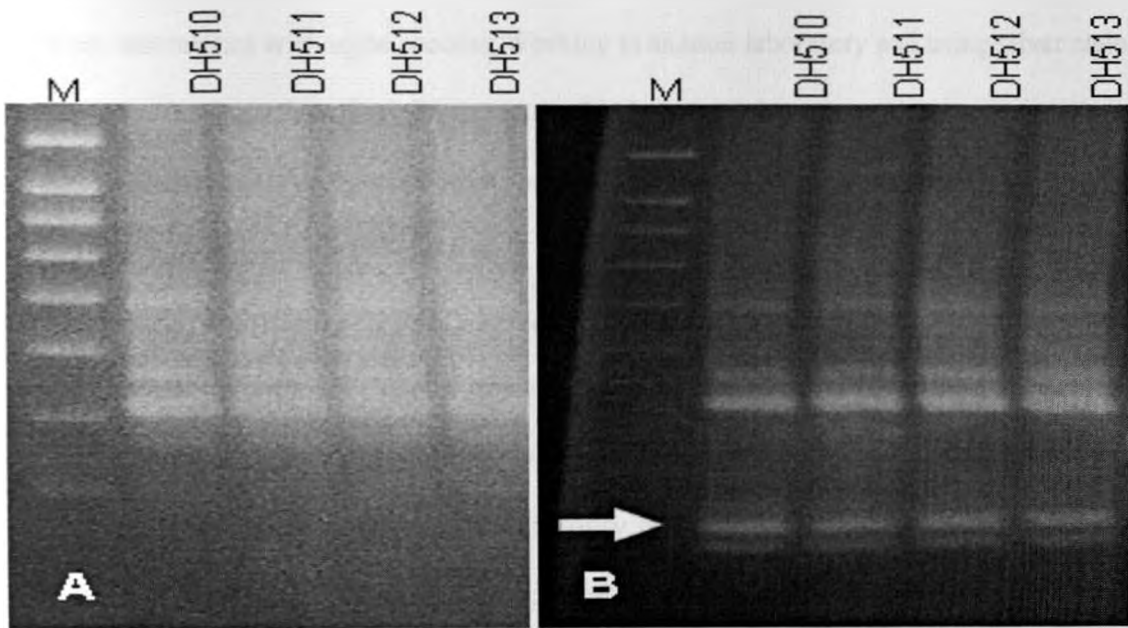
Plate 6. Patterns obtained after digesting SCAR products amplified from four *C. canephora* DH plants (510, 511, 512 and 513) derived from a RAPD marker for CBD resistance $N18_{250}$ with the restriction enzyme *Bfa* I. Panel A shows the pattern before digestion and panel B after digestion. M is a 100 base pair ladder. It can be observed that digestion generated extra bands (arrowed) but they were not polymorphic.

5.1.5   DISCUSSION

The work of this section had direct relationship with those of some earlier workers such that regeneration of their results was required. It was possible to regenerate AFLP markers identified by other researchers in the same laboratory (Lashermes *et al.*, 2000a; Noir *et al.*, 2003, Ansaldi, 2003). However, this was possible by using the same model of thermocycler as the one that they used. The use of another model of thermocycler led to slight differences from the reference patterns obtained by the above researchers, and this reduced the confidence of identifying the targeted marker bands. This highlights the problem of sharing of AFLP markers between laboratories or even their repeatability within the same laboratory with change of facilities, or even with a change of performance of machines. On the contrary, no effect of the model of thermocycler was observed with the specific primers (SCARs) designed from the sequenced AFLP bands. This demonstrates the need for developing sequence-based primers to enhance the

transferability of non-sequence based markers. AFLP markers can however be reproduced in different laboratories with some success. Working in another laboratory and using silver staining instead of radioactive labelling, Diniz *et al.* (2005) were able to reproduce some markers of nematode resistance (*Mex*-1) identified by Noir *et al.* (2003).

The analysis of the accessions of *C. arabica* cvs Catimor and SL28, their $BC_1 F_1$ and $BC_1 F_2$ progenies backcrossed to cv Catimor, showed that while there is a large uniformity in cv SL28, there is diversity between and within the cv Catimor lines. Similarly, Agwanda *et al.* (1997) and Pearl *et al.* (2004) observed higher heterozygosity in the cv Catimor accessions that they used compared to accessions of un-introgressed Arabicas. There is therefore a high potential for selection within these lines. The selection would depend on the objectives and knowledge of genetic segments to select for. For instance, one lineage of cv Catimor 88 seemed to be particularly low in markers of introgression including T4 (Table 2), and this could be either advantageous or disadvantageous depending on what genes are located on the introgressed fragments. For example, the absence of T4 markers that have also been identified as markers for resistance to the nematode *Meloidogyne exigua* (by collaboration of work by Noir *et al.*, 2003 and Ansaldi, 2003) makes this line less favourable for breeding. Markers of the fragments T2 and T3 were present in all the accessions analysed. This may be speculated to be a reflection of their association with characters that were selected for during the breeding history of these genotypes, first in Colombia and later in Kenya. The introgressed chromosomal fragments T1 and T5 were not identified but it may not be certain if they were selected against during selection stages or they were absent even in the parental accessions of HDT derivatives at the start of the breeding. Out of the total HDT derived markers, it is probable that potential markers for genes of resistance to CBD and CLR (at least the genes related to races of CLR present in Kenya) are present in all the cv Catimor and $BC_1 F_1$ plants analysed in this study because they are all

resistant to these diseases. Consequently, the identified *C. canephora* chromosomal fragments and any unmapped HDT derived markers are potential candidate markers of the introgressed resistance genes. Another category of common markers in the cv Catimor lines would be those related to the dominant compact growth. However, this is a mutation within *C. arabica* to give rise to cv Caturra (Jones, 1956), which was crossed with HDT to give rise to cv Catimor. This character is therefore not of HDT origin.

Different accessions of HDT derivatives have different levels of introgressed *C. canephora* genome (Lashermes *et al.*, 2000a). Some introgressed *C. canephora* fragments may affect beverage quality of *C. arabica* (Bertrand *et al.*, 2003) and lines of cv Catimor in Kenya are of lower cup quality than SL28 (van der Vossen and Walyaro, 1981) It is therefore of interest to identify undesirable fragments and possibly use plants with the smallest fragments carrying desirable gene(s) as the donor parent in breeding. Such plants may be obtained from accessions/progenies of either cv Catimor or HDT using DNA markers. Apart from the diversity due to the number of mapped *C canephora* fragments present in the various lineages, the fragments may also be different in size compared to those in the T5296 x ET6 cross that was used for mapping by Ansaldi (2003). This could be particularly true for the fragment T4 because there were polymorphic bands that co-segregated with markers of this fragment but are unmapped by Ansaldi (2003). The fragment T4 was not present in all BC_1 F_1 plants as would be expected for a backcross to homozygous dominant parent. This may indicate lack of strict selection for or against this fragment during the breeding programmes both in Colombia and Kenya. This is interesting while noting that these materials were selected for resistance to CLR in Colombia and for resistance to CLR and CBD in Kenya. The absence of this fragment in the BC_1 F_1 plants which were resistant to both CBD and CLR in the field at CRF makes it a lower priority candidate for resistance to these diseases at least under the particular field conditions.

However, it may be a complement of some type to other fragment(s). It should be noted that by collaboration of the results of Noir *et al* (2003) and Ansaldi (2003), this fragment (T4) carries the resistance gene to the nematode *Meloidogyne exigua*. Future breeding and conservation work should therefore pay attention to this aspect and ensure that it is not lost.

Breeding programmes in Kenya have been based on bulking pollen from several similar plants of paternal variety and using it to pollinate several similar plants of the maternal variety. It was therefore not possible to identify individual parents of the BC_1 F_1 plants. The four parental representatives used in this study (P1-P4) are therefore not the real parents but were representatives of the parental cultivars. During AFLP analysis of BC_1 F_2 progenies, four bands were observed to be polymorphic in these progenies but they were not polymorphic in the parents such that two were present and the other two were absent in all parental representatives. This may therefore be due to the fact that the accessions designated P1, P2, P3 and P4 were not identified as the actual parents of the progenies. Other researchers have also observed polymorphic bands in progenies that were not observed in the parents such as Yang *et al.* (2000) in soybean. Explanations could be PCR errors or restriction artefacts, genomic mutations and contaminations, but the repeatability and frequency of the bands observed in this study rules out these possibilities. Yang *et al.* (2000) could not explain their observations but ruled out the above explanations also. Recently, Lolle *et al.* (2005) demonstrated by use of single nucleotide polymorphism in Arabidopsis, that some characters can be observed in a progeny and not in the parents but in earlier generations, possibly by storage as extra-genomic information in RNA. However, this is not likely to be the explanation in this study. The observations of this study are likely due presence of extra polymorphism within the cultivars which was not present in the four representative plants (P1, P2, P3, P4). However, such bands that were not polymorphic between the parents were not considered as potential makers of disease resistance.

In this study, seven plants lacking the introgression fragment T4 were identified from the BC_1 F_2 seedlings and were preserved alongside a sample with the fragment as a resource for future research. These plants will be useful in comparative studies with the plants with T2 and T3 to identify candidate fragments for different phenotypes, the same way inbred lines (ILs) are used (Zamir, 2001; Jeuken and Lindhout, 2004, Von Korff *et al.*, 2004). The intention was to have a collection of plants with or lacking one of the possible introgression fragments in various combinations to serve as differentials for different phenotypes. In this endeavour, preservation of plants identified to have unique combination of the introgressed *C. canephora* genomic fragments in later studies was envisaged.

AFLP markers of different *C. canephora* chromosomal fragments introgressed into *C. arabica* were cloned and sequenced from different HDT derivatives i.e. cv Catimor lines which were initially bred from HDT accession 1343 and a Sarchimor line T5296 bred from HDT accession 832/2. The sequences of AFLP bands from T5296 x ET6 and cv Catimor x cv SL28 progenies were indistinguishable (Figure 1). This agrees with the suggestion that the genomic fragments introgressed from *C. canephora* through HDT share the uniqueness of the original introgression in the original HDT (Orozco-Castillo *et al.*, 1994). It was evident that knowledge generated from either of the progenies was valid for the other. It was also possible to identify markers mapped by Ansaldi (2003) in cv Catimor accessions from Kenyan although an F_2 population of T5296 x ET6 was used to map them. This was useful in reducing the need to repeat mapping of introgressed fragments unless later studies showed that the fragment(s) carrying the CBD resistance gene(s) was not mapped.

The sequences of AFLP marker of genomic introgression from *C. canephora* into *C. arabica* also displayed high similarity to the sequences of SCARs amplified from DH plants with primers

designed from the introgressed fragments (Figure 2), with a range of 1.73 to 8.08 % difference on a base per base comparison. Lashermes *et al.* (2000a) and Anthony *et al.* (2002b) observed that HDT derivatives are more similar to *C. canephora* accessions from Central Africa than those from West Africa. The *C. canephora* clone IF200 that was used to generate the DHs belong to an intermediate genetic group (Lashermes *et al.*, 1994) and this may explain the variability of the similarity of the DH sequences to the introgressed fragments.

When some cloned bands were sequenced, they gave highly dissimilar sequences between themselves. This could be due to a chance of co-migration of non-homologous bands (Robinson and Harris, 1999; Zhang and Stommel, 2001) or poor delimitation of the gel containing the band when cutting such that an adjacent band was included. Such dissimilar bands were not used to design SCAR primers. The least number of similar sequences used for primer design was two, in two cases, and this was due to inadequate number of positive clones with the right size of inserts. There were some cultures that yielded multiple sequences and they could not therefore be sequenced in full. This could have been due to an error when picking the positive (white) colonies such that mixed colonies were picked or, though less likely, due to a colony picking more than one plasmid during transformation. Poor results necessitated repetition of the experiments but this did not improve on some bands like A2. Absolute confirmation that the bands cloned were the targeted ones would be if the designed primers amplified products with polymorphism matching that of the parent AFLP bands and several bands subsequently mapped on the parent fragment. Similarly, confirmation of the chromosomal location of the fragments in the DH populations would be possible if more than one of the SCARs developed from AFLP markers of the same fragment generated co-segregating polymorphism. However this was not achieved in this study, and judging from the rate of success, it would require much more cloning. There was extremely low polymorphism of the SCARs within genotypes assessed (Table 4,

Figures 2 and 3). The probability of success also needs to consider the number of markers available for a fragment and of large size (preferably more than 150 bp) to enhance the probability of getting useful polymorphism both by size or restriction. The SCAR primer pair A2 did not amplify clear bands. Unspecific amplification may be due to poor primer design, poor sequence results or duplication of homologous regions in the genome. Once amplification of clear bands in agarose was obtained, PCR conditions were not further optimised because the intention was to be able to use the same programme to analyse many primer pairs.

The results obtained with the various AFLP SCAR primers (Table 4, Plate 4) clearly demonstrated the existence of allelic specificity to *C. canephora* and *C. eugenioides* genomes in the *C. arabica* genome. This is in agreement with the reported origin of the *C. arabica* genome, as a rather recent combination of *C. canephora* and *C. eugenioides* or their close relatives (Raina *et al.*, 1998; Lashermes, *et al.*, 1999). In addition, the results demonstrated the existence of conserved regions across the three *Coffea* species. These can be explored further for use as anchor markers within wider taxa as also found by Poncet *et al.* (2005). The results are also in agreement with those of Lashermes *et al.* (2000b) and Hererra *et al.* (2002), who using molecular markers in *C. arabica* and *C. canephora* inter-specific hybrids concluded that the chromosomes in their genomes are very similar to allow random pairing and cross-overs. There was duplication of some sequences even in DHs (Plate 4). Some DNA fragments such as T3 seemed to be more conserved than others such as T1 and T2. Multiplicity of RFLP loci in the same DH population was earlier reported by Paillard *et al.* (1996). It would be interesting to investigate if this is related to the function of genes in these genomic regions that necessitated differential evolution. Three sequences each of fragments T2 and T3 were compared for their content of the nucleotides A and T (AT). Two markers of each fragment were from the same cluster or within 3 cM from the cluster. The markers from T2 had 50.7%, 55.6% and 58.2% AT, while those from T3 had

77

60.36%, 61.49% and 64.0% AT. From these observations, it would appear that sequences on T2 are more likely to be putatively of the coding type as explained by Poncet *et al* (2005) whereby putative expressed sequences have AT content averaging 55%, but this is entirely subject to further study.

The SCAR primer J3 demonstrated the existence of two loci in *C. canephora* using the DH population (Plate 4 B). From the results of this study, it can be argued that allele (c) is the introgressed allele of fragment T1 due to its occurrence in introgressed population T5296 x ET6 (Plate 4 B: sample 7). This would mean that *C. arabica* has only one allele (by size) in locus 2 and introgression from *C. canephora* introduced the second allele while locus 1 is either absent or has the same allele as locus 2. The fragment T1 is therefore located on the linkage group 8 which putatively correspond to chromosome 8 (Lashermes *et al.*, 2001). IAPAR 59 is a cultivar derived from HDT but possibly the accession used in this study did not have the T1 fragment as was also observed with cv Catimor from Kenya. SCAR markers may not exhibit the polymorphism of the markers from which they are cloned. The segregation of the SCAR marker J3 in the DHs was observed and scored as co-dominant markers while the parental AFLP marker was scored as dominant. The successful conversion of a dominant AFLP marker to a more informative co-dominant marker will be very useful in future studies on the chromosomal fragment T1. Polymorphism of an AFLP marker band largely reflects polymorphism related to the enzyme cutting site and/or primer annealing sequences and not absence of homologous fragments. On the other hand, SCAR primers are designed to match sequences of the AFLP fragments usually interior to the site(s) affecting AFLP polymorphism. This affects both the presence and/or type of polymorphism. Similar loss of target polymorphism upon conversion of AFLP bands into SCAR markers was reported by Shan *et al.* (1999) in barley and wheat and by Poncet *et al.* (2005) in *Coffea* genus. In this study, products of the SCAR primers variably

included unspecific amplifications, monomorphism, genome specific polymorphism and co-dominance. The work of other researchers has revealed similar results (Paran and Michelmore, 1993. Shan *et al.*, 1999; Zhang and Stommel. 2001; Weiland and Yu. 2003; Boukar *et al.*, 2004; Diniz *et al.*, 2005).

It was not possible to regenerate the RAPD markers of CBD resistance identified by Agwanda *et al.* (1997). This is not unexpected since the PCR conditions affect the repeatability of RAPD polymorphisms (Rafalski *et al.*, 1996). Genomic DNA concentration, temperature profile of thermocycler, magnesium chloride concentration and the type of DNA polymerase affect reproducibility of RAPD. In this study all these parameters would have affected the results since the chemicals (except for the primers) and the thermocycler were from different manufacturers while the estimation of DNA concentration and its quality might have differed from that of Agwanda *et al.* (1997). This has been one limitation of earlier markers that led to the need for more repeatable markers of high reproducibility and easily transferable between laboratories (Rafalski *et al.*, 1996). The banding pattern of the product amplified by the primers designed from the sequence of the RAPD marker $M20_{830}$ amplified two bands in *C. arabica* one of which was the introgressed allele due to its presence in introgressed accessions. However, in *C. canephora*, it was clear that there were at least two alleles which were monomorphic in all accessions even the DH plants, and were therefore not in the same locus (Plate 4). This SCAR was amplified in 60 of the samples used by Ansaldi (2003) to determine if it is linked to any mapped *C. canephora* genomic fragments. It was observed that there was some kind of competition between the two alleles in *C. arabica* accessions having the introgressed allele. The competition was such that the non-introgressed allele was poorly amplified or not at all. Moreover, there was poor amplification in several samples causing unclear scoring. When the marker was scored alongside the data generated by Ansaldi (2003), it was evident that it was

linked to the fragment T2 but due to the above observations, it could not be clearly mapped. Despite some repetitions, poor amplifications were observed which could not be explained.

When the SCAR derived from RAPD $N18_{250}$ marker was digested, some extra bands were observed but not polymorphism. This was rather unexpected on the assumption that each DH plants had one allele and based on the occurrence of a single band in acrylamide gel (Plate 5). These results implied that there were mixtures of sequences in the products. This indicated repetition of the sequences. In a haploid genome, it would be expected that sequences occur singly unless they are duplicated and thus the maximum number of alleles expected in DH population derived from a single diploid plant is two. The occurrence of multiple sequences of the SCARs could be explained by existence of duplicated regions. This observation was true for SCARs derived from both AFLP and RAPD. Paillard *et al.* (1996) also observed restriction fragment length polymorphisms (RFLP) indicative of repeated DNA in the DH plants from the *C. canephora* clone IF200. The authors suggested that the duplicated loci probably referred to gene families dispersed on different chromosomes. The repetition of sequences therefore seems to be of common occurrence in coffee.

In conclusion, this phase of study set a firm starting point in the search for CBD resistance gene in a F_2 population that was being bred when these studies were being carried out. It highlighted an inventory of candidate targets in terms of *C. canephora* chromosomal fragments introgressed into *C. arabica* and as AFLP markers. In reference to the fragments, the most probable ones were T2 and T3. This phase also indicated the expectations for instance, in the analysis of SCARs due to their lack of polymorphism and duplication. This phase also enhanced the information available on the introgressed *C. canephora* fragments that is useful for any subsequent studies aimed at their utilization and conservation especially in cv Catimor in Kenya.

SECTION 5.2 ESTABLISHMENT OF POPULATIONS FOR MAPPING RESISTANCE TO CBD

5.2.1 INTRODUCTION

In order to be able to map markers of a trait, it is necessary to have a collection of a segregating progeny (population) derived from parents that differ in that trait and then characterise the phenotypes of the individuals of the population. Such a population is called a mapping population and such populations are the foundation of genomics research. The choice of the type of mapping population to use is affected by the reproductive mode of the plant to be analysed (self-fertile or not) and the relative ease of raising the population. Analysis of correlation between phenotypic data of the mapping population and marker data, prove or disapprove potential candidate genes controlling mono- or polygenic traits. An ideal mapping population should be derived from parents with a large variation in the trait to be analysed (Schneider, 2005). Self-fertile naturally inbreeding plants such as Arabica coffee can attain a high degree of homozygosity, and obtaining well-varied pure line parents for generating mapping populations is possible. The mapping populations possible for such plants are F_2 plants, recombinant inbred lines (RIL), backcross (BC) plants and doubled haploid lines (DH).

F_2 populations are the simplest form of mapping populations and are the basis of Mendelian laws of inheritance. Two pure or DH lines are crossed to give rise to F_1 generation that ideally is uniform. An individual F_1 plant is selfed to produce an F_2 population that segregates for the differentiating traits of the parents. F_2 plants are therefore the outcome of one meiotic event hence one recombination event. It is of advantage if the F_2 plants can be preserved as they are or as clones, for any future analysis as a permanent source of DNA since propagation of F_2 plants by crossing or selfing changes their genotypes. Some plant species do not allow easy selfing or cloning, but fortunately coffee is a perennial plant that can be cloned even by simple methods

like cuttings and the potential yield from one plant is in thousands of seeds. To produce a genome wide overview map, a population of about 100 F_2 plants is a good compromise between linked loci and cost/feasibility (Schneider, 2005). This size can then be increased for higher resolution of selected genomic regions whereby many plants are analysed with fewer markers. The use of an F_2 population was thus highly rated for this study.

Distorted segregation of some markers, meaning markers that do not obey laws of inheritance, is an often encountered problem in mapping populations. This maybe due to selection of gametes in favour or against some particular gametal genotypes, selective fertilization of particular gamete genotypes or other mechanisms operating during seed development, seed germination or plant growth (Lashermes *et al.*, 2001; Schneider, 2005). The complexity of a fragment may also be a cause of segregation distortion as observed by Nikaido *et al.* (1999) using AFLPs in *Cryptomeria japonica*. The authors were able to reduce or eliminate the distortion by adding more selective bases to the primers. Different methods of analysis may also cause interpretational problems that may be erroneously referred to as segregation distortion. This may include occurrence of "slippage" products in microsatellite analysis (Robinson and Harris, 1999).

In coffee breeding fields at Coffee Research Foundation (CRF) (Kenya), there are mature F_1 plants of crosses between different lines of cv Catimor and cv SL28. These trees can be used to rapidly develop F_2 generations by selfing. The F_2 populations would be expected to segregate in traits that present major differences between the parents such as resistance to CBD and CLR and compact growth. However, since the earlier breeding protocols was not designed with molecular studies in mind, some technical hitches might be encountered since the crosses were made using bulked pollen from several trees of a cv Catimor line to pollinate several cv SL28 trees and bulking the F_1 seed. Individual parents that donated the gametes cannot therefore be traced and

this might result in some polymorphism in progenies that cannot be explained by polymorphism between representative accessions of the parental varieties. In this study, individual parents of the F_1 plants could therefore not be identified. However, segregation of traits that are divergent between the parents is expected in the F_2 and these populations can therefore be used to map a trait such as CBD resistance that is uniform and contrasting between the parental cultivars. The first trait targeted was resistance to CBD but other traits could also be analysed in the same populations alongside the studies and/or after field establishment, so long as the traits are divergent between the parents and segregate predictably in the population. The particular population(s) used for the study of CBD resistance may not at the end be the most appropriate on 'as is' basis for all future studies due to lack of identity of individual parent plants, but they can generate very useful highlights. In addition, quantitative trait loci (QTL) may also be identified for composite traits like yield and quality by analysing mapping populations derived from these crosses, despite the lack of identity of individual parents. This would be done by raising a large mapping population of about 150 individuals from one of the F_1 trees that would cover the range of variation in the trait. The use of these parents also increases the level of polymorphism which otherwise is much lower between un-introgressed *C. arabica* varieties, making the generation of dense maps more difficult (Lashermes *et al.*, 1999; Anthony *et al.*, 2002a, Pearl *et al.*, 2004).

5.2.2 OBJECTIVE

The objective of this phase of the study was to develop an F_2 generation between cvs SL28 (susceptible) and Catimor (resistant) suitable for genetic mapping and verify its segregation.

5.2.3 MATERIALS AND METHODS

5.2.3.1 Establishment of seedlings

The plan of activities in establishing two segregating F_2 populations for mapping CBD resistance gene is presented schematically in Figure 4. The span of the activities presented (Figure 4) covered the entire period of this study with molecular studies being carried out when the plant materials were in the appropriate stage or after appropriate treatment such as screening for CBD resistance. It should be noted that the search for candidate markers for CBD resistance (Section 5.1) was done while the F_2 seeds were maturing in the field. Two F_1 plants, one from a cross between of cv Catimor line 127 and cv SL28 and another from a cross between cv Catimor line 88 and cv SL28 were selfed in October 2003, and the seeds harvested in June 2004 to give rise to populations D and E from the first and second trees respectively. Harvesting was done on two different dates (two weeks apart) as the berries ripened. On the first date, 128 and 134 seeds were harvested from the two trees respectively while 134 and 160 seeds were harvested on the second date.

The seeds harvested on the two dates were planted separately as replicates. Seeds of susceptible *C. arabica* cv Caturra were harvested on the second date from the same field and subsequently treated in the same way as the seeds of populations D and E. This was to provide a susceptible control to verify success of infection and comparative disease score during inoculation tests. After pulping by squeezing the berries, the seeds were partially dried, the parchment was removed by hand and then the seeds were germinated at room temperature in moist sterile sand in plastic boxes with a capacity of 200 seeds each. The seeds were watered frequently but lightly to avoid water logging.
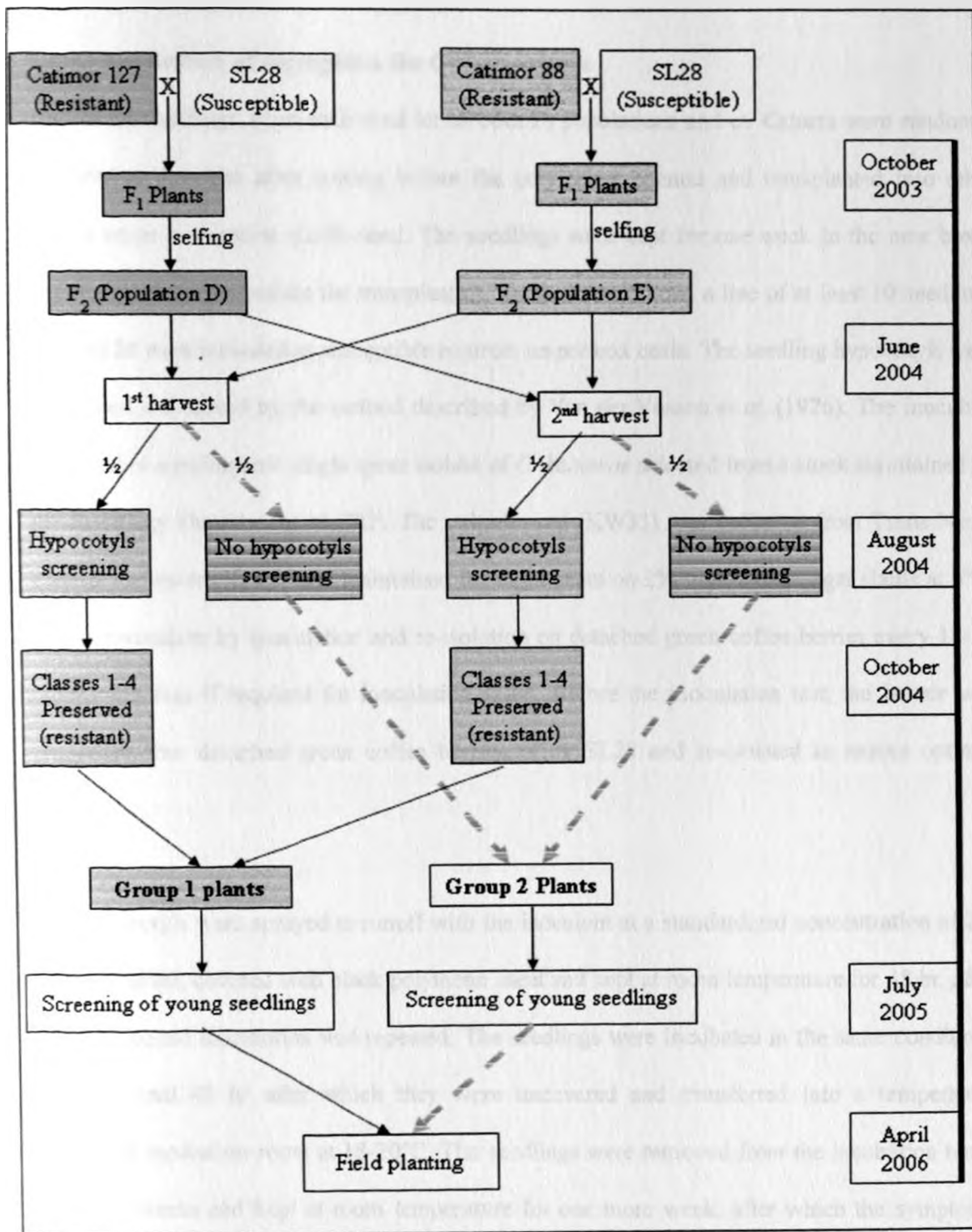
Figure 4. Schematic diagram of the plan adopted to establish and verify resistance to CBD in the F_2 populations obtained by selfing two F_1 plants available in the field.

5.2.3.2 Verification of segregation for CBD resistance

Half of the seedlings from each seed lot of both F_2 populations and cv Caturra were randomly uprooted at 5 weeks after sowing before the cotyledons opened and transplanted into other plastic boxes with moist sterile sand. The seedlings were kept for one week in the new boxes before inoculation to reduce the transplanting shock. In each new, a line of at least 10 seedlings of cv SL28 were included as susceptible controls on per box basis. The seedling hypocotyls were inoculated and scored by the method described by Van der Vossen *et al.* (1976). The inoculum consisted of a pathogenic single spore isolate of *C. kahawae* selected from a stock maintained by the Pathology Department of CRF. The culture used (KW33) was collected from Trans Nzoia District in western Kenya and maintained for eight years on 2% malt extract agar slants at 4°C, with rejuvenation by inoculation and re-isolation on detached green coffee berries every 18-24 months (or less if required for inoculation tests). Before the inoculation test, the isolate was inoculated into detached green coffee berries of cv SL28 and re-isolated to ensure optimal virulence.

The hypocotyls were sprayed to runoff with the inoculum at a standardized concentration of 2×10^6 conidia/ml, covered with black polythene sheet and kept at room temperature for 48 hr, after which a second inoculation was repeated. The seedlings were incubated in the same conditions for additional 48 hr after which they were uncovered and transferred into a temperature controlled incubation room at 18-20°C. The seedlings were removed from the incubation room after two weeks and kept at room temperature for one more week, after which the symptoms were scored. The scores were on a 1-12 scale where 1 is the most resistant with no visible symptoms which progressively become more severe from minute brown lesions in class 2, to girdling by deep black active lesions in Classes 11 and 12, which results into seedling death (van der Vossen *et al.*, 1976, Appendix 1). The infection scores were analysed for inter and intra-

86

population similarity and goodness of fit for Mendelian segregation by Chi square (χ^2) tests (Steel and Torrie, 1981)

Seedlings from the two populations that were classified into classes 1 to 4 (which are highly resistant) were transplanted into plastic bags containing a mixture of subsoil, sand and cattle mature (3:2:1). The decision of selection only seedlings in classes 1-4 was based on routine practice in breeding programmes at CRF. These seedlings were meant to be the resistant counter-checks from the F_2 populations. The seedlings of the two F_2 populations and cv Caturra, which were not inoculated by the hypocotyls inoculation method, were also transferred into the polythene bags. All these seedlings were then transferred into a nursery under 25% shade nylon netting until when required for subsequent studies. Apart from frequent watering, two rounds of foliar fertilizer were applied but no fungicides were applied to the seedlings.

5.2.3.3 Molecular verification of segregation

When the seedlings established in the nursery at six months old and most had two to three pairs of leaves, two to three leaves were sampled from 20 seedlings of each of the two F_2 populations (D and E) for DNA analysis. This sampling was from the lot of seedlings that were not screened by hypocotyls inoculation (Group 2). The leaves were sent to IRD, Montpelier, France where they were lyophilized and genomic DNA was extracted as presented in section 5.1.3. The DNA was analysed for presence and segregation of *C. canephora* chromosomal fragments T1, T2 and T3 using microsatellites primers Sat 32, Sat 207, and Sat 11 respectively, by the methodology described by Combes *et al.* (2000) with minor modifications. Six plants from population D, 5 from population E and a *C. canephora* accession (clone IF200) were used in the analysis. Amplification was in 25 µl PCR reaction mix containing 5 µl of 1 ng/µl genomic DNA, 2.5 µl of buffer (10X, Promega), 2.5 µl of MgCl$_2$ (25 mM, Promega), 1.0µl of SSR dNTPs (dNTPs stock

with a little dATP, Appendix 3), 2.5 µl each of right and left primers (2 µM, Eurogentec,), 0.1µl of *Taq* DNA polymerase (5U/µl, Promega), 8.8 µl of PCR grade water and 0.08 µl of αdATP P[33] (10 µCi/µl, Amersham Biosciences, UK). The PCR programme consisted of an initial denaturation of 2 min at 94 °C followed by 5 cycles of 45 sec of denaturation at 94 °C, 1min primer annealing at 60 °C reducing by 1 °C every cycle, elongation for 1 min at 72 °C and 30 cycles of 45 sec of denaturation at 90 °C, primer annealing at 55 °C for 1 min and elongation at 72 °C for 1 min 30s and final extension of 8 min at 72 °C. Electrophoresis and gel treatment was as for AFLP in Section 5.1.3.4 and revelation was by digital scanning using Phosphor storage screen (Amersham Biosciences) as in Section 5.1.3.5.

5.2.4 RESULTS

The hypocotyls inoculation results displayed distribution consistent with segregation in resistance to CBD (Plate 7, Figure 5). The populations also segregated in visible traits like vigour (height, girth and leaf size) and the colour of the young leaves and tips (Plate 7). There were more stunted seedlings in population D than in E. The distribution of the seedlings in the classes displayed some differences both between the populations and between seed lots of the same population. The success of infection was evaluated by comparison with severity in the susceptible check cv Caturra. Majority (97.5%) of cv Caturra seedlings were in the highly susceptible classes 11 and 12 (Figure 5 A) with a mean score of 11.8, and therefore infection was highly successful. On the other hand, the F_2 populations segregated into all the classes, except class 1 (Figure 5 B, C). The distribution of the seedlings within the infection classes was similar between the seed lots and populations, though Population D appeared to be more evenly spread out. However, there were higher counts of seedlings in the highly susceptible classes (11 and 12) within the lots of seeds harvested at the onset of ripening (Lot 1) than in the later lot (Lot 2). The mean infection grades of the first and second seed lots of Population D were 7.6 and 6.5

respectively and an overall population mean of 7.1. In Population 2, the mean infection grades were 7.3 and 6.9 respectively for first and second lots and an overall average of 7.1. By comparing the infection results the F_2 populations with those of the susceptible cultivar, seedlings in classes 11 and 12 were considered as susceptible and the rest of the seedlings were considered to express some degree of resistance. Using this criterion, it was observed that there were neither significant differences in the distribution of the seedlings between lots of the same population ($\chi^2 = 1.05$; $p = 0.300$ and $\chi^2 = 0.85$; $p = 0.336$ for Populations D and E respectively) nor between the two populations ($\chi^2 = 0.21$; $p = 0.646$). However, the distribution was highly significant between the two mapping populations and the susceptible cultivar (cv Caturra). The ratios of resistant to susceptible seedlings were 96:35 and 103:44 in populations D and E respectively, which fitted a 3:1 ratio for a major gene action ($\chi^2 = 0.206$; $p=0.650$ and $\chi^2 = 1.907$; $p=0.167$ for the respective populations). Despite the above categorisation, only seedlings within classes 1 to 4 were preserved for use in subsequent studies as resistant sub-populations so as to facilitate direct comparison with observations of breeding programmes at CRF. Susceptible seedlings were completely killed without a chance of obtaining DNA from them (Plate 7).

The presence and segregation of three microsatellite markers of introgressed *C. canephora* DNA fragments was evaluated, and two fragments (T2 and T3) were present in both populations and appeared to segregate in the expected 3:1 ratio while one (T1) was not observed (Plate 8). Sat 11 generated a pattern with an allele in the *C. canephora* accession IF200 similar to the highest allele in the Populations D and E (Plate 8 C), but comparison with cv Catimor accessions (P1 and P2) demonstrated that the introgressed allele from *C. canephora* was another one (Plate 8 D). The difference in the details of the autographs of Plates 8 C and 8 D is the level of separation which was due to electrophoretic conditions in which the gel for Plate 8 D was migrated for longer distance thus attaining better separation of the bands.
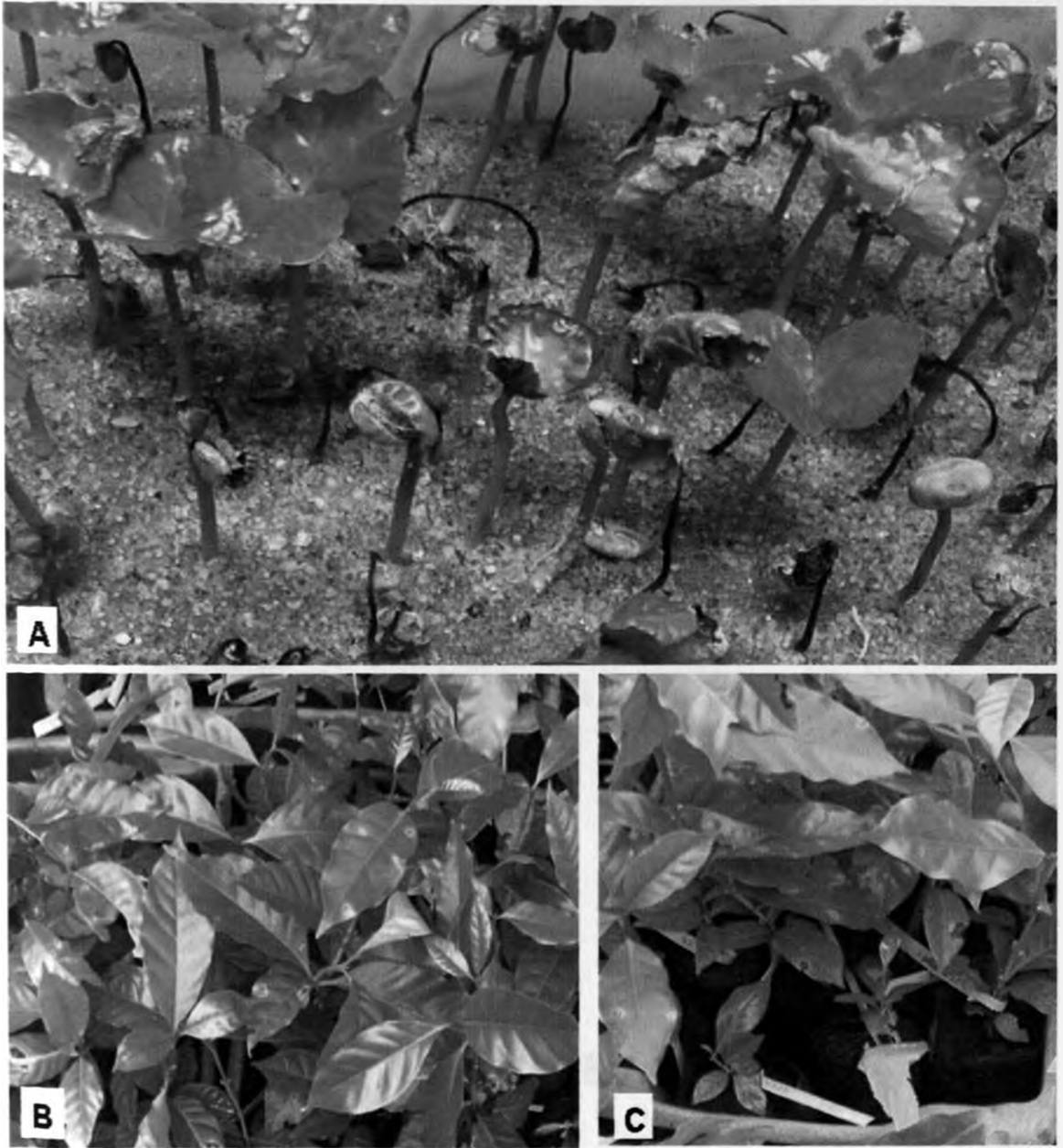
Plate 7. Some phenotypic traits that were observed to segregate in the two F_2 populations of cv Catimor x cv SL28; (A) resistance to CBD by hypocotyls inoculation test and (B, C) colour of young tips and vigour of young seedlings.
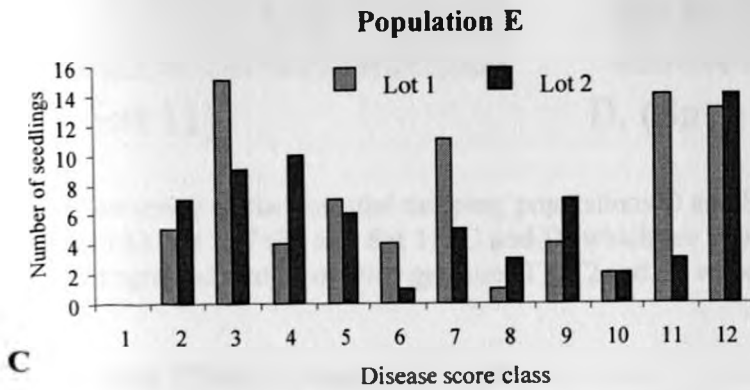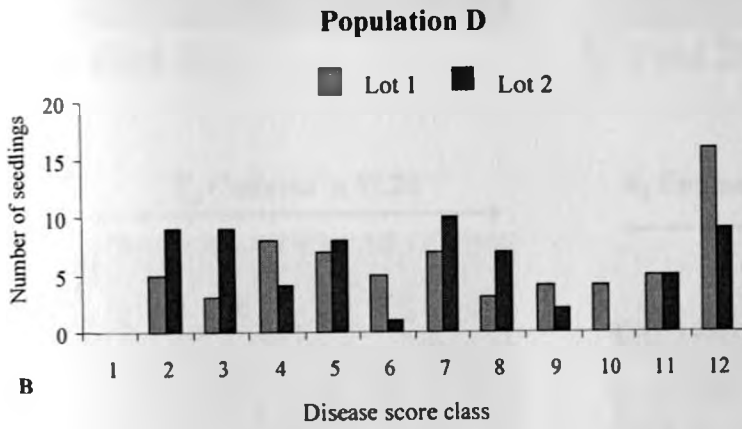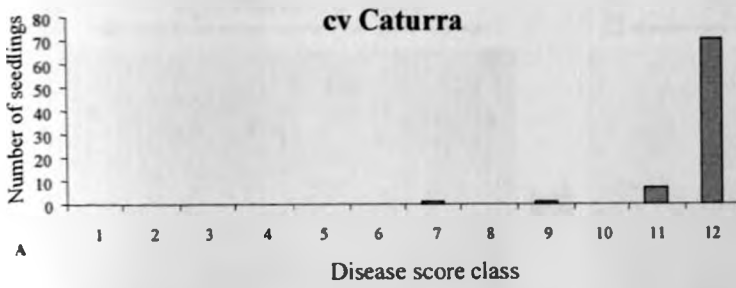
Figure 5. Bar graph presentation of infection scores of seedling hypocotyls of the two replicates of F_2 populations of after inoculation with *C. kahawae*.

Notes

Lots 1 and 2 refer to first and second harvest batches of each population: (A) Population D (Catimor 127 x SL28) and (B) population E (Catimor 88 x SL28).
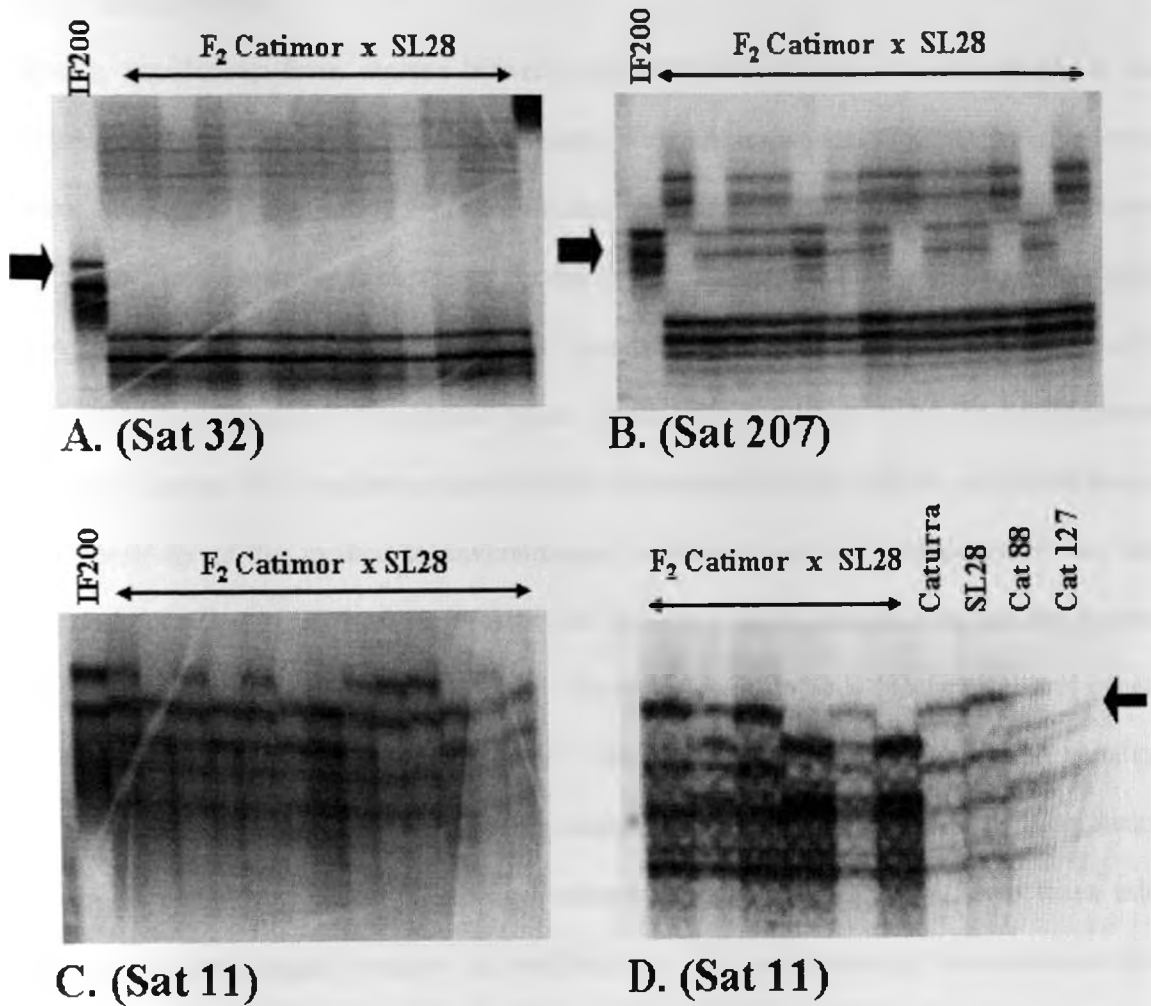
Plate 8. Screening of the potential mapping populations D and E using three microsatellites: Sat 32 (A), Sat 207 (B) and Sat 11 (C and D) which are markers of *C. canephora* fragments introgressed into *C. arabica* genome: T1, T2 and T3 respectively (arrowed).

Notes

    Sample IF200 is a clone of *C. canephora*

    Cultivars SL28 and Caturra are susceptible to CBD and CLR

    Cat 88 and Cat 127 are cv Catimor lines 88 and 127 which are resistant to CBD and CLR

5.2.5 DISCUSSION

Two F_2 populations from crosses between cvs SL28 and Catimor were realised. It was demonstrated that the populations were segregating in resistance to CBD by hypocotyls inoculations tests. The infection results of the two populations exhibited high similarity between and within them. However the tendency for the first lots of the two populations to have slightly more sensitive plants (Figure 5) indicated the possibility of the action of factors that may not be limited to the occurrence of resistance genes. These were not likely to be the environmental conditions during the inoculation tests because all treatments were random and simultaneous. The sensitivity of the method to environmental conditions especially temperatures has been observed for a long time (van der Vossen and Waweru, 1976; Masaba and van der Vossen, 1982). The possible cause of the observed tendencies could include biochemical and genetic composition of the seeds. The former would affect both inter- and intra-population variation, while the second would affect the inter population variation. The interplay of multiple factors during inoculation of hypocotyls generates results that may differ over time even when using seeds of the same origin. However, the verification of the involvement of factors such as seed biochemistry and physiology is subject to further investigations. Despite the inconsistencies and arguments of scaling and data interpretation, the method is valuable especially in screening populations to obtain resistant seedlings or using the averaged results to deduce the CBD reaction phenotype of the mother plant or line (Van der Vossen, *et al.*, 1976; van der Graaff, 1978, 1982; Dancer, 1986; Owour and Agwanda, 1990).

The distributions of the seedlings within the infection classes were similar between the seed lots and populations, though Population 1 appeared to be more evenly spread out. The mean infection grades of the first and second lots of Population 1 were 7.6 and 6.5 respectively and an overall mean of 7.1. In Population 2, the mean infection grades were 7.3 and 6.9 respectively for first

and second lots with an overall average of 7.1. There were no significant differences in the distribution of the seedlings between lots of the same population ($\chi^2 = 1.05$; p = 0.300 and $\chi^2 = 0.85$; p = 0.336 for Populations 1 and 2 respectively), nor between the two F_2 populations ($\chi^2 = 0.21$; p = 0.646). By comparing the infection results of the F_2 populations with those of the susceptible cultivar, seedlings in classes 11 and 12 were considered as susceptible and the rest of the seedlings were considered to express resistance. Using this criterion, the ratios of resistant to susceptible seedlings were 96:35 and 103:44 in Populations 1 and 2 respectively. The ratios fitted a 3:1 ratio for a major gene action ($\chi^2 = 0.206$ p=0.650 and $\chi^2 = 1.907$; p=0.167 for Populations D and E respectively). However, it was still borne in mind that the spread of the reaction phenotypes suggested lack of strict dominance, presence of other modifying genes or gene by environment interaction.

In routine breeding programmes at CRF, preselection by inoculation of hypocotyls results into plants with high resistance to CBD and CLR, with very few exceptions that are discarded as escapes. In this study, no seedling of the cv Caturra was classified within the resistant classes (1-4) which demonstrated that the infection was effective. However, the two seedlings in intermediate susceptible classes indicated the possible ambiguity of the classes and this demonstrates the advantage of retaining the highly resistant classes. Another advantage is that these seedlings have higher survival chances in the nursery than the more infected seedlings. Finally, by adopting the routine CRF procedure, the expected field resistance could be directly related to results of previous breeding programmes. The disadvantage of the method is killing susceptible plants as was demonstrated in this study (Plate 7). In fact, the seedlings were completely killed even before their cotyledons opened and they could not be used for DNA extraction.

Molecular analysis using microsatellite markers revealed that two *C. canephora* chromosomal fragments (T2 and T3) were present and segregating in the two F_2 populations (Plate 8). The results of these tests and those of Section 5.1 made fragments T2 and T3 the most probable candidates for disease resistance in the cv Catimor accessions, though not exclusively. T1 was not observed in this phase of the studies and its absence in cv Catimor accessions introduced into Kenya from Colombia is therefore further confirmed because it was also not observed in Section 5.1. In this case, if an accession with this fragment is encountered (either in cv Catimor or HDT accessions); it should be preserved as source of extra diversity. Analysis of the DNA markers in these populations coupled with traits like disease resistance or agronomic traits like compact growth habit in these or similar populations would generate information on their possible genetic linkages. The polymorphic microsatellite markers of *C. canephora* introgressed fragments were considered to be priority candidate markers for CBD resistance gene alongside the AFLPs identified in section 5.1. The seedlings classified as resistant (Classes 1-4) by the hypocotyls inoculation test (Group 1) were maintained as counter checking population for later studies during alternative screening method(s) and genetic mapping.

In conclusion, two segregating F_2 populations between a donor (cv Catimor) and a recipient (cv SL28) were realised as demonstrated both by phenotypic and molecular traits. This was an important prerequisite for subsequent work in deciphering the genetic basis of resistance to CBD, derived from *C. canephora* that is introgressed into *C. arabica* via HDT. The populations will also avail a collection of plants that at the end of the study will constitute an important source of breeding material for coffee improvement. This will be in form of plants identified to be of differential genetic assortment and can be used as breeding parents or even developed into single-tree selection cultivars.

SECTION 5.3 DEVELOPMENT AND USE OF YOUNG SEEDLINGS INOCULATION METHOD TO SCREEN COFFEE PLANTS FOR RESISTANCE TO CBD

5.3.1 INTRODUCTION

In order to be able to identify and map a certain phenotype, the phenotypes of the individuals of a mapping population have to be determined. In regard to this study, the overall objective was to identify and map markers for CBD resistance. Noting that there was no mature mapping population and the need to gain on time, a young mapping population had to be screened early and DNA obtained from all the plants irrespective of susceptibility to the disease. One widely used method to characterise resistance to CBD in immature coffee plants is the inoculation of seedling hypocotyls (van der Vossen *et al.*, 1976). However as observed in Section 5.2 above (Plate 7 A), this method results in the death of susceptible seedlings and they cannot be available for later studies in living form. The resultant population is thus biased towards resistance and factors that affect its expression. The susceptible seedlings also die very early before extraction of DNA can be done and the dead seedlings are necrotic and colonised by the fungus, such that extraction of DNA from them would be little, contaminated by the DNA of the pathogen and degraded. Another disadvantage of the method is that the results of inoculations of seedlings of the same source may give different results in different repeats overtime thus creating some inconsistency. This is partly due to the sensitivity of the method to temperature that necessitates the need for a temperature-controlled room for such inoculations (van der Vossen and Waweru, 1976).

However, the method as it was developed or with minor modifications especially on data interpretation and analysis, is very valuable especially in screening populations or using the averaged results to infer the phenotype of the mother plant or line (Van der Vossen, *et al.*, 1976; van der Graaff, 1978, 1982; Dancer, 1986; Owour and Agwanda, 1990). An alternative screening

method that was also developed by Van der Vossen *et al.* (1976) is the inoculation of shoot tips of young seedlings. This method has the advantage that leaves can be sampled from the entire population for DNA extraction, and at least part of the susceptible plants can survive for later studies. However, as described by the authors, the method requires selection of only seedlings with tips at the right stage (1-2 cm long with unopened leaves). It is also not clear if the authors chose their plants irrespective of vigour such that even weak and stunted seedlings were included. Furthermore, environmental conditions were not controlled which implies that the success of infection depends on the occurrence favourable weather conditions, and therefore can only be done within specific periods of the year. Moreover, the climatic conditions have changed over the last three decades at the site where the method was developed (Ruiru, Kenya), such that the average maximum and minimum temperatures have increased while rainfall seasons have become shorter (Gichuru, 2005). This is also the site where the mapping population for this study was to be screened and therefore there was concern of not achieving results similar to those of the authors.

The *in vitro* selection methods proposed by Nyange *et al.* (1995, 1997) require development of technical skills and capital investment, and may not be the most suitable for screening large populations. Since the authors used calli generated from seedling hypocotyls, the methods are destructive which would eliminate a population if all individual were to be screened. This is also true also for biochemical and histochemical methodologies reported by Gichuru (1993, 2001) and Gichuru *et al.* (1996, 1999). The option of raising the plants to maturity for observations tests in the field or laboratory tests on the berries or the seeds that they produce is time consuming. There was consequently a need to develop a method for early screening of resistance to CBD, which addresses the above limitations. The method would have to give a high value to the individual seedling reaction as opposed to the average value of the progeny. In this study, a

modification of the shoot tip inoculation method was assessed. The modifications were aimed at enhancing the infection and disease progress and then develop a scoring scale based on the details of infection.

It was recognised that it might be crucial to confirm the results of laboratory screening by testing in the field at least for some plants, especially those with unique genetic assortments of value either in refining the genetic map or as candidates for breeding lines. The subsequent confirmatory tests on mature plants would either be by inoculation of their progenies (F_3), field infection (natural infection or artificial inoculation) or a combination of both field and hypocotyls tests. In addition, F_3 populations would generate further recombination which may be important for fine mapping while field infection would generate information directly related to the actual mother plant genotype without genetic recombinations. One foreseen problem regarding natural field infection was that the plants were first to be established in the fields at CRF's main station in Ruiru, where natural CBD infection has been low for several years in the past (personal observation). This problem may be solved by introduction of clonal materials into research substations in more favourable regions, but this would require even more time. To address this issue, the effectiveness of artificial field inoculation under current field conditions was assessed at CRF Ruiru, so that the initial berries borne could possibly be inoculated in the field and therefore save time.

5.3.2 OBJECTIVE

To develop and use a suitable method to screen the F_2 populations for CBD resistance at an early stage, while allowing availability of DNA from the entire population and enhancing the survival of susceptible plants.

5.3.3 MATERIALS AND METHODS

5.3.3.1 Preliminary testing of young seedlings inoculation method

Two lots of ten seedlings each were randomly used per the cultivars SL28 and Catimor line 88. The seedlings were picked irrespective of visual growth status of the tips from a nursery at CRF, Ruiru in early June 2004. The two lots of each cultivar differed in age such that they were 6 and 12 months old respectively. The seedlings had been raised on a mixture of soil, sand and cattle manure (3:2:1) as outlined in Section 5.2. The seedlings were picked randomly irrespective of the visual assessment of the physiological state of the growing tip and then taken to the laboratory for inoculation tests. Inoculation was by spraying the top part of the seedlings (up to the third node from the tip) with *C. kahawae* conidial suspension at 2×10^6 conidia/ml. The fungus had been freshly isolated from berries infected by CBD in the field at Coffee Research Station, Ruiru. The seedlings were incubated in the dark (covered with dark polythene sheet and humidified) for 48 hr at room temperature (22-24 °C) and then transferred into a cold room at 18-20 °C for three weeks, after which they were transferred back to room temperature. They were maintained in this condition for two months with frequent watering and observation twice a week for progress of infection and recovery processes.

5.3.3.2 Field inoculation tests

Field inoculation tests were carried out on expanding green coffee berries on four trees of cv SL28 and eleven trees from different lines of cv Catimor by the method of van der Vossen *et al.* (1976). Berries on three selected branches per tree with at least thirty (30) berries per branch were sprayed to run-off with the same inoculum used to inoculate the young seedlings. The inoculations were done in the evening and the inoculated branches were subsequently covered with translucent polythene tubes for 4 days. The berries were then uncovered and infection was

left to progress under natural conditions with weekly observations but final records were taken after three weeks.

5.3.3.3 Screening of F_2 populations by the young seedlings inoculation method

The seedlings established in the nursery as explained in Section 5.2, were maintained for one year up to late June 2005, without any application of fungicides and only two applications of foliar fertilizer. Fungicides were not applied even to control nursery diseases such as brown eye spot (*Cercospora coffeicola*) so as to avoid any possible interference of the fungicide residues with inoculation with *C. kahawae* later and also to allow natural infection by CLR, a trait that was expected to segregate. One week before inoculation, leaves were sampled from all the seedlings and sent to IRD, Montpellier where they were lyophilized or stored at -80°C as described in Section 5.1. The one-week interval was a precaution to be sure that the samples had reached Montpellier before carrying out inoculations.

The seedlings were arranged in boxes for ease of handling during the inoculation process and each box could hold 32 potted seedlings. In each box, two seedlings of the susceptible cv Caturra were placed randomly among the F_2 seedlings as checks for the effectiveness of inoculation and infection. In addition, one box with only seedlings of cv Caturra and another box containing ten seedlings each of cvs SL28 and Catimor line 88 were included as extra controls, although these seedlings were older. The seedlings were inoculated as outlined in Section 5.3.3.1 but using the single spore isolate of *C. kahawae* (KW33) used in Section 5.2 to inoculate seedling hypocotyls of the two F_2 populations D and E. They were then covered at room temperature with black polythene sheet for 48 hr and the enclosure was kept humid by placing dishes with water inside plus three daily water sprays with a hand atomiser between mid-morning and late afternoon. The temperature of the room was monitored with a maximum-minimum thermometer. After the 48

100

hr, the seedlings were then transferred into a cold room used for inoculation of hypocotyls (Section 5.2), where they stayed for three weeks and the temperature monitored. During this time, they were observed at least twice a week for infection and those that were infected with likelihood of drying were removed from the room in attempt to save them. After three weeks, all the seedlings were transferred from the cold room back to the nursery for two more weeks after which the symptoms were recorded. Then a disease scoring scale was described considering all aspects of infection process observed during the entire screening process. The seedlings were thereafter maintained in the nursery for establishment in the field later.

5.3.4   RESULTS

5.3.4.1 Preliminary test of the young seedlings inoculation method

The initial symptoms on the seedlings of the susceptible cv SL28 were dark lesions mainly at leaf bases (Plate 9 A, B). This was observed in the two age groups (6 and 12 months) and it led to either leaf fall or the leaves dried but remained attached. Initial infection was also observed in the internode areas. The lesions then progressed rapidly in the susceptible cultivars and all the 6-month old seedlings of SL28 finally dried completely by the fourth week. The lesions in older seedlings also progressed down the main stem and most leaves dropped. There was a leading yellow halo ahead of the dead areas (between the dead and healthy areas) as a sign of progressing infection that had not been arrested (Plate 9 C). The number of infected nodes varied from 2 to 4 on the older seedlings of cv SL28. However, the nodes below the infected areas started sprouting and by the third month, they had developed new shoots (Plate 9 D). The infection had stopped by this time possibly due to unfavourable weather since the average ambient temperature had also increased and humidity reduced in the months of August and September. One of the 12 months old seedlings of cv SL28 failed to be infected and it was

observed to have a dormant tip. This highlighted the possibility of occurrence of failed infection in some susceptible genotypes.

Unlike the cv SL28 seedlings, there were no marked differences between the 6 and 12 months old seedlings of cv Catimor. Some of these seedlings did not have any symptoms of infection and even the leaves on the tips were not affected. Some seedlings were slightly infected and dropped the young leaves on the tip while some infections on the young tips progressed downwards but stopped at the first node below the young leaves (Plate 8 E). There was no halo below these nodes and the leaves at these nodes did not fall. Young shoots then developed from the node below the dead tips. Seedlings of cv Catimor did not have the general defoliation observed in SL28. Aspects of pathogenesis which were considered to be of potential in differentiating resistance and susceptible plants included the number of nodes or length of infected area, the rate of lesion progress, lesion type/characteristics and defoliation or drying of leaves. Death of the very young shoot tips was observed in both cvs Catimor and SL28 and was considered to be ambiguous in terms of genetic interpretation.

5.3.4.2 Inoculation of attached coffee berries in the field

The infection on attached berries of cv SL28 inoculated in the field ranged from 84-96 % on per tree basis (100-120 berries per tree) with most of the berries turning black and others falling off the branches (Plate 10 A). This was despite the intermittent cloudy and sunny weather observed during that period and open tree canopies. These factors were unfavourable for the disease progress and this was evident from the lack of natural infection on the non-inoculated branches on the same trees (Plate 10). In cv Catimor, the range of infection was 0-20%. However, even in the more infected plants, the lesions were superficial and berry drop was not observed as for cv

SL28 (Plate 10 B, C). This was despite the fact that canopies of the cv Catimor trees were thicker than in cv SL28 due to growth habit, close spacing and lack of CLR infection.
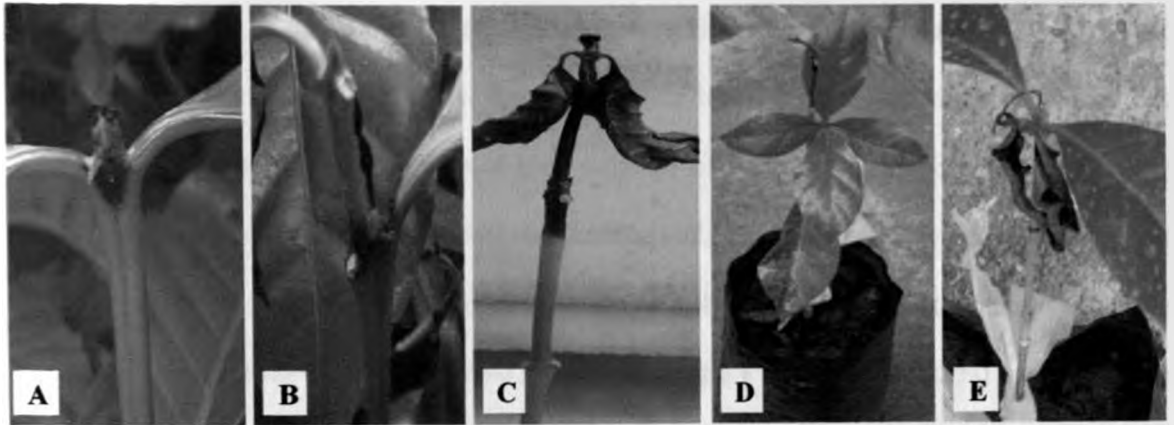
Plate 9. Symptoms observed on young coffee seedlings after inoculation with *C. kahawae*. (A and B); early symptoms of infection on cv SL28 seedlings, (C); an active lesion on infected a cv SL28 seedling showing the dead top and halo zone ahead of the necrotic area (D) a cv SL28 seedling after regeneration of a young shoot during recovery after infection. (E); a cv Catimor 88 seedling showing a dead young tip and infection arrested at the first node.

Plate 10. Symptoms of infection on attached green coffee berries in the field three weeks after inoculation with *C. kahawae*. (A); blackened berries of SL28 (note the stalks from which berries had fallen indicating susceptible infection), (B); inoculated attached berries on a cv Catimor tree, (C); close-up of the infected berries of cv Catimor showing the limited progress of the lesions (resistance).

5.3.4.3 Screening of the F_2 mapping populations by young seedlings inoculation method

The F_2 seedlings were inoculated after 10 months in the nursery. The seedlings exhibited large differences in growth vigour, with some being just 4-6 cm high, especially in population D, and some being as tall as 50-60 cm. Some seedlings were also very thin in relation to their height. Some of the small seedlings had few internodes while others had several but short internodes. This was in contrast to the seedlings of pure cultivars of both cv SL28 and cv Catimor which were used for pre-testing the method and the seedlings of cv Caturra which were raised alongside the F_2 populations, all of which had exhibited highly uniform growth. Most of the F_2 seedlings had brown eye spot disease (*Cercospora coffeicola*), while 9 (9%) and 4 (3.2%) seedlings from populations D and E respectively were observed to have at least one sporulating lesion of CLR. One of the CLR infected seedlings was very small and was growing under the canopy of the other seedlings. To estimate the disease pressure of CLR based on incidence, 50 seedlings of cv Caturra grown alongside the two F_2 populations were randomly observed for CLR infection. Infection was recorded as being present where at least one lesion with some sporulation was observed. All the observed seedlings of cv Caturra were infected hence an incidence of 100%. It was also observed that the severity of the disease on individual seedlings was higher in cv Caturra than in the F_2 populations.

The ambient temperature of the room at the time of inoculation ranged from 18.5 °C to 21 °C for the two days (48 hr) that the seedlings were kept there for infection before being transferred into the cold room. They were then incubated in the cold room for three weeks when infection was judged to be satisfactory from observations of the seedlings of cv Caturra which were severely infected. During this period, temperatures in the cold room ranged from 15 °C to 18 °C. The weather during the whole inoculation period was cool and humid with occasional light rains. The initial infection symptoms were the typical ones observed during the pre-testing stage, though

infection on several nodes per seedling was more common. The infection progress was variable in these populations resulting in a continuum of severity at the time of recording. Some seedlings did not exhibit any infection symptoms at all while in the most susceptible cases; there was complete seedling death or infection of a large part of the plant, more than 3 internodes on medium height plants with or without sporulation (Plate 11).

Most of the small seedlings were infected and killed, mostly within the first three weeks thus casting doubt on their reliability in phenotype identification. However on some of these stunted seedlings, the severity of infection was low or even none especially those with short internodes and thick stems (Plate 11 C). Although some seedlings were infected at the nodes resulting into girdling, the other parts of these seedlings remained visually healthy without even signs of wilting up to five weeks after inoculation when symptoms were recorded (Plate 11 C). Seedlings that were severely infected before the full incubation period in the cold room (3 weeks) were removed in attempt to rescue them (Plate 11 D). It was observed that even when the seedlings were taken to the nursery, further progression of infection occurred which killed some more seedlings. When scoring the symptoms, all the aspects of the pathogenesis were considered. However, the time of first symptoms appearance was not found to correspond to the final severity of infection, and was therefore disregarded as a criterion for phenotype identification.
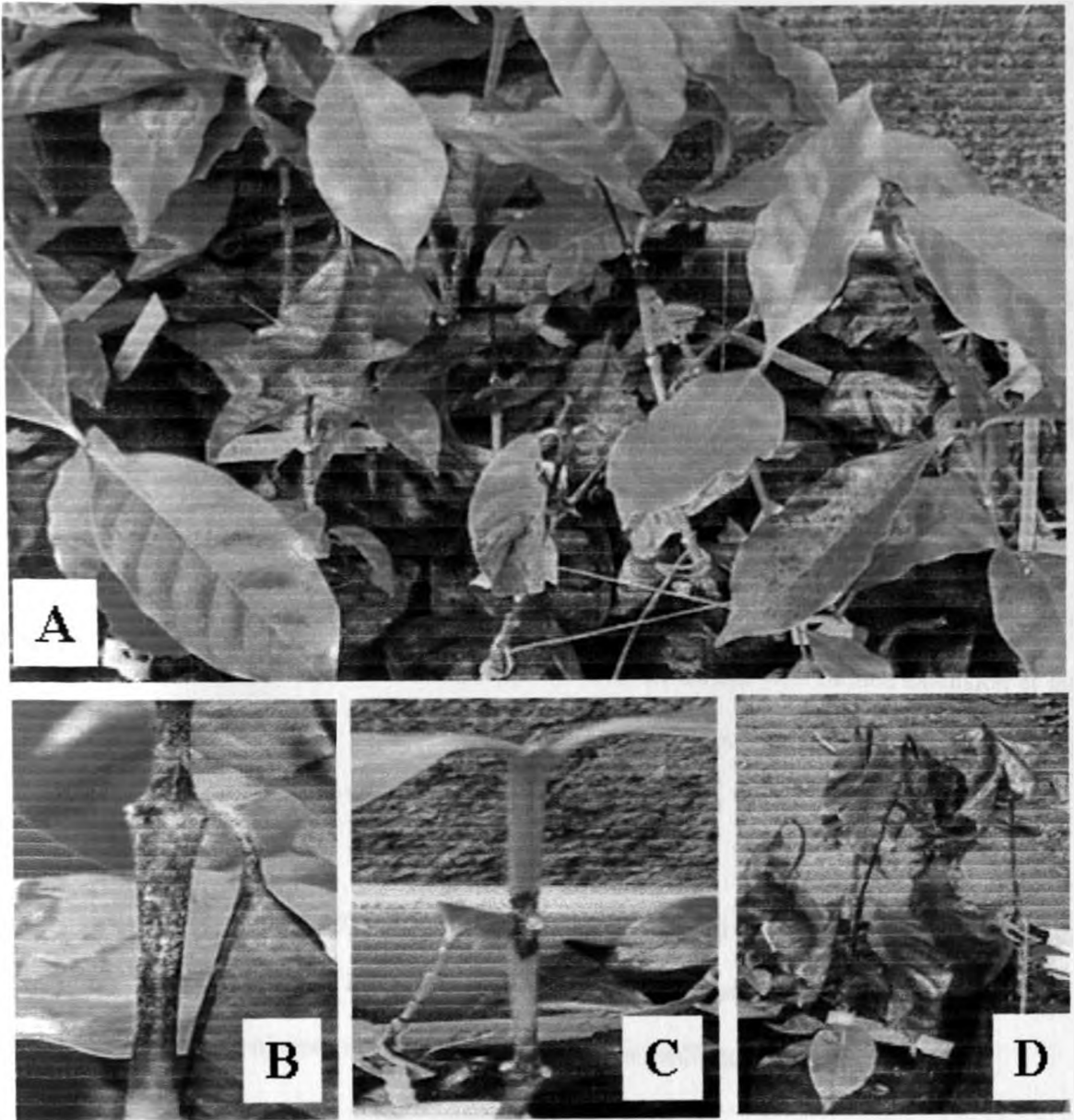
Plate 11. Symptoms observed on F_2 seedlings (Catimor x SL28) five weeks after inoculation with *C. kahawae*

(A)   A mixture of symptoms in one of the inoculation boxes

(B)   An active lesion with heavy sporulation of the pathogen

(C)   Two seedlings whereby one had no symptoms of infection except defoliation (in addition to leaves sampled for DNA extraction) and another with restricted infection on a node resulting in girdling but not wilting of upper part,

(D)   a group of severely infected seedlings which were removed from the inoculation room before the end of incubation period as an attempt to save them.

In consideration of the symptoms observed, a five class disease scale was described and adopted as described below:

0   No visible symptoms of infection

1   Infection on leaves and very small brown lesions on the stem. The lesions were superficial and inactive. Infection signs occurred mainly on the young shoot above the fully expanded leaves.

2   Larger lesions (than in Class 1), on stem above first node but not active. The variable symptoms include scabs, brown superficial lesions small, small deep black lesions with restricted borders lacking a leading yellow halo

3   Large black lesions sometimes mixed with scab lesions, total collapse of the young shoot tips, girdling at the points where fallen leaves detached but with limited extension of the subsequent lesion into the inter-node areas. Dead internode areas that did not exhibit the blackening associated with colonisation by the pathogen but results from wilting due to girdling at the nodes.

4   Very active lesions progressing into the inter-node areas with leading halo zones, dead areas are characterised by the typical tissue blackening associated with tissue colonisation by the pathogen, death of multiple nodes or whole seedling, pathogen sporulation.

The distributions of seedlings into the various classes are presented in Table 5. There were differences between the populations and between the groups screened and not screened by hypocotyls inoculation method i.e. groups 1 and 2 respectively (Table 5). Eight out of eleven small (non-vigorous) seedlings were rapidly killed within the first three weeks and were not subsequently put into any phenotypic class. These included one seedling from Group 1 of Population D. Two seedlings whose reaction was doubtful due to mechanical damage and signs of root infections were also not categorised. However, the plants that were not categorised into

CBD resistance phenotype were included in molecular analysis of entire populations, to avoid segregation distortion of markers.

Table 5. Disease infection scores of one year old coffee seedlings of two F_2 populations (D and E) from crosses between cvs SL28 and Catimor, five weeks after inoculation with *C. kahawae*.

| Class | Group 1* | | Group 2# | | |
	Population D	Population E	Population D	Population E	cv Caturra
0	5	14	12	26	0
1	10	7	17	22	3
2	5	6	9	10	2
3	6	8	24	37	10
4	3	0	33	26	36
Total	29	35	95	121	51

Notes
* Group 1 were the seedlings which were screened by hypocotyls inoculation method and identified as resistant
# Group 2 were the seedlings that were not screened by hypocotyls inoculation method

The first three classes (0-2) were considered to be resistant and the fifth one (Class 4) to be susceptible. Plants in Class 3 could not be clearly categorised into resistant or susceptible due to presence of mixed symptoms which were observed in both the resistant and susceptible parents (cvs Catimor and SL28/Caturra respectively) both during preliminary testing of the methodology and in this phase. This class also had many plants that had low vigour especially in girth and were girdled. However, when the infection was observed as continuous tissue colonisation, these low vigour plants were placed in Class 4. Although no elaborate data was taken, the low vigour plants (small or thin) were more frequent in Population D than in Population E. Attempts to subdivide Class 3 further was too laborious and un-rewarding. Five plants of cv Caturra (9.8%) were in classes 0-2 and were interpreted as failed infection. Two of these plants had dormant shoot tips though the topmost leaves were dark green and fully expanded. On the other hand, there were three plants (10.3%) of Group 1 of Population D that were classified as susceptible

although they were resistant by hypocotyls screening test. These plants had thin stems as explained above. In population D, the number of seedlings in the resistant category (Classes 0-2) was almost equal to that in the susceptible category (Class 4) i.e. 38 and 33 respectively while in population E, the number of seedlings in the resistant classes 0-2 was a little more than twice that in the susceptible class i.e. 58 and 26. In both cases these figures appeared to reflect higher susceptibility than expected, but no definite conclusions could be drawn due to the relatively large numbers in Class 3.

5.3.5 DISCUSSION

Coffee has a long generation interval of about 5 years. On the other hand, CBD is a disease of the mature crop since it affects the fruits. It is therefore consequential that if field/natural screening for resistance to the disease has to be done, a long time is required to pass from one generation to another. However, there are artificial methods of screening which have been developed to detect CBD resistance in early stages of coffee, notably the inoculation of seedling hypocotyls and shoot tips (van der Vossen *et al.*, 1976). In order to be able to relate molecular results to phenotypes for genomic mapping, individual plants have to be phenotyped and DNA extracted from them. The methods reported above had some shortcomings in this respect. The hypocotyls inoculation method diminishes the chance of obtaining DNA from susceptible plants because they are killed and eliminated from later studies in living form. The shoot tips inoculation method is unsuitable for whole population screening in view of the fact that it requires selection of seedlings in a particular growth stage. In this study, the hypocotyls inoculation method and a modification of the shoot tips inoculation method was co-utilized so as to overcome the disadvantages of each. Inoculation of shoot tips of seedlings at about one year was considered a suitable screening method allowing early screening without high loss of susceptible plants, and it was pre-tested and then used. The methodology was modified by

provision of suitable environmental conditions that were anticipated to enhance infection beyond the shoot tips and irrespective of the stage of growth of the tips. This would facilitate screening of the entire population because the seedlings do not need to be selected as described by van der Vossen *et al.* (1976). While selection of only the seedlings with appropriate shoot tips may not affect the random distribution of resistance genes, it would necessitate starting with much larger populations or carry out multiple inoculations on subsets of the same population at different times. The seedlings were incubated in a cooled inoculation room rather than nursery conditions as was done by van der Vossen *et al.* (1976). The length of period that the seedlings were kept in the inoculation room was determined by the incidence and severity of infection on the susceptible controls (cvs Caturra and SL28). While pre-screening was done in plants of highly uniform genotypes and phenotypes, the screening of the F_2 population was more challenging because of the mixed phenotypes especially with regard to plant vigour that resulted into more variable disease expressions.

A five-scale classification of the symptoms was proposed by grouping symptoms that were perceived as similar levels of expression of resistance. The first three classes (0-2) reflect categories which were considered to result from incompatibility (resistance) while the fifth (Class 4) was considered to be due to lack of resistance (susceptibility). Class 3 was perceived as ambiguous in terms of possible genotype as it appeared to be transitory reflecting either the physiological status or moderated gene action that may have been due to genotype, environment or interaction of the two. It was clear from the results of both pre-testing and F_2 screening (Table 4) that there were factors other than genotype, which affected the final disease symptoms whereby with some seedlings of the susceptible cultivars even failed to be infected. There was an effect of age of the seedlings in view of the fact that no misclassification was observed on the young seedlings of cv SL28 in the pre-testing because all of them were infected and ultimately

killed. On the contrary one of the older seedlings of the same variety failed to be infected. By comparison with the seedlings screened by hypocotyls inoculation method and found to be resistant, there were also some resistant plants which were as much infected as the susceptible ones either in Classes 3 or 4 (Table 4). In the F_2 populations, non-vigorous plants were observed to be particularly susceptible which was suspected to be unrelated to absence of the resistance gene. It was also evident that it would be difficult to save susceptible plants if they are inoculated at a young stage or are of low vigour. Recording of the symptoms and development of disease scale considered different attributes of pathogenesis. One of the difficult aspects was to recognise plants that were infected only at the nodes but the girdling caused wilting and death of the upper part. Infection at the nodes may have been enhanced by accumulation of the inoculum run-off at these points and presence of natural entry points like hydathodes. Such infection could then cause defoliation that in turn would avail additional entry points even for secondary pathogens such as *Fusarium* spp.

Rescuing of susceptible seedlings was improved by transferring them from the incubation room to ambient room temperature that was unfavourable for CBD development, though the absolute conditions can be affected by weather conditions depending on the season and may approach favourable conditions for CBD development. Salvaging of the susceptible plants could be further improved where necessary by cutting the infected part if the growth structure allows, since some seedlings had small compact stems. Upon recovery the seedlings sprouted later from nodes below the infected area (Plate 8). However, it can be expected that some plants would be lost because sometimes the infection progressed very rapidly and if the ambient temperatures are favourable, infection can continue even when the seedlings are outside the cold room. Infection by *C. kahawae* can also facilitate secondary infections by other pathogens such as *Fusarium* spp that can accelerate seedling death. Infection below the grey mature part of the stem was not

111

easily noticeable which led to some seedlings appearing to be healthy towards the based but were infected. To ensure analysis of DNA from all the plants, sampling of leaves for DNA analysis was done before inoculation which is an advantage of using this method.

In consideration of the results of inoculation of young seedlings, Population E was chosen for marker identification and mapping purposes and Population D as a cross-checking population especially in respect to resistant seedlings obtained after screening by hypocotyls inoculation method. This was because population D potentially had more plants misclassified as susceptible comparing with group 1 plants (Table 5). It was anticipated that later studies would detect misclassified plants and the significance of the intermediate class (Class 3).

Inoculation of attached berries in the field was successful and clearly differentiated resistant and susceptible genotypes despite the prevailing climatic conditions that were unfavourable for natural infection (Plate 10). This will be very useful in confirming the results of the early screening methods especially in clarification of results of the subsequent molecular studies that may require phenotypic confirmation, especially of recombinant plants without progeny advancement that would lead to genetic reconstitution. Inoculation of attached berries in the field would also save on time that would be required for the berries to mature and germinate seedlings for hypocotyls inoculation tests.

The infection of coffee by *C. kahawae* is highly dependent on temperature such that at low temperatures of 15°C and below, even resistant genotypes are infected while at temperatures higher than 22°C even susceptible plants exhibit resistance phenotype (Nutman and Roberts, 1960; Van der Vossen and Waweru, 1976; Masaba and van der Vossen; 1982). In fact, this was the factor that necessitated the construction of a temperature-controlled room (inoculation room)

at CRF to improve the efficiency of preselection by enhancing infection (van der Vossen and Waweru, 1976). The mode of temperature control in the inoculation room is based on cooling and not heating. This means that if the ambient temperatures fall below the optimum range of 18 °C to 20° C, as happens at night especially in the cold months of June and July in Kenya, the temperature of the inoculation room similarly falls below the optimum range and as a consequence expression of CBD resistance is inhibited. During very cold weather, the room is likely to experience even lower temperatures than in a normal closed room because the fan may blow in the air from outside into the room.

During the period that the F_2 populations were screened, the temperatures in the inoculation room ranged between 15-18° C. This factor might have enhanced infection of otherwise resistant seedlings as observed in this study. Another possible factor that could affect disease development in the room could be light. Plants in the inoculation room receive less light intensity than those outside, even if the curtains are opened and lights are switched on. As a practice, the electric lighting in the room is usually off unless someone is in the room. In totality, the seedlings receive less light hours and of less intensity, which could affect their photosynthetic rate and hence nutritional status. This could have weakened the seedlings especially the less vigorous ones leading to their succumbing to the infection. While hypocotyls are largely non-photosynthetic and rely on stored food, the young seedlings are photosynthetic. This aspect might cause some differences in the efficiency of the two methods. It is possible that the ultimate reaction to infection by *C. kahawae* is affected by multi-genetic and environmental factors that may be related to plant vigour and general defence systems. While major gene actions are important in hypersensitive reactions like cell death at infection points, other subsidiary mechanisms such as lignifications of cell walls and formation of cork barrier are also

involved (Masaba and van der Vossen, 1982; Gichuru *et al.*, 2001). The net effect of the potential mechanisms and their interaction with abiotic factors are subject to investigation.

It was nonetheless anticipated that even under these circumstances, plants with the resistance gene(s) would display some phenotypic difference from the ones without the gene(s). This was why all aspects that could reflect resistance such as lesion type, rate of lesion development and sporulation were considered in developing the disease scale and classification of reaction type. Seedlings in class 3 were not categorically classified as resistant or susceptible although a bias in opinion was indicated as (R?) or (S?) for resistance and susceptibility respectively. Efforts to separate this class further did not help to clear ambiguity even by enlarging the range of classes and it was impossible to avoid misclassification. It was observed that even some cv Caturra seedlings displayed symptoms within the resistant classes. Despite the limitations, it was anticipated that misclassifications would not be very high and make it impossible to detect DNA markers linked to resistance gene(s) and that the misclassified plants would be revealed by molecular studies. A further precaution for detection of markers was by using plants classified as resistant by hypocotyls inoculation method (Group 1) as controls in mapping work. Previous experience at CRF with this pre-screening method laid a lot of confidence on detecting resistant plants. This strategy also had the advantage that Group 1 plants and Group 2 plants could be directly compared since they were derived from the same segregating populations hence no cross-specific concerns.

Although no artificial inoculation with *H. vastatrix* for screening purpose was done, natural CLR pressure in the nursery was high enough to effect selection for the disease. The seedlings were raised in a space surrounded by many older coffee seedlings and mature coffee trees that were infected by CLR. The seedlings stayed in the nursery for about 8 months including two

favourable CLR infection periods in October-December 2004 (short rains) and from March-June 2005 (long rains). Furthermore, all the examined seedlings of cv Caturra growing in the same nursery bed with the F_2 populations were infected. Chances of disease escape either due to lack of inoculum or unfavourable infection conditions were arguably low and the main reason for non-infection was most likely due to genetic factors. The observation of CLR infection on a small seedling growing under un-infected seedlings indicated further the lack of physical protection. No CLR infection was observed on seedlings that were selected for CBD resistance by hypocotyls inoculation method (Group 1). This agrees with the routine observations in breeding programmes in Kenya whereby selection for CBD resistance generates populations that are also highly resistant to CLR. CLR infection on HDT derivatives is characterised by occurrence of small to medium size yellow spots with low sporulation and it occurs particularly on plants of poor nutritional status, overbearing plants or senescing leaves (Gichuru, 2005). It is also of interest to note that most accessions selected for resistance or tolerance to CBD (such as Rume Sudan, K7, Catimor and accessions of HDT) exhibit similar reactions to CLR, at least under Kenyan conditions. This would indicate some genetic linkage of the two traits. Seedlings with CLR infection were therefore included in the mapping studies in order to observe any similarity in their genotypes that may indicate potential candidate markers for CLR resistance.

In conclusion, the inoculation of seedlings of F_2 populations at an age of one year facilitated their screening for CBD at a relatively early stage with a degree of success that was considered sufficient for identification and mapping of DNA markers of the resistance. This method also allowed DNA to be obtained from both susceptible and resistant individuals especially by sampling leaves before inoculation tests. Seedlings of the two F_2 populations that were screened by hypocotyls inoculation method and identified to be highly resistant were preserved for use as confirmatory controls during subsequent molecular studies. It was anticipated that there would

be a need to confirm the CBD phenotype of some plants after maturity in the field. To facilitate this in the event of low natural infections, effectiveness of phenotyping by inoculation of attached green coffee berries was successfully demonstrated.

SECTION 5.4 IDENTIFICATION AND MAPPING DNA MARKERS LINKED TO CBD RESISTANCE AND POSSIBLE CANDIDATE MARKERS FOR CLR RESISTANCE

5.4.1 INTRODUCTION

This phase of the studies utilized the results of the previous phases in order to identify the markers of CBD resistance. The candidate markers analysed in Section 5.1 were tested on the F_2 populations that were established in Section 5.2 and screened for CBD resistance in Section 5.3. There were two possible situations that were expected after identification markers linked to CBD resistance. One possibility was that such marker(s) could be located on one of the *C. canephora* chromosomal fragments already mapped by Ansaldi (2003), in which case there would be no need for remapping the fragment but rather walking on it to locate the gene. Secondly, it was possibly that the identified marker(s) might be unmapped since some polymorphic unmapped bands were observed in Section 5.1. In this case, it would be necessary to map the chromosomal fragment carrying the gene. In section 5.1, the priority was AFLP due to its versatility to detect polymorphism but in this section, microsatellites were given first priority due to their relative ease of use and higher repeatability compared to AFLP (Vos *et al.*, 1995; Rafalski *et al.*, 1996). However, it was envisaged that due to the low number of microsatellites, AFLP analysis would be required to refine the region around any identified microsatellite marker(s). There are three accessions of HDT i.e. 832/1, 832/2 and 1343 that have been widely used in different breeding programmes over the world. Previous molecular studies have demonstrated that these materials are similar as shown in Section 5.1 and also by Lashermes *et al.* (2000a) and Noir *et al.* (2003). It was therefore of interest to evaluate if this is also true for markers of CBD resistance.

Development of SCAR markers enhances reproducibility of their parent markers such as AFLP or RAPD (Paran and Michelmore, 1993; Shan *et al.*, 1999). In relation to this aspect, it was an

aim within this stage of study to develop SCAR markers from identified markers for CBD resistance such as AFLP which are not sequence based, and try to establish any relationship shared with those which were analysed in Section 5.1, including those based on RAPD markers of CBD resistance (Agwanda *et al.*, 1997). The SCARs designed from AFLP bands developed in Section 5.1 may be useful indirectly even if they were not polymorphic. One way would be to use them as probes in physical mapping or chromosome walking. Monomorphic SCARs can be used as probes to screen genomic DNA libraries, the positive clones are end sequenced and the sequences are in turn used to design more SCAR primers that may be polymorphic and validate the initial sequences. This procedure also helps in saturation of the map with more markers that are sequence based and therefore more reproducible. For the SCARs to be useful for such a purpose, they have to be of single or low copies, and if physical mapping targets a specific trait, they preferably have to be tightly linked to the trait. After development of a SCAR marker, there is need to validate it because there could be an error during cloning or duplication in the genome in some other loci that are not associated with the trait. In this study, it was endeavoured to assess characteristics of SCARs located on the chromosomal fragment carrying the resistance gene as possible starting points for chromosome walking or physical mapping.

5.4.2 OBJECTIVES

The general objective of this phase of study was to identify and characterise markers of CBD resistance with specific sub-objectives being:-

i. To identify and map DNA markers linked to the gene(s) for CBD resistance and highlight possible candidate markers for gene(s) resistance to CLR.

ii. To develop SCAR markers from AFLP markers and/or establish relationship with SCARs already developed

iii. To assess the repetitive characteristics of SCARs developed from AFLP bands in *C. arabica* genome

iv. To assess the prevalence of any identified markers in germplasm bred from different accessions of HDT

5.4.3   MATERIALS AND METHODS

5.4.3.1 Plant materials and DNA extraction.

Plants from two F_2 populations D and E used for this study were established, sampled and screened for CBD resistance as in Sections 5.2 and 5.3, while DNA was extracted from their leaves as described in Section 5.1.

5.4.3.2 Identification of molecular markers of CBD resistance

5.4.3.2.1 Identification of microsatellite markers of CBD resistance

The methodology for microsatellite analysis is described in section 5.2. A total of fifty-seven (57) microsatellites including those tested in Section 5.2 (Sat 11, Sat 32 and Sat 207 that are markers of *C. canephora* chromosomal fragments T3, T1 and T2 respectively) were screened for polymorphism. Twenty seven of these microsatellites had not been tested by Ansaldi (2003). Fifteen plants were used for the initial screening which included five plants from each of the two F_2 populations (D and E), one accession each of cvs Catimor line 88, Catimor line 127, Sarchimor line T5296, SL28 and Caturra. Out of these, seven microsatellites had common alleles in HDT derivatives that were also polymorphic in the F_2 populations. These microsatellites were selected for further analysis as priority candidate markers for CBD resistance. In order to assess the segregation behaviour of the markers in population E that was chosen for mapping, the seven microsatellites were amplified in 95 Group 2 plants (which were not inoculated at hypocotyls stage). These plants consisted of 47 resistant plants, 18 susceptible plants and 30 plants in Class

3. For further verification of linkage to CBD resistance, the microsatellites were also amplified in fifty-six (56) Group 1 plants comprising of 29 and 27 individuals from populations D and E respectively. Microsatellites that appeared to be linked to CBD resistance were tested for segregation fitness by Chi square tests (Steel and Torrie, 1981). Eight Group 2 plants from population D that were observed to be infected by CLR in the nursery were also included to highlight possible markers for CLR resistance. These CLR susceptible plants ranged from Class 3 to 4 in CBD resistance as screened by young seedlings inoculation method. In addition, 18 randomly selected Group 2 plants from population D, which had their DNA extracted for tests of segregation in section 5.2, were amplified with Sat 11 and Sat 207 to highlight the segregation pattern in this population, and compare it with that of population E.

5.4.3.2.2 AFLP analysis of the chromosomal fragment conferring resistance to CBD

The plants analysed with microsatellites were also amplified with a T4 marker (AFLP-17) to reveal the segregation pattern of this fragment and if it was related to CBD resistance. From the results and those of the tests with microsatellites above, a fragment linked to CBD resistance was identified and AFLP markers mapped on this fragment (T2) were selected for further confirmation and refining the location of the gene. In this endeavour, all Group 1 plants (resistant by hypocotyls inoculation method) that were analysed by the microsatellites were further analysed with the AFLP primer pair EACT-MCTT that amplifies two markers of the T2 fragment (AFLP-21 and AFLP-22) and one marker of T3 fragment (AFLP-23) (Ansaldi, 2003, Appendix 2). Based on the combined results of this test and those of microsatellites, 30 plants were chosen for further analysis with more AFLP markers of the T2 fragment for recombinations and assessment for possible linkage to CLR resistance. The selected plants consisted of: 3 resistant Group 2 plants which were homozygous for the HDT derived allele of Sat 207, 4 resistant plants from both Groups1 and 2 that were heterozygous for Sat 207, 7 resistant plants

from both Groups 1 and 2 that lacked the HDT allele of Sat 207, 5 CBD susceptible plants and 11 plants which had some CLR infection (from both populations D and E). The CLR susceptible plants were either in classes 3 or 4 for CBD reaction by young seedlings inoculation method. These plants were analysed for AFLP markers of the T2 fragment spanning Sat 207 i.e. AFLP-32, AFLP-28 (on one side of Sat 207), AFLP-22, AFLP-27, AFLP-33, AFLP-34, and AFLP-36 (on the other side) and mapped in that respective order (Ansaldi, 2003, Appendix 2). Due to lack of recombinants on one side of Sat 207 in the above selection of plants, all remaining Group 1 plants that were heterozygous for this marker (20 plants) and 10 heterozygous resistant Group 2 plants were analysed for three AFLP markers (AFLP-33, AFLP-34 and AFLP-36). This was done with an aim of revealing the limits of the location of the resistance gene of this side.

5.4.3.2.3 Mapping Sat 235.

From the analysis of microsatellites in Section 5.4.3.2.1, it was observed that one of the un-mapped microsatellites (Sat 235) co-segregated with Sat 207 and was linked to CBD resistance. To facilitate mapping of this marker, it was amplified on the samples used by Ansaldi (2003) to map the introgressed *C. canephora* fragments and then mapped using Mapmaker programme Version 3.0b. An initial LOD score of 5.0 was used to ascertain linkage of markers and mapping was done at an LOD value of 3.0. This marker was also analysed for polymorphism in the DH population with an objective of identifying an associated linkage group corresponding to the basic coffee genome. In these mapping experiments, Sat 262 was also included because out of the priority candidate markers it was the only one that was not tested by Ansaldi (2003).

5.4.3.3 Analysis of SCARs derived from AFLP markers of the chromosomal fragment conferring resistance to CBD

The SCARs developed from AFLP markers of the introgressed *C. canephora* fragment T2 in section 5.1 were amplified on a set of six CBD resistant and four CBD susceptible plants. Primers N2-1R, N2-2R and W3 were amplified at an annealing temperature of 55 °C and AA4 at 60 °C, which were their theoretical optimum annealing temperatures. All other conditions were as in section 5.1. SCAR AA4 revealed intensity in 2% agarose that appeared to be differential between resistant and susceptible samples. Further tests were then done to determine if the difference in intensity was due to temperature dependent efficiency of amplification or was due to amplification of multiple products in resistant plants that were not separated in agarose gel. This was investigated by amplifying the SCAR at annealing temperatures of 55 °C, 62 °C and 64 °C and separated in agarose, and then at 60 °C followed by separation in 6% denaturing poly-acrylamide gel as described in Section 5.1.

The multiplicity characteristics of three SCARs (A2, W3 and AA4) that were derived from AFLP markers of T2 fragment were investigated in a BAC library for *C. arabica* genome. The SCARs were used to hybridize high-density membranes containing DNA from the BAC library constructed by Noir *et al.* (2004). SCAR N2 was not used because the results of Section 5.1 had demonstrated that it is a repeated sequence but A2 was analysed because although it did not amplify specific products, it could be a single copy but the primers were poorly designed. Probes of SCAR DNA were produced by amplification of thawed stock cloning bacterial cultures (prepared in Section 5.1) using universal primers of the cloning vector. The PCR mixtures and conditions were as presented in Section 5.1 for testing positive clones. Then the DNA quality was assessed in 2% agarose gel and its quantity estimated by comparing with lambda standards.

Hybridization followed the methodology described by Sambrook *et al.* (1989). Twenty-five (25) ml of pre-hybridization solution per membrane was prepared by mixing 2.5 ml of Denhardt Reagent (50X), 6.25 ml of SSC (20X), 1.25 ml of SDS (Sodium dodecyl sulphate, 10%), 1.25 ml of KPB (Potassium Phosphate Buffer, pH 6.5, 25 mM), 0.25 ml of herring sperm (10 mg/ml; after denaturation at 95 °C for 10 min). Composition of these reagents is shown in Appendix 3. Before adding herring sperms, the pre-hybridization solution was heated for 10 min at 65 °C. The solution was then added to the membranes each in a hybridization tube and left to hybridize for at least 2 hr at 65 °C in a hybridization incubator (Hybridization oven, Stuart Scientific, UK). The probes were labelled with α-P^{32} dCTP 3000 Ci/mMol (Amersham Biosciences, UK) following Megaprime DNA labelling system (Amersham Biosciences, UK; Appendix 6), denatured and added into the hybridization tubes and left to hybridize overnight at 65 °C. The hybridized membranes were washed in three series of 15 minutes each in SSC 2X-SDS 0.1%, SSC 1X-SDS 0.1% and SSC 0.5X-SDS 0.1%. The washed membranes were placed between plastic paper holders and put into cassettes with the hybridized side up. X-ray films were placed on top, incubated for three days and then developed to reveal the images.

5.4.3.4 Determination of association between RAPD $M20_{830}$ SCAR and the identified markers of CBD resistance.

A random sample of 12 plants (6 plants from each population) was amplified with primers designed from the RAPD marker $M20_{830}$ for CBD resistance identified by Agwanda *et al.* (1997) by radioactive PCR as described in Section 5.1. The results encouraged the samples to be increased by additional 60 plants which included recombinant plants observed from the molecular analysis above and the results were used to evaluate linkage to markers of the T2 fragment.

5.4.3.5 Survey of markers of CBD resistance in various HDT derivatives

Seeds were obtained from twenty-one (21) individual trees of advanced progenies of crosses between *C. arabica* cultivars and Hibrido de Timor derivatives in a germplasm collection maintained at Cicafé (Costa Rica) and one Catimor line (129) from Kenya (Table 9). These materials have been bred using three different accessions of the original Hibrido de Timor collections (832/1, 832/2 and 1343). Due to limited seed availability and germination percentage, only 10 to 25 seedlings were available for inoculation. Seedlings were screened for CBD resistance at CIRAD (Montpellier, France) by dipping into a spore suspension of a pathogenic *C. kahawae* isolate from Cameroon at a strength of 2×10^6 conidia/ml. The seedlings were incubated in moist boxes at 20 °C and symptoms were scored after 14 days on a 0-5 scale. Class 0 had no visible symptoms of infection and the lesions enlarged progressively up to class 5, in which the top halves of the hypocotyls were dead. Classes 0 to 3 were considered resistant while classes 4 and 5 were susceptible. A mean infection score was calculated from the inoculated seedlings and used to classify the accessions as resistant (mean score ≤ 2), moderately resistant (mean score of 3), moderately susceptible (mean score of 4) and susceptible (mean score of 5). Genomic DNA was extracted from lyophilized leaves from the mother plants and amplified with Sat 11, Sat 207 and Sat 235.

In addition, DNA was extracted from four accessions of Hibrido de Timor with field resistance to CBD in Kenya and included in these studies. A search was also made in young F_2 plants of crosses between HDT and either SL28 or K7 that are established in field, but are not yet in full bearing, for plants infected by CLR. Three plants of HDT x SL28 cross and one plant of HDT x K7 cross were obtained. A tree of cv Catimor which was observed to be infected by some CLR and CBD in some years in the field was also included. DNA was extracted from these plants and amplified with the three microsatellites (Sat 11, Sat 207 and Sat 235).

124

5.4.4 RESULTS

5.4.4.1 Analysis of microsatellites

A total of fifty seven (57) microsatellites were screened in fifteen individuals which included five each from the two F_2 populations (D and E), accessions of cvs Catimor line 88, Catimor line 127, Sarchimor line T5296, SL28 and Caturra. Twenty-three of these microsatellites were variously polymorphic either within or between lineages/accessions while the rest were monomorphic. There was also differential polymorphism between the two F_2 populations such that one and three microsatellites were polymorphic only in populations D and E respectively. The most probable candidate marker alleles for CBD resistance were expected to be common in all the Hibrido de Timor derivatives, absent in cvs SL28 and Caturra but polymorphic in both F_2 populations. Seven microsatellites (Sat 11, Sat 41, Sat 162, Sat 172, Sat 207, Sat 235 and Sat 262) satisfied this criterion and were chosen for analysis in the F_2 populations. The microsatellites were analysed in 95 Group 2 plants from population E, fifty-six (56) plants from Group 1 (29 and 27 plants from populations D and E respectively), eight (8) Group 2 plants from population D observed to be infected by CLR in the nursery and (18) Group 2 plants from population D.

During the experiments with enlarged F_2 samples, some microsatellites (e.g. Sat 11; Plate 12) displayed a pattern that was slightly different from the pattern observed during tests for pre-selection and verification of segregation in the F_2 populations (Section 5.2; Plate 8). However the interpretation of genotypes in the various tests was the same, even by comparing amplification of the same samples but of different DNA extractions. Sometimes one allele was observed in the middle band of this microsatellite (Sat 11) in heterozygous plants, but it was evident from homozygous plants that the introgressed allele was slightly bigger (Plant 12 A). Sat 172 also displayed uniform intensity of all its four bands during the pre-selection tests while in the large

sample analysis, the intensity of the two bigger bands was weak (Plate 12 B). It also seems that there could have been size differences in the middle alleles (arrowed) but they could not be reliably separated and scored in different experiments and were therefore scored as one allele. An alternative explanation would be presence of "slippage" products, which may explain the lowest band in the sample marked with an asterix (*) (Plate 12 A). Some other microsatellites (e.g. Sat 41) had 'slippage' products that co-migrated with the lower bands and were fairly strong in some cases where the larger allele was homozygous (Plate 12 C). In heterozygous situations, the top band was weaker but it was always scored as present because there was no evidence of 'slippage' products that were bigger than the main (parent) bands. A unique allele of Sat 41 was observed in one and three seedlings of populations D and E respectively and these plants were concluded to be off-types and excluded in analysis.

A (Sat 11)

B (Sat 172)

    (i)   (ii)

C (Sat 41)
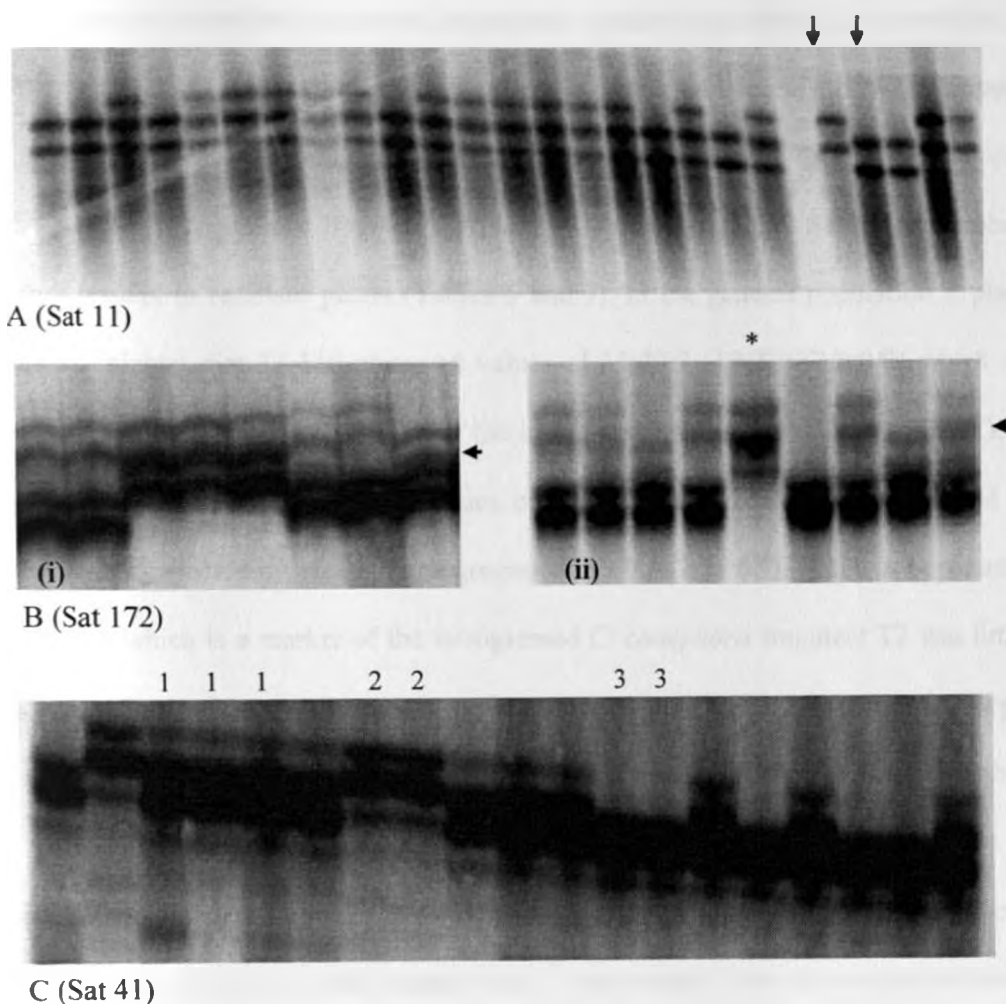
Plate 12. Autoradiographs of three selected microsatellites analysed in an F_2 population of a cross between cvs Catimor (line 88) and SL28 (Population E) demonstrating analytical experiences.

Notes

    (A)   Sat 11 showing a pattern that is different from that in Plate 7. Comparison of homozygous samples (arrowed) reveals that the bands at the middle of the pattern are slightly different in size: the sample on the left is homozygous un-introgressed while the one on the right is homozygous introgressed,

    (B)   Two radiographs from different experiments with Sat 172 (i and ii) showing products which appeared to be polymorphic alleles (arrowed)

    (C)   A pattern of Sat 41 showing 'slippage' products that were smaller than their "parental" bands: (1) heterozygous samples; (2) homozygous samples for the bigger allele and (3) homozygous samples for the smaller allele

127

Six of the microsatellites displayed segregation patterns conforming to Mendelian inheritance while one (Sat 11) was distorted in favour of the presence of the allele that is introgressed from *C. canephora* (Table 6). Introgressed alleles of Sat 11, Sat 207 (which are mapped onto the introgressed *C. canephora* fragments T3 and T2 respectively) and Sat 235 were observed to be highly present in resistant plants (Tables 6 and 7). In the general population E plants (total of Group 2 plants), Sat 11 had observed values of 35:49:9 (T3/T3:T3/0:0/0) which significantly deviated from a 1:2:1 ratio in favour of the introgressed allele (χ^2=14.806; p=0.001). In the same population, Sat 207 had observed values of 21:48:26 (T2/T2:T2/0:0/0) that fitted the ratio of 1:2:1 that is expected by Mendelian segregation (χ^2=0.537; p=0.764). It was apparent that the Sat 207 allele which is a marker of the introgressed *C. canephora* fragment T2 was linked to CBD resistance by its higher presence in resistant plants and higher absence in susceptible plants compared to the general population. The distribution of Sat 11 was not affected by the phenotype of reaction to infection by *C. kahawae* and it displayed the distorted pattern in all sub-populations. The distribution of this microsatellite in the random sample of population D was not clear possibly due to the small sample size. It was evident from the results that Sat 235 which was un-mapped co-segregated with Sat 207 and was linked to CBD resistance (Table 7, Plate 13).
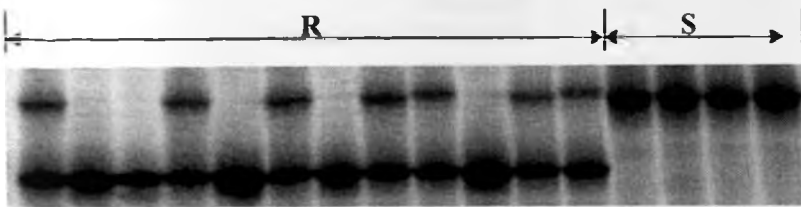
Plate 13. An example of the pattern of Sat 235 in F_2 plants (cv Catimor x cv SL28) that were resistant (R) and susceptible (S) to infection by *C. kahawae*.

Table 6. Summary of the occurrence of two microsatellite markers of two *C. canephora* chromosomal fragments [T2 (A) and T3 (B)] that are introgressed into *C. arabica*, as analysed in two F_2 populations between cv SL28 and two lines of cv Catimor i.e. line 127 (Population D) and line 88 (Population E).

(A) Sat 207 (marker for the introgressed *C. canephora* chromosomal fragment T2)

Population E			Molecular genotype			
	CBD phenotype		**T2/T2**	**T2/0**	**0/0**	**Total**
Group 1	Resistant	Observed	10	15	2	27
		Expected	6.75	13.5	6.75	
Group 2	Resistant	Observed	13	29	5	47
		Expected	11.75	23	11.75	
	Susceptible	Observed	0	5	13	18
		Expected	4.5	9	4.5	
	Others	Observed	8	14	8	30
		Expected	7.5	15	7.5	
	TOTAL	Observed	21	48	26	95
		Expected	23.75	47.5	23.75	
Population D						
Group 2	Resistant	Observed	14	13	1	28
		Expected	7	14	7	
Random		Observed	6	8	4	18
		Expected	4.5	9	4.5	

(B) Sat 11 (marker for the introgressed *C. canephora* chromosomal fragment T3)

Population E			Molecular genotype			
	CBD phenotype		**T3/T3**	**T3/0**	**0/0**	**Total**
Group 1	Resistant	Observed	8	14	5	27
		Expected	6.75	13.5	6.75	
Group 2	Resistant	Observed	14	27	5	46
		Expected	11.5	23	11.5	
	Susceptible	Observed	8	9	1	18
		Expected	4.5	9	4.5	
	Others	Observed	13	13	3	29
		Expected	7.25	14.5	7.25	
	TOTAL	Observed	35	49	9	93
		Expected	23.25	46.5	23.25	
Population D						
Group 1	Resistant	Observed	9	19	1	29
		Expected	7.25	14.5	7.25	
Group 2	Random	Observed	4	11	3	18
		Expected	4.5	10	4.5	

Note: Some discrepancy in the entries of the total number of samples between the two markers was due to failed amplification of some samples in the different PCRs.

Table 7. Percent incidence of markers of three *C. canephora* chromosomal fragments (T2, T3 and T4) in two F_2 populations (Catimor x SL28) screened by inoculation of seedling hypocotyls with *C. kahawae* (Group 1) and in a general sub-population (Group 2)

				C. canephora chromosomal fragments introgressed into *C. arabica*								
				Markers of T2						Markers of T3		Marker of T4
		Number of seedlings	CBD Reaction	Sat 207	AFLP-22	AFLP-33 and 34	AFLP-36	Sat 235		Sat 11	AFLP-23	AFLP-17
Pop 1	Group 1	29	Resistant	96.43	100.00	96.55	96.55	100.00		96.56	93.67	72.67
Pop 2	Group 1	27	Resistant	92.60	100.00	100.00	96.30	100.00		81.48	83.30	69.85
	Group 2*	95	All phenotypes	72.63	nt	nt	nt	73.91		90.32	nt	70.23

Notes

Pop 1: - Population 1 derived from cv Catimor line 127 x cv SL28
Pop 2: - Population 1 derived from cv Catimor line 88 x cv SL28
*   This figure includes seedlings in the intermediate phenotype class (Class 3) and those which were not classified due to low vigour or mechanical damage
nt   not tested
T2 markers Sat 207, AFLP-22, AFLP-33, AFLP-34 and AFLP-36 are arranged in their mapped order by Ansaldi (2003) (Appendix 2)

5.4.4.2 Analysis of AFLPs

All the plants which were analysed by microsatellites above were also analysed with an AFLP primer combination EAAG x MCGT which amplifies a marker of the T4 fragment (AFLP-17) to evaluate if this fragment was related to CBD resistance. This marker was observed to be present in 70.23% of the Group 2 plants suggesting random Mendelian inheritance and did not appear to be affected by CBD phenotype (Table 7). The fragment (T4) was therefore concluded to be unlinked to CBD resistance. This fragment was however conspicuously absent in all plants that had some CLR infection (Table 8). No other polymorphic bands amplified by this AFLP primer combination co-segregated with CBD resistance.

All Group 1 plants (56 plants) were analysed with the AFLP primer combination EACT x MCTT, which amplifies two T2 markers (AFLP-21 and AFLP-22), and one T3 marker (AFLP-23). AFLP-21 was not clear in many samples and was therefore not considered. It was observed that all the samples had AFLP-22 marker even in samples without the introgressed Sat 207 allele (Table 7), while the T3 marker (AFLP-23) displayed the same pattern as the Sat 11 allele. Furthermore, these results demonstrated that the resistant plants of Group 1 that lacked the introgressed allele of Sat 207 were recombinant on one side of this marker. Further analysis of the T2 fragment was done on a selection of 30 plants with AFLP markers that are mapped on both sides of Sat 207. All the resistant plants had T2 markers on one side of Sat 207, except one plant (E102) that did not have any T2 markers on both sides of Sat 207, and was therefore considered as misclassified by young seedlings inoculation method (Group 2) (Table 8, Plate 14). Conversely, two plants of the susceptible samples (from Group 2 of population E), had T2 markers and were considered misclassified. One Group 2 plant from population D (D84) had T2 markers on one side of Sat 207 similar to resistant plants and was also considered most likely to be resistant though it was classified in Class 3 (intermediate category).

There were no recombinants observed in this set of plants on the side of the fragment carrying the resistance gene relative to Sat 207. This necessitated analysis of additional thirty (30) resistant plants that were heterozygous for Sat 207 (20 from Group 1 plants of both populations and 10 plants from Group 2 plants of population E) with three AFLP markers (AFLP-33, AFLP-34 and AFLP-36) which revealed three (3) recombinant plants. Two of these recombinant plants were resistant by hypocotyls method (Group 1), one of which lacked the three markers while the other lacked only AFLP-36. The other recombinant plant belonged to Group 2 and lacked all the markers and this confirmed earlier results that since it had been observed to lack AFLP-22. This result showed that the resistance gene is certainly not beyond AFLP-33 and

Table 8. Ordered AFLP markers and a microsatellite (Sat 207) of the *C. canephora* chromosomal fragment (T2) and one marker each for fragments T3 and T4.introgressed into *C. arabica* analysed on selected F_2 plants obtained from crossings of cv Catimor lines 88 and 127 to cv SL28 (Populations E and D)

	E2	E5	E11	E83	E132	E135	E144	E1	E12	E90	E102	E150	E151	D113	E41	E50	E84	E111	E122	E39	E75	E79	E103	D27	D47	D61	D68	D76	D84	D85
CBD reaction	R	R	R	R	R*	R*	R?*	R	R	R	**R**	R*	R*	R*	S	S	S	S	**S**	S?	S?	**S**	R	S	S	S	S	S?	S?	S?
Class	0	0	1	2	2	0	3	1	1	1	0	2	1	2	4	4	4	4	4	3	4?	4	0	4	4	4	4	3	3	3
AFLP-32	1	1	1	?	1	1	1	0	0	0	0	0	?	0	0	0	0	0	1	0?	0	1	0?	0	0	0	1?	?	0	0
AFLP-28	1	1	1	1	1	1	1	0	1?	0	0	0	1?	0	0	0	0	0	1	1	0	1	1	0	0	0	1	1	0	0
Sat 207(T2)	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	1	0	0	1	0	1	1	1	0	1	0	0	1	1	0
AFLP-22	1	1	1	0?	1	1	1	1	1	1	0	1	1	1	0	0?	0	0	1	0	0	1	1	0	0	0	0	0	1	0
AFLP-38	1	1	1	?	1	1	1	1	1	1	0	1	1	1	0	0	1	?	1	0	1?	1?	1	?	0?	?	1?	0?	1?	0
AFLP-27	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0?	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0
AFLP-33	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0	0	0	0	1	0	0	1	1	0	0	0	0	0	1	0
AFLP-34	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0	0	0	0	1	0	0	1	1	0	0	0	0	0	1	0
AFLP-36	?	1	?	1	1	1	1	1	1?	1	0	1	1	1?	0	0	0	0	1	0	0	1	1	0	0	0	0	0	1	0
Sat 11 (T3)	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	0	1	0	1	0	0	1	1
AFLP-17 (T4)	0	0	1?	0	1	0	1	1	?	1	0	0	1	0	1	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0

Notes

i. * Seedlings of Group 1 and were classified by the hypocotyls inoculation test as resistant, however the disease classification shown in the table is that of young seedlings inoculation method.

ii. Microsatellite data is inform of presence (1) or absence (0) of the introgressed allele and not homo- or heterozygous.

iii. The letter in front of the plant number refers to the populations D (Catimor 127 x SL28) and E (Catimor 88 x SL28).

iv. The shaded samples were observed to have some CLR infection in the nursery.

v. The plants in bold were considered as misclassified for CBD resistance.

vi. ? indicates that the score or classification was not clear/certain
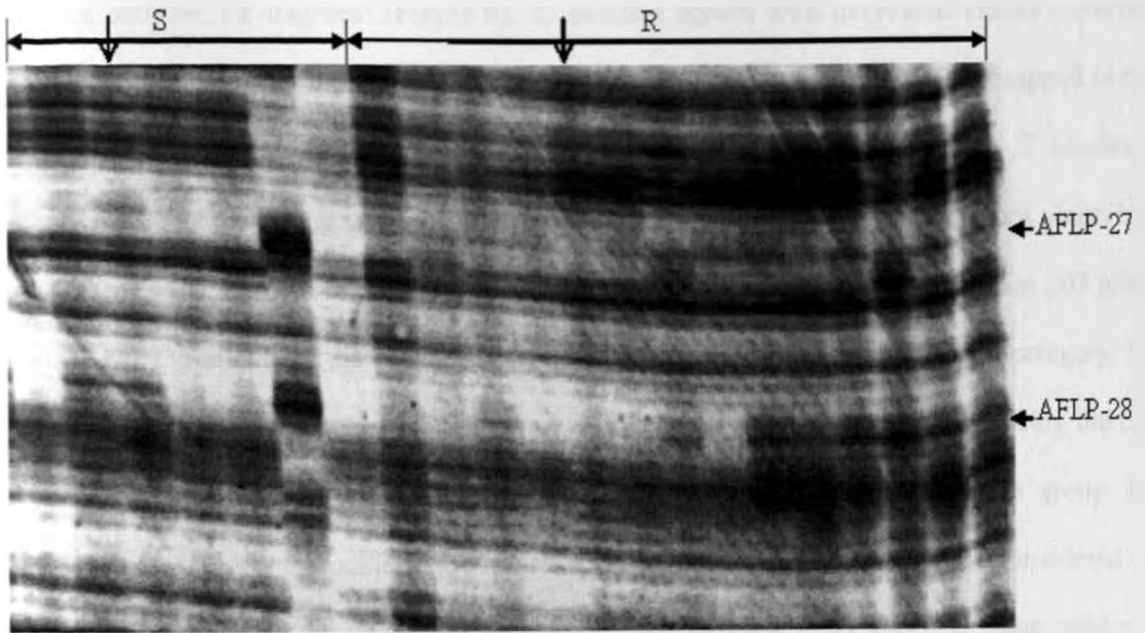
Plate 14. Autoradiograph of AFLP amplification products of selected F$_2$ plants derived from crossing cvs Catimor and SL28, that are resistant and susceptible to infection *by C. kahawae* respectively. Some recombinant resistant plants (R) with AFLP-27 but without AFLP-28 are visible, and vice-versa for one susceptible plant (S). The arrowed plants were misfits that were considered as misclassified by the young seedlings inoculation method.

possibly not beyond AFLP-22, but since this was observed in only one Group 2 plant it could not be taken as confirmatory.

5.4.4.3 Mapping and analysis of Sat 235 data

Sat 235 was mapped by amplification on the same DNA samples that were used by Ansaldi (2003) and analysing the data against the data that she generated. Mapping was done using MapMaker programme Version 3.0b with a LOD score of 5.0 to identify linked markers. Polymorphic AFLP bands were named by the three selective nucleotides of the primer combinations (*Eco*RI followed by *Mse*I) and a letter in increasing order from the largest band except two bands that were named by numbers because they were observed as additional polymorphic bands during mapping and were not observed when screening parents. Sat 235

mapped onto the T2 fragment (Figure 6). Its position agreed with the results earlier observed using the other mapped markers above. It was interestingly observed that Sat 262 mapped in the same position as Sat 172 of Ansaldi (2003). On comparison with population E seedlings screened only by the young seedlings inoculation method (Group 2), four (4) resistant plants had the Sat 207 allele but not the Sat 235 allele, one (1) susceptible plant without the Sat 207 allele had the Sat 235 allele and six (6) recombinant plants were observed in the Class 3 category. By considering resistant plants lacking both markers as misclassified, two plants (including the one reported earlier in Section 5.4.4.2) were identified as misclassified into resistant group. By applying the converse argument to the susceptible plants, four plants were considered as misclassified including the two plants reported in Section 5.4.4.2. By applying these results to those of Sat 207 in Table 5, it was deduced that out of the resistant plants in Group 2 lacking the introgressed allele of Sat 207, two were misclassified while the other three were recombinant. Similarly, of the five susceptible plants with Sat 207 allele, four were misclassified while one (E50) unexpectedly amplified with this allele yet it did not have the flanking AFLP markers (Table 8). This plant was further tested using the same DNA solution to amplify both Sat 207 and Sat 235. Even then, the results showed presence of the introgressed allele of Sat 207 but not the introgressed allele of Sat 235. Double recombination on both of Sat 207 was considered to be the possible explanation.

The percentages of the two markers in the resistant and susceptible plants screened by the young seedlings inoculation method (Group 2) were then recalculated excluding the misclassified plants and the corrected results are shown in Table 9. The resistant plants i.e. Group 1 plants from both Populations and resistant plants of Group 2 from Population 2 (after correction for misclassifications), were compared by genotype ratios of homozygous introgressed: heterozygous: homozygous non-introgressed in regard to Sat 235, and the observed values were

17:12:0, 11:16:0 and 15:26:2 respectively. There was therefore no pronounced favour for the homozygous introgressed genotypes compared to the heterozygous ones especially in Group 2 plants.
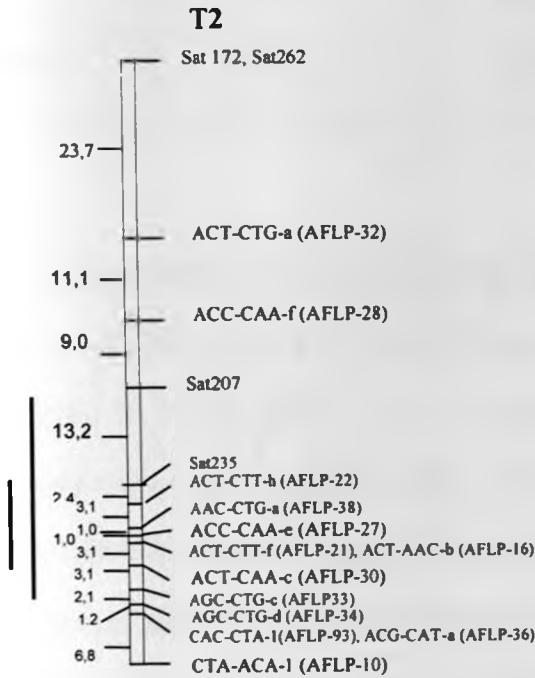
Figure 6. Genetic linkage map of the *C. canephora* chromosomal fragment T2 introgressed into *C. arabica* genome.

Notes: The identities of AFLP markers as named in the map by Ansaldi (2003) are shown in brackets. The values on the left are the distances between the markers in cM. The larger rectangle on the left shown the established limits of the location of the *Ck*-1 locus while the smaller one show the speculated limits (10.6 cM) but further phenotypic confirmation of the indicator plants is required.

Table 9. Percent occurrence of two introgressed microsatellite marker alleles for CBD resistance in seedlings screened by young seedlings inoculation method (Group 2). The   data   is presented before and after correction by exclusion (not transfer) of seedlings which were considered as misclassified. The corrected   values are in parenthesis.

CBD Phenotype	Number of seedlings	Sat 207	Sat 235
Resistant	46 (44)	89.36 (93.02)	91.10 (95.35)
Susceptible	18 (14)	27.78 (7.14)	35.71 (7.14)

5.4.4.4 Analysis of SCARs derived from AFLP markers of T2 fragment

The SCARs N2 (first and second primer designs), W3 and AA4 which were developed from AFLP markers the introgressed *C. canephora* fragment T2 (Section 5.1) were amplified on a set of six resistant and four susceptible plants and revealed in 2% agarose gel. There were no observable differences between the amplification products of SCARs N2-1R and N2-2R and W3. No further investigations were therefore deemed to be necessary in addition to what was done before in Section 5.1. The amplification products of SCAR AA4 appeared to be more intense in samples with the parent AFLP marker (AGC-CTG-c/AFLP-33) than in samples without this marker including one recombinant resistant plant that lacked this marker (Plate 15 A), although one plant with the marker also displayed weak amplification. Further tests at different annealing temperatures and electrophoresis in denaturing poly-acrylamide gel were done to confirm if the difference was due to primer mismatch which can be exploited by altering the annealing temperature or due to multiple products in plants with the parent marker. Reduction of the annealing temperature to 55 °C resulted into PCR products that did not appear to have differences between the two categories of samples (Plate 15 B). At an annealing temperature of 62 °C, only samples with the parent AFLP marker were amplified (Plate 15 C), while at higher

temperature of 64 °C, the intensity decreased and one sample, which had low intensity at 60 °C, did not amplify (Plate 15 D).

When the products were amplified with a radioactive nucleotide and separated in denaturing poly-acrylamide gel, only one band was observed and there was no visual evidence of differential intensity as observed in agarose under the same amplification conditions (Plate 15 E). The marker was designated AGC-CTG-c-$_{AA4}$ in regard to its parental AFLP marker (AGC-CTG-c) and the code AA4 of the clone culture used in primer design. To test its reliability, it was amplified in all the samples amplified with Sat 207 and Sat 235, and electrophoresed in 2% agarose. By comparison with position of the parent AFLP marker (AGC-CTG-c) relative to Sat 235, the SCAR amplified as expected except in four plants that were assumed to be recombinant. Three of these plants had the SCAR but lacked the introgressed Sat 235 allele, while one plant lacked the SCAR but had the introgressed Sat 235 allele.

The multiplicity characteristic of three SCARs (A2, W3 and AA4) derived from three AFLP markers of T2 fragment were investigated by using them as probes to hybridize high-density membranes containing BAC DNA from *C. arabica*. The results are presented in Plate 16. The SCAR AA4 hybridized to seven (7) clones only. W3 hybridized more strongly to 27 clones and weakly to 33 clones while A2 hybridized uncountable colonies showing that it matches very many sequences in the *C. arabica* genome.

A (60°C)   B (55°C)

C (62°C)   D (64°C)

E   A radiograph (60°C)
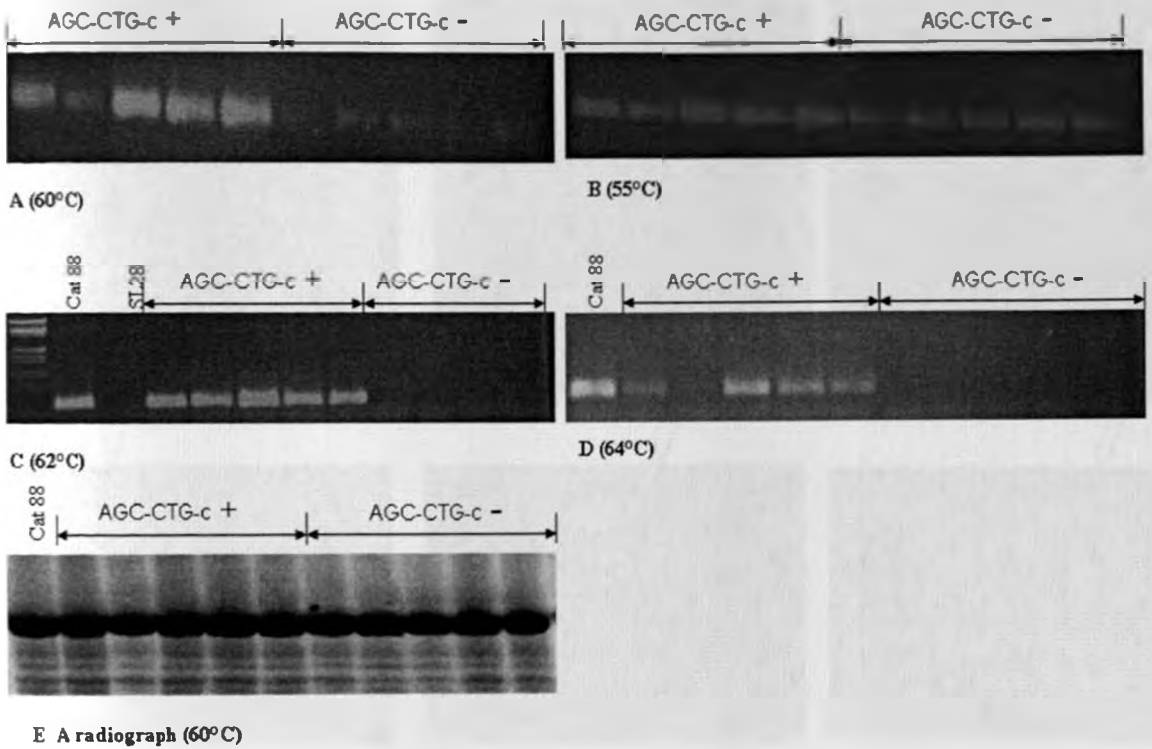
Plate 15. Different PCR products of AGC-CTG-c-$_{AA4}$ SCAR at different annealing temperatures exhibiting temperature dependent polymorphism between plants with the parent AFLP marker (AGC-CTG-c +) and those without the marker (AGC-CTG-c -) in 2% agarose (A, B, C and D). Plate E shows the PCR products of the same samples amplified with radioactive labelling at 60 °C and separated in denaturing polyacrylamide gel.

A (AA4)   B (W3)   C (A2)
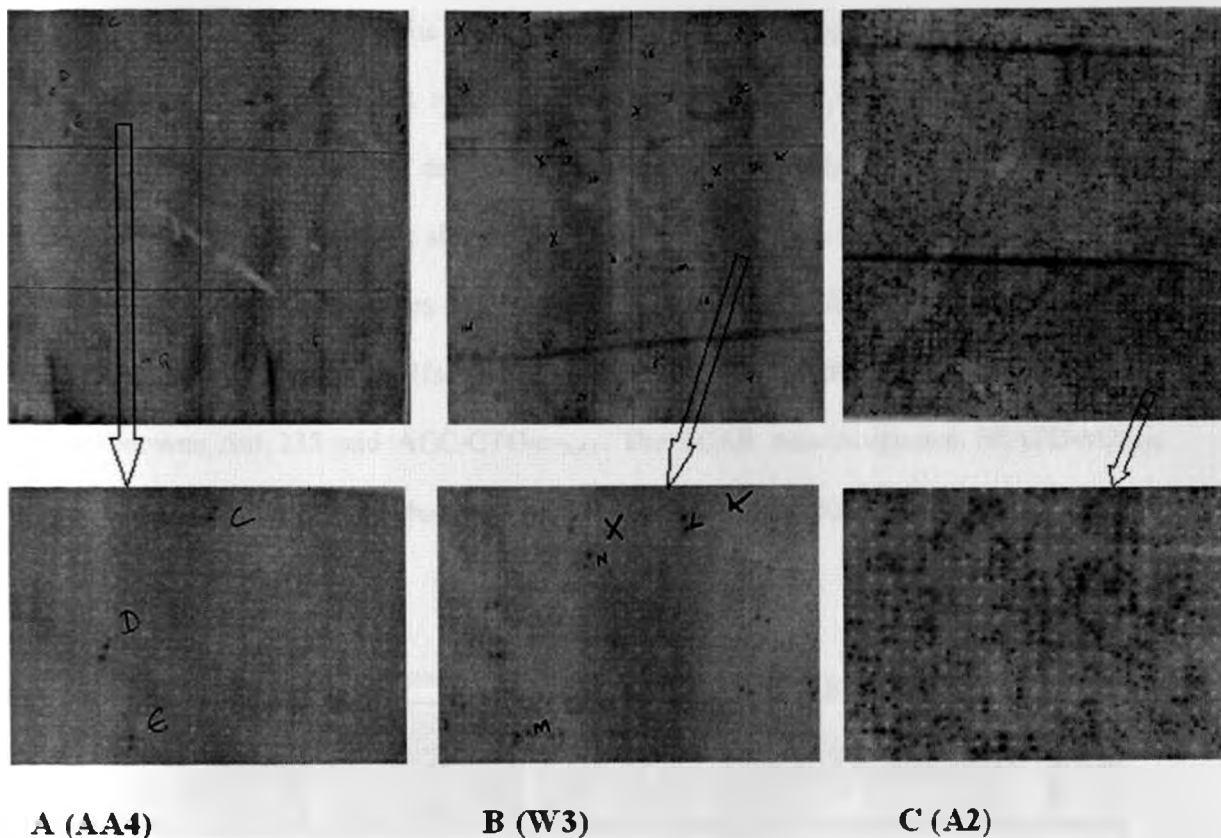
Plate 16. Hybridization patterns of high density membranes spotted with BAC DNA of *C. arabica* genome that were probed with sequences of three AFLP markers of the *C. canephora* chromosomal fragment T2 that is introgressed into *C. arabica*. The lower panel shows close-ups of selected quadrants as indicated by the arrows.

Notes

SCARs AA4, W3 and A2 are designed from AFLP markers AGC-CTG-c (AFLP-33), ACG-CAT-a (AFLP-36) and ACT-AAC-2 (AFLP-16) respectively (Figure 6).

5.4.4.5 Analysis of the SCAR derived from RAPD marker of CBD resistance ($M20_{830}$)

Twelve plants comprising of resistant and susceptible plants from the two F_2 populations were amplified with SCAR primers designed from a RAPD marker for CBD resistance ($M20_{830}$) by radioactive PCR as described in Section 5.1. The amplification results were fair and the sample was increased by sixty additional plants, which included recombinant plants observed from the molecular analysis above. A satisfactory quality of amplification was obtained (Plate 17), unlike what was observed with Ansaldi's population in section 5.1. However the competitive nature of

139

the alleles was still evident and the amplification of either the introgressed allele alone or both alleles did not to a large extent agree with the heterozygous or homozygous introgressed genotypes identified with Sat 207 and Sat 235 (Plate 17). It is interesting to note that this aspect is more pronounced on the right side of this panel. However, the introgressed allele was not amplified in plants lacking markers of T2 fragment and it was therefore considered that where it was not amplified, it was absent. Using this argument on recombinant plants, RAPD-M20$_{830}$ was located between Sat 235 and AGC-CTG-c-$_{AA4}$. The SCAR was designated SRAPD-M20$_{830}$ where 'S' was added to indicate that it is a SCAR from the original RAPD marker M20$_{830}$.
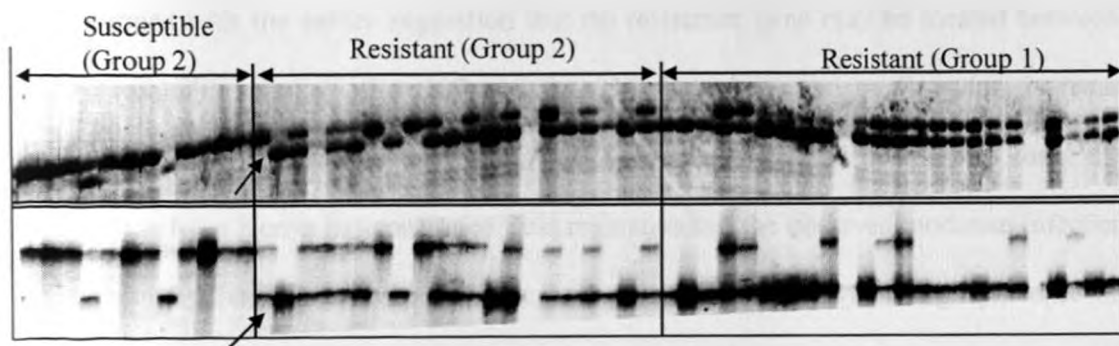
Plate 17. Alignment of radiographic patterns of SRAPD$_{830}$ SCAR marker (top panel) and Sat 235 (bottom panel) amplified on the same panel of F$_2$ plants from a cross of cvs Catimor and SL28.

Notes
1. The lower alleles in each panel are introgressed from *C. canephora*.
2. Many samples on the right amplified two alleles of the SCAR but only one allele of Sat 235 leading to a discrepancy of genetic interpretation. One recombinant plant is arrowed in both panels.
3. The two plants with the introgressed alleles but classified as susceptible were misclassified during the screening test as explained in text.

140

5.4.4.6 Survey of markers of CBD resistance in diverse HDT derivatives

Twenty-two progenies bred from different accessions of Hibrido de Timor (832/1, 832/2 and 1343) were screened for CBD resistance by inoculation of hypocotyls raised from their seeds. DNA from the parents was analysed with two markers of CBD resistance (Sat 207 and Sat 235) and Sat 11 that is a marker of the T3 fragment. It was observed that plants with introgressed alleles of these two microsatellites in homozygous state were either resistant or moderately resistant by their mean scores (Table 10). Plants that were recombinant between the two markers were either resistant or susceptible while two plants (T17940 and T18131) lacked both markers but were rather resistant. Heterogeneity of accessions of cultivars like IAPAR was observed. The results agreed with the earlier suggestion that the resistance gene may be located between the two microsatellite markers (Sat 207 and Sat 235) but phenotype confirmation is required, particularly for the recombinant plants. Of the accessions analysed in this step, only the cv Catimor line from Kenya has confirmed field resistance and the observed moderate infection on it demonstrates the delicateness of this screening method. There was a high presence of T3 fragment even in plants that were susceptible to CBD (Table 10), which further confirmed that this fragment is not linked to CBD resistance. It was also noticed that the accession of T5296 used in this test was susceptible and lacked the markers.

In addition, DNA was extracted from four accessions of Hibrido de Timor with field resistance to CBD in Kenya, one Catimor tree which has been noticed to be infected by some CLR and CBD, and four F_2 plants of crosses between HDT and either SL28 or K7 but infected by CLR were included in these studies. It was observed that the CBD and CLR resistant plants had the introgressed alleles of Sat 207 and Sat 235 (fragment T2) but with or without the introgressed allele of Sat 11 (fragment T3). However, the CLR susceptible plants lacked the introgressed alleles of both microsatellites (Table 10).

Table 10. Occurrence of introgressed alleles of Sat 207, Sat 235 and Sat 11 in different HDT derivatives screened for CBD resistance by hypocotyls inoculation method or observed in the field for CBD and CLR infection

	Accession	Cultivar	Parent CIFC HDT	Origin	CBD [1]	Sat 207[2]	Sat 235[2]	Sat 11 (T3)
1	IAPAR59-43	Sarchimor	832/2	Brazil	MS	h	h	1
2	IAPAR	Sarchimor	832/2	Brazil	MS	1	0	1
3	T5175	Catimor	832/1	Brazil	S	0	0	1
4	T5296	Sarchimor	832/2	Brazil	S	0	0	1
5	T17926	Catimor	1343	Colombia	S	0	0	1
6	T17930	var Colombia	1343	Colombia	R	1	1	1
7	T17931	var Colombia	1343	Colombia	R(?)	h	0	1
8	T17933	Var Colombia	1343	Colombia	MR	1	1	1
9	T17940	Catimor	1343	Colombia	MR/MS	0	0	1
10	T18121	Catimor	832/1	Brazil	MS	0	0	1
11	T18122	Catimor	832/1	Brazil	S	0	0	0
12	T18123	Catimor	832/1	Brazil	MS	0	h	h
13	T18126	Catimor	832/1	Brazil	R	0	1	0
14	T18127	Catimor	832/1	Brazil	S	0	0	1
15	T18130	Catimor	832/1	Brazil	MR/MS	1	0	1
16	T18131	Catimor	832/1	Brazil	MR/MS	0	0	1
17	T18137	Sarchimor	832/2	Brazil	S	0	0	0
18	T18138	Sarchimor	832/2	Brazil	S	1	0	0
19	T18139	Sarchimor	832/2	Brazil	MS	0	0	h
20	T18140	Sarchimor	832/2	Brazil	MR	1	1	1
21	T18141	Sarchimor	832/2	Brazil	MS	h	h	h
22	Catimor 129	Catimor	1343	Kenya	MR	1	1	1
23	Caturra	Caturra	-	Costa Rica	S	0	0	0
i	HDT progeny		1349/269	Kenya	FR	1	1	0
ii	HDT progeny		1349/269	Kenya	FR	1	1	0
iii	HDT progeny		1349/269	Kenya	FR	h	h	h
iv	HDT progeny		1349/269	Kenya	FR	1	1	?
v	Catimor	Catimor	1343	Kenya	CLR/CBD	0	0	0
vi	F$_2$ HDT x SL28		1349/269	Kenya	CLR	0	0	0
vii	F$_2$ HDT x SL28		1349/269	Kenya	CLR	0	0	?
viii	F$_2$ HDT x SL28		1349/269	Kenya	CLR	0	0	0
ix	F$_2$ HDT x K7		1349/269	Kenya	CLR	0	0	?

Notes

[1]   R: resistant, MR: moderate resistant; MS: moderate susceptible; S: susceptible

[2]   1: - homozygous presence of introgressed allele;

    h – heterozygous

    0 – absence of introgressed allele

FR   Field resistant to CBD and CLR

CLR/CBD   Susceptible to CLR and/or CBD in the field in Kenya

5.4.5 DISCUSSION

Different DNA based marker systems were used in this study. Each marker system had different characteristics related to its repeatability and transferability over time, space and facilities. Microsatellites and SCARs are rated as some of the best in these attributes (Rafalski *et al.*, 1996). This however does not mean that they do not exhibit problems in these attributes. For example there were differences in the pattern of some microsatellites in different experiments especially in regard to the number and relative intensity of bands (e.g. Sat 11 in Plates 8 and 12). This could have been due to PCR or electrophoresis conditions or both. However, the genotypic interpretations were the same despite these differences and the extra bands were interpreted as "slippage" products or due to differences in resolution. Some microsatellites such as Sat 41 (Plate 12) had 'slippage' products that co-migrated with major products. Keen evaluation was therefore necessary during scoring of data and mistakes in judgement could have caused some error. Such aspects can create data interpretation problems in the use of microsatellites (Robinson and Harris, 1999) that can even cause inaccurate conclusions like segregation behaviour or un-linkage of a marker to a trait. Another problem encountered was the possibility of 'missed' polymorphism, as might have been the case with Sat 172, in which there appeared to be two alleles that were poorly separated and one was somewhat faint to be reliably scored (Plate 12). This can potentially lead to loss of some information.

The low genetic diversity of *C. arabica* was demonstrated by the fact that out of a total of 57 microsatellites tested; only 23 exhibited any polymorphism at all. Out of these microsatellites, only seven fitted the expectation of candidate markers of the resistance by having alleles that were common in the CBD resistant derivatives of HDT and absent in the susceptible cvs SL28 and Caturra. From the exploratory experiments with candidate microsatellites linked to CBD resistance, it was apparent that the introgressed *C. canephora* allele of Sat 207, that is mapped on

fragment T2 (Ansaldi, 2003; Appendix 2), is linked to resistance to CBD (Table 5). This conclusion was possible after considering all the infection categories of the seedlings after screening the F_2 populations by both the hypocotyls method (Group 1) and young seedlings method (Group 2). This was because from the resistant groups alone, the introgressed *C. canephora* allele of Sat 11 (fragment T3) also appeared to be equally linked to the resistance. However in the susceptible seedlings, T2 was markedly absent while T3 was still highly present. It was also clear that in the intermediate category (Class 3), there was no bias for or against T2 fragment meaning that the presence or absence of this fragment did not determine the appearance of this class. Furthermore, the total score of the two fragments revealed that while T2 fitted the expected distribution by Mendelian laws of inheritance, the presence of T3 was higher in the whole population than would be expected and therefore had segregation distortion. It was not clear whether this marker was equally distorted in population D because even though the resistant plants by hypocotyls inoculation method (Group 1) indicated so, the random sample of eighteen (18) plants did not appear to be distorted, probably due to the small size.

Segregation distortion of some markers is an often-encountered problem in mapping populations. This maybe due to selection of gametes, selective fertilization of particular gamete genotypes or other mechanisms operating during seed development, seed germination or plant growth (Lashermes *et al.*, 2001; Schneider, 2005). For this particular fragment, Ansaldi (2003) did not observe segregation distortion in a cross between a Sarchimor line (T5296) and a wild accession of *C. arabica* from Ethiopia (ET6). This would imply that the segregation distortion was specific to the cross of this study. Segregation distortion is commonly observed in coffee. Pearl *et al.* (2004) observed segregation distortion of 25% of markers in a mapping population between the *C. arabica* cvs Catimor and Mokka. Coulibaly *et al.* (2003) observed that in BC_1 (*C. canephora* x *C. heterocalyx)* x *C. canephora*, sixteen percent of both microsatellite and AFLP markers had

distorted segregation all in favour of the *C. canephora* alleles. Lashermes *et al.* (2000b) noted from their data on HDT derivatives that introgression into *C. arabica* may involve asymmetric chromosome segment exchange mechanisms which indicates the occurrence of atypical genetic mechanisms in these lineages. Marker distortion might also be due to data recording, which can in turn depend on the type of markers e.g., recording of microsatellites with 'slippage' products or null alleles (Robinson and Harris, 1999). The complexity of a fragment may also be a cause of segregation distortion as observed by Nikaido *et al.* (1999) using AFLPs in *Cryptomeria japonica*. The authors were able to reduce or eliminate the distortion by adding more selective bases to the primers. However in this study, both microsatellite (Sat 11) and AFLP (AFLP-23) data displayed the same pattern. It is therefore clear that the distortion was fragment based and not marker specific. Saliba-Colombani *et al.* (2000) reported a similar type of fragment based segregation distortion in tomato in-bred lines.

The method of hypocotyls inoculation method (van der Vossen *et al.*, 1976) is very efficient in isolating a group of resistant genotypes especially when the medium classes are discarded as has been the practice at CRF. No potentially misclassified seedlings were observed in the resistant sub-populations (Group 1) obtained by this method as was the case with young seedlings inoculation method (Sections 5.2 and 5.3). This is supported by the inoculation results of molecular analysis in this Section. However, the method leads to recovery of DNA from only resistant plants and as observed in this study, it would have been difficult to distinguish true markers from distorted makers like T3 (Table 6). The resistant plants obtained by the two methods were valuable counter checking populations during mapping of the DNA markers of resistance. Normally, observation of a few plants of susceptible varieties in resistant classes is taken as an error due to failed infection, whose detection is the objective of including susceptible varieties in inoculation tests. However, some of these plants can be actually resistant whose

presence in the susceptible lot could be due to pollen, seed or seedling contamination during the entire breeding and screening process up to scoring. Three plants with unusual pattern of Sat 41 were observed in population E and one in population D. Such illegitimate plants may have been due to the same reasons. While such atypical plants can affect mapping studies, they are still useful in confirming markers. Other researchers like Coulibaly *et al.* (2003) also observed illegitimate plants in their mapping population and also reported that microsatellites are more efficient in identifying such plants than AFLP.

AFLP analysis was undertaken to define the location of the gene in relation to Sat 207 and increase the number of markers linked to the gene. The initial AFLP test with Group 1 plants proved that all the three seedlings that did not have the introgressed allele of Sat 207, were recombinant on one side of this marker since they all had the marker AFLP-22. The analysis of the 30 selected plants confirmed these results and furthermore demonstrated that three of the four resistant Group 2 plants that lacked the introgressed allele of Sat 207 were similar recombinants. However, the results of this set of plants did not reveal any recombinant plants that would help to identify a limit of the location of the gene on the opposite side to Sat 207. An extra sample of 30 resistant plants (from the two screening methods) were therefore analysed and three 3 recombinant plants were identified. Two plants were of Group 1 and one of them was recombinant from AFLP-33 onwards while the other lacked only AFLP-36. The third plant was from Group 2 and lacked all the markers from AFLP-22. In reference to the molecular results obtained from samples screened by the hypocotyls inoculation method, all the Group 1 plants had the middle segment of the introgressed fragment T2. Judging from the extensive use of the hypocotyls inoculation method, the effectiveness of infection in this study and the molecular results, the possibility of having misclassified plants among the Group 1 plants was negligible. It was therefore conclusive that the gene conferring resistance to CBD in this cross is located

between Sat 207 and AFLP-33 markers, which is a genetic distance of 26.9cM (Figure 6). The AFLP results of the selected plants showed close linkage between Sat 235 and AFLP-22 and this was confirmed by mapping Sat 235 (Figure 6). The distance from Sat 235 to ACT-CAA-c (AFLP–30) is 10.6 cM and it is evident that the gene is located within or just outside this fragment. Since more plants were analysed by the microsatellites, more recombinant plants were revealed. There were four resistant plants of Group 2 that lacked the introgressed allele of Sat 235 but had the introgressed allele of Sat 207. This could suggest that the resistant gene is located between the two microsatellites, which a distance of 13.2 cM, but most likely nearer to Sat 235. A precaution was necessary in that these plants were not analysed for other markers of this fragment for confirmation and that they were screened by the young seedlings inoculation method, which had some errors in terms of misclassification and therefore less confident. Nevertheless, judging from the fact that three out of five plants that were similarly screened and lacked Sat 207 marker allele were recombinant, it is unlikely that all the four plants are misclassified. Ultimate phenotypic confirmation will be available when the plants mature in the field and are assessed by natural infection, artificial field inoculation and hypocotyls inoculation on their progeny.

A pair of primers was designed from the sequence of an AFLP marker (AGC-CTG-c/AFLP-33) and it amplified a SCAR (coded as AGC-CTG-c-$_{AA4}$) that identified the presence of the parent marker in a dominant manner. The primers were sensitive to annealing temperature and the optimum temperature to detect the polymorphism in this study was 62 °C. At lower annealing temperature of 55 °C, there was amplification also in samples without the marker, with no appreciable difference of intensity (Plate 15). For successful use of this marker, pre-testing to optimise and ascertain the difference in amplification is due to genetic factors and not due to technical factors would therefore be required. This is because the temperature regimes of

different thermocyclers might affect the results. In addition, it is necessary to ensure that lack of amplification is not due to technical attributes of the DNA sample. The conversion of AFLP markers into SCAR markers is not often successful as observed in Section 5.1 and by other workers such as Shan *et al.* (1999) in wheat and barley and Diniz *et al.* (2005) in coffee. An interesting observation was that this marker was missed out in Section 5.1 and retrials in this section revealed that there was utilizable polymorphism. This was because in Section 5.1, few samples of each genotypic category were used and the difference in intensity of the amplified product was ignored as a technical aspect. The most effective parameters in optimization of PCR results are annealing temperatures and concentration of Mg^{++} ions (Paran and Michelmore, 1993; Zhang and Stommel, 2001). The success of achieving polymorphism by alteration of annealing temperature depends on the degree of mismatch between the primers and DNA sequences. An optimum is achieved between low temperatures that amplify all samples and higher temperatures that lead to unreliable results as observed in this study. When successful, the conversion of these markers may even yield the more informative co-dominant markers as was observed by Boukar *et al.* (2004) in cowpea. Though AGC-CTG-c-$_{AA4}$ is dominant and therefore less informative than co-dominant markers, it potentially has the advantage of direct revelation after amplification by addition of ethidium bromide into the reaction tubes without the need for electrophoresis as demonstrated by Wang *et al.* (2002). However such a procedure requires the evaluation of the limit of successful detection, so that weakly positive amplifications are not missed out.

The utility of the SCAR marker AGC-CTG-c-$_{AA4}$ is especially high in breeding programmes due to its position in the chromosomal fragment carrying the CBD resistance gene in relation to Sat 207 and Sat 235. Use of the three markers will enable selection of recombinant plants on both sides of the gene, which will be useful both for MAS breeding and collection of recombinant plants that can be used later for chromosome walking. Trials demonstrated that Sat 235 can be

separated in 2% agarose. It is hoped that a simple method for analysis of Sat 207 will also be developed either using agarose or silver staining of polyacrylamide gels to facilitate its routine use in low technology molecular laboratories especially in the developing laboratories.

The multiplicity of sequences of three SCARs from AFLP bands was tested in *C. arabica* genome by hybridization of BAC library of *C. arabica* genome. It was observed that A2 is highly repeated while W3 is less repeated in *C. arabica* genome. The SCAR coded as AA4 hybridized only seven clones, which fits a single copy sequence in this library (Noir *et al.*, 2003). These results agree with those obtained in Section 5.1 meaning that the unclear amplification by primers designed from A2 was most probably not due to poor primer design but multiplicity of the sequence. This means that out of the three, only AA4 would be useful as a starting point for chromosome walking but it might be far from the location of the CBD resistance gene for this purpose.

Although the consistency of amplification of the SRAPD-M20$_{830}$ marker (SCAR amplified with primers designed from the sequence of RAPD marker of CBD resistance identified by Agwanda *et al.* (1997)) was not high, the results clearly demonstrated that it is associated with the resistance and is located on the T2 fragment. The complication appeared to be allelic competition in the presence of the introgressed allele, usually resulting in poor or no amplification of the non-introgressed allele. Amplification in samples used by Ansaldi (2003) was not good enough for clear mapping but association with T2 fragment was demonstrated. Furthermore, the results obtained in the F$_2$ plants in this study indicated that the allele is located between Sat 235 and AFLP-33. The demonstrated association of this SCAR with the identified markers in this study supported the results of Agwanda *et al.* (1997) who had recommended further testing of the markers they identified in a segregating population. At the same time, this association provided

further validation of the markers identified in both studies because the plants used were independently screened for CBD resistance both in the laboratory and field.

Diverse accessions of HDT derivatives (Table 9) were assessed for CBD resistance and analysed with Sat 11, Sat 207 and Sat 235. The results exhibited a correlation between the microsatellite markers and CBD resistance (Sat 207 and Sat 235), despite the limited number of seedlings assessed and the fact that the infection results obtained in this particular test were less stringent than those obtained when screening the F_2 populations. Overall, the results agreed with those from F_2 populations on the supposed location of the resistance gene to be between the two microsatellites, but this is not conclusive. This is because the two plants that lacked the two microsatellite markers but were resistant maybe double recombinants or the gene maybe located beyond Sat 235 on the opposite side of Sat 207. Further confirmatory studies are required. These observations mean that the two markers can be used for MAS across different derivatives of HDT and they also demonstrate high similarity between these progenies. The results further confirmed that the *C. canephora* fragment T3 (Sat 11) is not linked to CBD resistance because it was also present in susceptible plants (Table 10). It was also noticeable that Sat 11 was absent in only 4 of the 21 HDT derivatives analysed, meaning that it is highly present in these material and it will be of interest to focus some attention to this fragment. Observations on the accessions of HDT derivatives from Kenya that were either resistant or susceptible to CBD and/or CLR were similar (Table 10). It was however observed that the CLR susceptible plants were lacked the markers of introgressed fragments tested. This raised the question of whether the introgressed *C. canephora* chromosomal fragment (T3) is involved in CLR resistance. Similar to the results of this study, heterogeneity of the cultivar IAPAR has also been reported by Crochemore *et al.* (2004) using RAPD. There is thus a lot of selection potential within and between cultivars derived from HDT as was also observed in lines cv Catimor in Kenya (Section 5.1)

The results of this study support the action of a major CBD resistance gene in one locus. The number of plants identified by both screening methods was not rigorously skewed towards homozygosity. However, the wide distributions of the phenotypes of the plants suggest deviation from a strict dominant behaviour. This could be genetic, possibly due to action of other genes that may be working through plant vigour, or the complex mechanisms of resistance. The possibility of dosage effects of the resistance gene cannot be ruled out especially by consideration of group 1 plants. Van der Vossen and Walyaro (1980) also described the CBD resistance in HDT to be controlled by one gene (T), of intermediate action and whose expression may be affected by presence of modifying genes. Earlier, van der Vossen et al. (1976) had speculated the action of minor genes even in susceptible varieties. However, it may be argued that several other factors such as pathogenicity of the inocula, environmental factors and seed conditions may have affected some of their results but these factors were highly unified in this study. It was evident that plants of low vigour were particularly susceptible to infection. From the molecular studies, the cv Catimor lines were polymorphic within themselves while the cv SL28 accessions were not. This is in agreement with the results of other researchers (Agwanda et al., 1997; Lashermes et al., 2000b; Steiger et al., 2002; Pearl et al., 2004) who observed higher diversity within HDT derived cultivars than in un-introgressed cultivars. Catimor lines used in the breeding of cv Ruiru 11 have been shown to have differences in specific and general combining abilities with the male parents in regard to CBD resistance (Omondi, 1994). It may be thus postulated that the differences in progenies between these two cultivars is due to the diversity within cv Catimor.

If it is speculated that the ultimate expression of CBD resistance is affected by some other genetic factors, then different progenies can be expected to display variations of absolute reaction types. Although no substantive data was taken, Population D was less vigorous since it

had more tiny plants and fewer tall plants compared to population E. A variation in seedling vigour or physiological state due to seed condition may also explain the difference in the slight differences in results obtained after inoculation of seedlings from the same tree and under same inoculation conditions but of different dates of harvest (Section 5.2). This may not be far fetched, considering the complexity of plants' resistance to infection by *Colletotrichum* (Esquerré-Tugayé *et al.*, 1992, Chongo *et al.*, 2002) and in Coffee-*C. kahawae* interaction in particular (Gichuru *et al.*, 1996; Gichuru, 1997, 2001; Rodrigues *et al.*, 1999; Chen *et al.*, 2004a, b, Silva *et al.*, 2006). The resistance mechanisms operate from pre-germination of the pathogen to its sporulation.

It can be perceived that each plant potentially may have a slightly different genetic constitution, which at the end modifies the final disease reaction. Van der Vossen and Walyaro (1980) observed that as a pure variety, HDT exhibited the same high degree of resistance as Rume Sudan. But after crossing with the susceptible varieties, the resistance appeared to be of intermediate type. The same question could be asked whether the change of genetic environment did affect the expression of the resistance. This variation may be more pronounced in young plants like the seedlings and less noticeable in the field on mature trees. Even the F_1 plants used to raise the F_2 populations for this study displayed the same level of CBD resistance as the parent Catimor lines over the years in the field. This maybe why some authors who have worked with HDT derivatives in the laboratory and field have also described the gene as dominant (Omondi, 1994). It was therefore concluded that the *C. canephora* chromosomal fragment T2 introgressed into *C. arabica* via HDT carries a major gene conferring resistance to *C. kahawae*. The designation *Ck-1* is hereby suggested, as the first mapped locus of resistance to *C. kahawae*. This locus is most likely synonymous to the T locus described by van der Vossen and Walyaro (1980).

For refining the map further in relation to the location of the CBD resistance gene, it is appears more prudent to screen seedlings from segregating populations of HDT derived donors screened by hypocotyls inoculation method of van der Vossen *et al.* (1976). Firstly, the method is more stringent in terms of isolating the resistant phenotype. Secondly, both phenotypic and molecular results can be obtained quite early because DNA can be isolated from the cotyledons or first true leaves after screening for CBD resistance, long before the plants can be analysed by either shoot tips or in field. Thirdly, the method would allow screening of much larger starting populations than either shoot tips or field screening, though more recombinant plants could be potentially lost with the discarded seedlings. However as with shoot tips inoculation method, plants with unique recombinations may require reconfirmation especially by field and/or subsequent progeny analysis. This aspect can be affected by the effectiveness of infection during the screening process.

While the experimental conditions were not designed to suit identification of CLR resistance by for example taking into account race specific interactions and no precise screening was done, some notes can still be advanced from the observations made in this study. There was high severity of CLR in the nursery where the seedlings were raised and all the seedlings of cv Caturra were severely infected. However, only a few seedlings of the F_2 populations, (nine from Population D and four from Population E), were observed to be infected by CLR. It was also visually clear that infection on these seedlings was less severe than on cv Caturra. Out of the eleven CLR infected plants tested by molecular markers, four had T2 fragment, seven had T3 fragment and none had T4 fragment. The conspicuous absence of T4 in these plants makes it a likely candidate for CLR resistance. As per previous breeding programmes, plants selected for CBD resistance normally also exhibit CLR resistance. Three of the four CBD susceptible plants that lacked T2 and were not infected by CLR had the T4 marker tested (Table 7) and the other

one lacked both fragment at as far as the tested markers are concerned. From these observations, an opinion can be formed that at least two *C. canephora* fragments may be involved in CLR resistance in Kenya i.e. T2 and T4. This may explain why selection for CBD resistance leads to simultaneous retention of CLR resistance. The absence of T4 in some CLR infected plants that had T2 may prompt the hypothesis that T4 confers resistance to all CLR races present in the location but there are some races that are able to exhibit some infection on plants protected by T2 only. There would therefore be a high pressure for the CLR pathogen to overcome resistance that is linked to CBD resistance, which is more prevalence in breeding materials that are selected for CBD resistance.

The involvement of T3 cannot be ruled out whether as major or minor. In fact such a function, along side possible general segregation distortion in its favour across progenies, may help in explaining why it is highly present in the cv Catimor as demonstrated in section 5.1 and in Table 10. Coincidentally, the seedling (E111) which lacked the other fragments had the fragment T3. The nursery where the seedlings were raised has been used for a long time to propagate breeding materials (some up to maturity) in different breeding programmes and therefore challenge to the pathogen by different assortments of genotypes can be anticipated. In the past, some infection has been observed on HDT derivatives but efforts to determine the races in collaboration with Coffee Rusts Research Centre (Portugal) have not been conclusive (Gichuru, 2005; Gichuru and Varzea, unpublished data). The failure to identify races of the isolates maybe due to their genotypes, inappropriate differential host ranges or the fact that recovery of viable spores from the lesions has been low.

In conclusion, a locus carrying a major gene locus (*Ck-1*) for resistance to infection by *C. kahawae* is definitely localised within a 26.9 cM segment of a *C. canephora* chromosomal

fragment introgressed into *C. arabica*, and possibly within distance of 10.6 cM or just outside the limits of this section. More certain and fine localization will be available once the analysed recombinant plants mature in the field at CRF. Microsatellites 207 and 235 will be very useful in MAS for this resistance especially if they can be analysed in agarose, which would make them of practical application in low technology molecular laboratories such as the one at CRF. Sat 235 was observed to be separable in 2% w/v agarose and it differentiated the parents and heterozygous plants. A dominant SCAR maker derived from an AFLP marker (AGC-CTG-c/AFLP-33) was also developed and designated as AGC-CTG-c-$_{AA4}$ and it will be very useful alongside the microsatellites. The distribution of these markers spans the *Ck-1* locus making their co-utilization of special interest in MAS breeding and selection of recombinant plants for further studies of the mapped region.

SECTION 5.5 VARIABILITY OF MICROSATELLITES AND SCARS RELATED TO GENOMIC INTROGRESSION FROM *C. canephora* INTO *C. arabica.*

5.5.1 INTRODUCTION

Microsatellites have characteristic genomic distribution and motif dependent dispersion in the genome with most of them being concentrated in centromeric chromosomal regions (Schmidt and Heslop-Harrison, 1996; Li *et al.*, 2002). It also appears that their expansions and contractions are restricted by counter selection, at least for some loci, because of their effects on aspects such as chromatin organization, regulation of gene activity, recombination, DNA replication, cell cycle, and mismatch repair system (Li *et al.*, 2002). Microsatellites and their flanking DNA sequences are rarely conserved in a whole genus, leave alone other genera in the family, although some may even be conserved across a genus (Hale *et al.*, 2005).

However, microsatellites developed from one *Coffea* species can be transferable to other species and even in the closely related genus *Psilanthus* (Combes *et al.*, 2000; Coulibaly *et al.*, 2003; Poncet *et al.*, 2004). For example, Poncet *et al* (2004) observed that 72.7 to 86.4% of 110 microsatellite primer pairs developed from *C. arabica* amplified in other *Coffea* species. Microsatellites also vary in the number of alleles in different *Coffea* species. Moncada and McCouch (2004) observed that diploid *Coffea* species averaged 3.6 alleles per microsatellite locus while wild tetraploid *C. arabica* averaged 2.5 alleles per locus and cultivated *C. arabica* had only 1.9 alleles per locus. In addition, 55% of the alleles found in wild *C. arabica* accessions were not shared with the cultivated genotypes. They also observed that the accessions of HDT in their study resembled *C. arabica* cultivars more than *C. canephora* accessions. On the other hand, Anthony *et al.* (2002b) identified four microsatellite alleles related to HDT introgression and observed closer similarity between the introgressed *C. arabica* lines and *C. canephora* from Central Africa than between them and a *C. canephora* accession from West Africa. Poncet *et al.*

(2004) observed a maximum of 9 and 8 alleles per locus in *C. canephora* and *C. pseudozanguebariae* respectively. The two species shared 30 polymorphic loci, which could indicate microsatellite evolution with shared ancestry.

Samples showing only one microsatellite allele are usually considered to be homozygous and this omits occurrence of null alleles. In *C. canephora* and *C. pseudozanguebariae*, Poncet *et al* (2004) observed more than 3 alleles per polymorphic locus and estimated null allele percentages of -9% and -11% respectively. In maize, Matsuoka *et al.* (2002) observed modest rates (less than 5%) of null phenotypes when analysing microsatellites derived from maize in diploid *Zea* spp. Microsatellite loci may also be duplicated in a genome but the duplicated loci may or may not amplify depending on conservation of the primer binding sites. Coulibaly *et al.* (2003) observed two microsatellite loci which were duplicated in both *C. canephora* and *C. heterocalyx*, and they also observed that unlike AFLPs, SSRs are not clustered and are randomly distributed in the coffee genome. Matsuoka *et al.* (2002) observed evidence of possibly duplicated microsatellite alleles in *Zea* spp. These workers observed amplification of more than two products in a plant at low frequencies (1.8% for teosinte and 0.02% for maize landraces) which could have been due to duplicated alleles, contamination of PCR or some other types of error such as inter well leakage. Such results may be treated as missing data and thus eliminate bias.

Between species, a given microsatellite may have different genomic location and therefore be subjected to different evolutionary forces. Diversity of microsatellites may also differ in relation to the focal species (from which they were developed). Hale *et al.* (2005) observed that there were generally more repeats in the focal species of the genus *Clusia* than in non-focal ones. Although they tested only 3 microsatellites, it seemed likely that there is a relationship between the size of the microsatellite and polymorphism. The diversity of microsatellites may also be

affected by factors other than the number of repeat units. By sequencing the amplification products, Matsuoka *et al.* (2002) observed that variability of microsatellite loci in *Zea* species was not restricted to repetition of the motifs but also included indels (insertions and deletions) in the regions flanking the repeat motifs. They demonstrated that 40 out of 46 microsatellites had allele distributions that did not strictly adhere to the simple model of allelic variation based on changes in the number of repeats. This high level of occurrence of indels prompted the authors to suggest the term Indel-Rich Regions (IRRs) to describe the maize microsatellites. Hale *et al.* (2005) reported that variability of microsatellite loci in *Clusia* species was affected by stepwise mutations and indels. Microsatellite data may differ with the method of analysis due to differences in sensitivity of detection or separation. Poncet *et al.* (2004) reported a discrepancy between amplification observed in agarose and that observed in fluorescent analysis in acrylamide gel, such that some samples that had detectable products in agarose were negative in the poly-acrylamide tests.

Microsatellites can be viewed as SCARs with highly variable segments in between the primer annealing sites. Other types of SCARs e.g. those developed from AFLP bands lack such a highly variable segment and are expected, at least in theory, to be less polymorphic. Poncet *et al.* (2005) tested the variability of 14 SCAR primers developed from AFLP markers specific to *C. pseudozanguebariae* and they were observed to amplify only one band with a size similar to the parental band in other coffee species. However there were some null phenotypes but the cross transferability was high with a minimum of 58%. They further observed that amplification in *C. arabica* was a juxtaposition of the patterns of its putative parental diploid species (*C. canephora* and *C. eugenioides*) and that the SCARs did not conserve the polymorphism of parental AFLP bands. The characteristics of a marker system including null alleles, low or lack of polymorphism, hyper-polymorphism and duplication in the genome present experimental

challenges such as their use in MAS or in chromosome walking. In this study, the amplification characteristics of microsatellites and SCARs derived from AFLP markers of *C. canephora* chromosomal fragments introgressed into *C. arabica* via HDT was assessed.

5.5.2 OBJECTIVE

To assess the diversity of microsatellites and SCARs derived from AFLP markers of chromosomal fragments introgressed from *C. canephora* into *C. arabica*, in *C. arabica* and its putative parents.

5.5.3 MATERIALS AND METHODS

Ninety-one (91) accessions were used in this study to represent diversity within and between *C. arabica*, its putative parents i.e. *C. canephora* (and its close relative *C. congensis*) and *C. eugenioides* (and its close relative *C. anthonyi*) (Table 11). DNA from these accessions was extracted from lyophilized leaves as explained in section 5.1. The leaves were obtained from plants maintained in five research centres namely: - IRD (Montpellier, France), CRF (Kenya), CATIE (Costa Rica), CICAFÉ (Costa Rica) and CIFC (Portugal). The samples were analysed with seven (7) SCARs developed from AFLP markers of *C. canephora* chromosomal fragments introgressed into *C. arabica* via HDT (developed in Section 5.1) and eighteen (18) microsatellites that were found to be associated with this introgression in Section 5.3 (Table 11). The SCARs were analysed radioactively as in Section 5.1. Microsatellites were analysed by "tailing" the 5' end of the forward microsatellite primer with the universal primer M13 (5'-CACGACGTTGTAAAACGAC-3') that was labelled with either of the infrared dyes IRD700 or IRD800 as described by Poncet *et al* (2004). Amplification was in 20 µl PCR reaction mixtures consisting of 1X reaction buffer (Promega), 2.0 mM of $MgCl_2$ 0.2 mM of dNTPs, 0.2 µM of forward primer tailed with M13 (Eurogentec, Belgium), 0.2 µM of reverse primer (Eurogentec,

Belgium), 0.2 μM of M13 primer labelled to either IRD700 or IRD800 infrared dyes (MWG-Biotech AG, Ebersburg, Germany), 0.02 U/μL of *Taq.* polymerase (Promega) and 20 ng of genomic DNA. After amplification with the PCR conditions described in Section 5.2, the samples were diluted two times with a loading buffer (LICOR loading buffer 2X, Appendix 3) and stored at 4 °C covered with aluminium paper to avoid exposure to light until when used for electrophoresis. For separation, the samples were denatured at 95 °C for 5 minutes and about 1 μl was loaded in a 25 cm gel of 6.5% *w/v* KBPlus (LI-COR, catalogue No. 827-05607) and electrophoresed using a LI-COR® DNA Analyser, Global edition IRD^2 system (LI-COR Inc., Nebraska, USA). The digital images obtained were scored visually as present (1) or absent (0).

Table 11. Accessions of *C. arabica, C. canephora, C. congensis, C. eugenioides* and *C. anthonyi* analysed with microsatellite markers and SCARs derived from AFLP markers for chromosomal fragments introgressed from *C. canephora* into *C. arabica*.

Serial No.	Code/Access No	Species	Genetic group	Source	Serial No.	Code/Access No	Species	Genetic group	Source
1	AR-02-06	C. arabica	Wild	Ethiopia (Wild)	25	BB59	C. canephora	Congolese	Central Africa (Wild)
2	AR-03-05	C. arabica	Wild	Ethiopia (Wild)	26	BC58	C. canephora	var Nana coffee	Central Africa (Wild)
3	AR-06-05	C. arabica	Wild	Ethiopia (Wild)	27	BC60	C. canephora	var Nana coffee	Central Africa (wild)
4	AR-08-06	C. arabica	Wild	Ethiopia (Wild)	28	BD68	C. canephora		Cameroon (wild)
5	AR-13-06	C. arabica	Wild	Ethiopia (Wild)	29	BD69	C. canephora		Cameroon (wild)
6	AR-15-05	C. arabica	Wild	Ethiopia (Wild)	30	S15-2	C. canephora	Gamey	Guinea (Wild)
7	AR-27-05	C. arabica	Wild	Ethiopia (Wild)	31	Gui-2	C. canephora		Guinea (Wild)
8	AR-30-PG-ABR-27-05	C. arabica	Wild	Ethiopia (Wild)	32	CA58	C. congensis		Central Africa (Wild)
9	AR-34-06	C. arabica	Wild	Ethiopia (Wild)	33	CB65	C. congensis		Cameroon (Wild)
10	AR-36B-05	C. arabica	Wild	Ethiopia (Wild)	34	CC54	C. congensis		Congo (wild)
11	AR-40-06	C. arabica	Wild	Ethiopia (Wild)	35	DA54	C. eugenioides		Kenya (Kimilili, Wild)
12	AR-50-06	C. arabica	Wild	Ethiopia (Wild)	36	DA63	C. eugenioides		Kenya (Kimilili, Wild)
13	AR-60-06	C. arabica	Wild	Ethiopia (Wild)	37	CRF 4	C. canephora		Kenya
14	CRF 1	C. arabica	cv SL28	Kenya	38	OD65	C. anthonyi	Moloundou population	Cameroon(Wild)
15		C. arabica	cv Caturra	Costa Rica	39	CRF 5	C eugenioides		Kenya (Nandi hills, wild)
16		C. arabica	cv Caturra	Costa Rica	40	OE56	C. anthonyi	Souanke population	Congo (Wild)
17	RS 5/21/4	C. arabica	var Rume Sudan	Kenya	41	CRF 2	C. arabica	Catimor 88/1343	Kenya ex Colombia
18	K7.2S	C. arabica	cv K7	Kenya	42	CRF 3	C. arabica	Catimor 127/1343	Kenya ex Colombia
19	K7.2R	C. arabica	cv K7	Kenya	43	(K)107	C. arabica	(Catimor x SL28) BC1F2	Kenya
20	IF200-DH 06	C. canephora	cv IF200	Cote d'Ivoire	44	(K)119	C. arabica	(Catimor x SL28) BC1F2	Kenya
21	IF200-DH318	C. canephora	cv IF200	Cote d'Ivoire	45		C. arabica	Catimor 129/1343	Zambia ex Colombia
22	BA53	C. canephora	Guinea	Cote d'Ivoire (Wild)	46		C. arabica	Catimor 86/1343	Kenya ex Colombia
23	BA55	C. canephora	Guinea	Cote d'Ivoire (Wild)	47		C. arabica	Catimor 90/1343	Kenya
24	BB56	C. canephora	Congolese	Central Africa (Wild)	48	IAPAR59-43	C. arabica	Sarchimor/832.2	Brazil

Table 11 continued

Serial No.	Code/Access No	Species	Genetic group	Source	Serial No.	Code/Access No	Species	Genetic group	Source
49	IAPAR	C. arabica	Sarchimor/832.2	Brazil	71	T17930	C. arabica	var Colombia/ 1343	Colombia
50	T3751	C. arabica	Nemaya	Costa Rica	72	T17931	C. arabica	var Colombia/1343	Colombia
51	T4389	C. arabica	Hibrido de Timor	Timor	73	T17932	C. arabica	var Colombia/1343	Colombia
52	T5159	C. arabica	Catimor 832/1	Brazil	74	T17933	C. arabica	Colombia/1344	Colombia
53	T5175	C. arabica	Catimor/832.1	Portugal	75	T17934	C. arabica	Catimor/1343	Colombia
54	T5296	C. arabica	Sarchimor/832.2	Portugal	76	T17936	C. arabica	Catimor/1343	Colombia
55	T5321	C. arabica	Catimor 832/1	Brazil	77	T17937	C. arabica	Catimor/1343	Colombia
56	T8666	C. arabica	Catimor/832.1	Brazil	78	T17938	C. arabica	Catimor/1343	Colombia
57	T12835	C. arabica	Catimor/832.1	Mexico	79	T17940	C. arabica	Catimor/1343	Colombia
58	T12856	C. arabica	Sarchimor/832.2	Brazil	80	T18121	C. arabica	Catimor/832.1	Brazil
59	T13229	C. arabica	Catimor 832/1	Brazil	81	T18122	C. arabica	Catimor/832.1	Brazil
60	T14718	C. arabica	Sarchimor/832.2	Brazil	82	T18123	C. arabica	Catimor/832.1	Brazil
61	T16785	C. arabica	Sarchimor/832.2	Brazil	83	T18126	C. arabica	Catimor/832.1	Brazil
62	T16786	C. arabica	Sarchimor/832.2	Brazil	84	T18127	C. arabica	Catimor/832.1	Brazil
63	T17531	C. arabica	Sarchimor/832.2	Brazil	85	T18130	C. arabica	Catimor/832.1	Brazil
64	T17543	C. arabica	Sarchimor/832.2	Brazil	86	T18131	C. arabica	Catimor/832.1	Brazil
65	T17924	C. arabica	Catimor/1343	Colombia	87	T18137	C. arabica	Sarchimor/832.2	Brazil
66	T17925	C. arabica	Catimor/1343	Colombia	88	T18138	C. arabica	Sarchimor/832.2	Brazil
67	T17926	C. arabica	Catimor/1343	Colombia	89	T18139	C. arabica	Sarchimor/832.2	Brazil
68	T17927	C. arabica	Catimor/1343	Colombia	90	T18140	C. arabica	Sarchimor/832.2	Brazil
69	T17928	C. arabica	Catimor/1343	Colombia	91	T18141	C. arabica	Sarchimor/832.2	Brazil
70	T17929	C. arabica	Catimor/1343	Colombia					

5.5.4 RESULTS

Ninety one (91) accessions of *Coffea* species consisting of *C. arabica*, its putative parents namely *C. canephora* (and its close relative *C. congensis*) and *C. eugenioides* (and its close relative *C. anthonyi*) (Table 11) were analysed with eighteen (18) microsatellite markers of introgression via HDT and seven (7) SCARs developed from AFLP markers of *C. canephora* fragments that are introgressed into *C. arabica*. Out the eighteen microsatellites analysed, four were not finally considered due to faint signals, poor resolutions or lack of amplification in many samples that could not be explained by null alleles. Different amplification characteristics of the microsatellites and SCARs were observed in the different *Coffea* species and the two marker systems namely microsatellites and AFLP-derived SCARs. Un-introgressed *C. arabica* accessions had either one or two microsatellite alleles that demonstrated high level of homogeneity (Plate 18). It was observed that in cases where *C. arabica* had two alleles per accession, there was amplification in all the species analysed. It was further observed that in such cases, there was either intersection of the alleles in the different species (Plate 18 A) or one allele of *C arabica* was similar to alleles of *C. canephora* and *C. congensis* while the other was similar to those of *C. eugenioides* and *C. anthonyi* (Plate 18 B). In cases where the un-introgressed *C. arabica* had one allele per accession, there was no amplification in all or most accessions of *C. eugenioides* and *C. anthonyi* accessions. In some cases, the alleles amplified in *C. canephora* and *C. congensis* spanned those in *C. arabica* (Plate 18 C) while in other cases there was clear distinction between the alleles in *C. arabica* on one side and *C. canephora* and *C. congensis* on the other side (Plate 18 D)

There were no null alleles observed in amplifications with the SCARs but there were species specific bands, as was also observed in section 5.1 with the same SCARs, and in many cases *C. canephora* and *C. congensis* had the same band, *C. eugenioides* and *C. anthonyi* had another

163

band while C. *arabica* combined the two. In other cases, all the accessions had the same band(s). The most polymorphic SCAR was J3 in which C. *canephora* had alleles spanning all the other species (Plate 19), but one C. *canephora* accession had three alleles of this SCAR. The maximum number of microsatellite alleles was seventeen and the minimum was three for Sat 227 and 255 respectively (Table 12). On the other hand, the maximum SCAR alleles were five and the minimum was one for SCARs J3 and G3 respectively (Table 12). It was further noted that the markers mapped onto the C. *canephora* chromosomal fragment T3 were among the least polymorphic. C. *canephora* had the highest number of alleles which might be attributed to both heterogeneity and the number of accessions used. For example all the alleles of Sat 254 were observed in C. *canephora*. However for Sat 280, C. *arabica* had more alleles than C. *canephora*. The least polymorphic group was C. *eugenioides/C. anthonyi*. The un-introgressed C. *arabica* accessions as a group had more alleles than the introgressed ones despite the introgressed accessions having extra alleles due to the introgression.

164
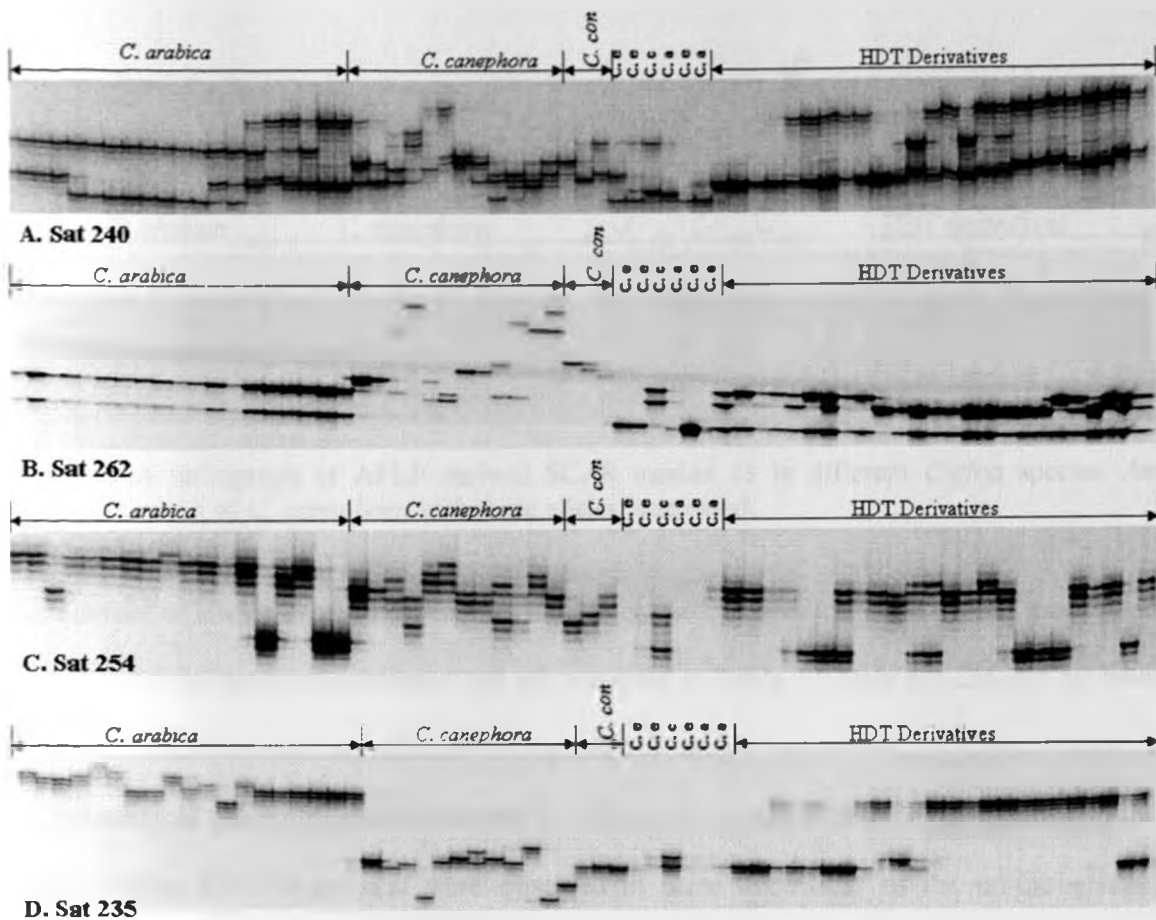
Plate 18. Banding patterns of four selected microsatellites in different *Coffea* species showing amplification patterns depicting either presence or absence of specificity to the constitutive sub-genomes in *C. arabica* (C^a and E^a).

NB: The samples are serialised from 1 to 64 as in Table 10.

Key:-   *C. con:- C. congensis*   *C. c:- C. canephora*

        C. e:- C. eugenioides   *C. a:- C. anthonyi*
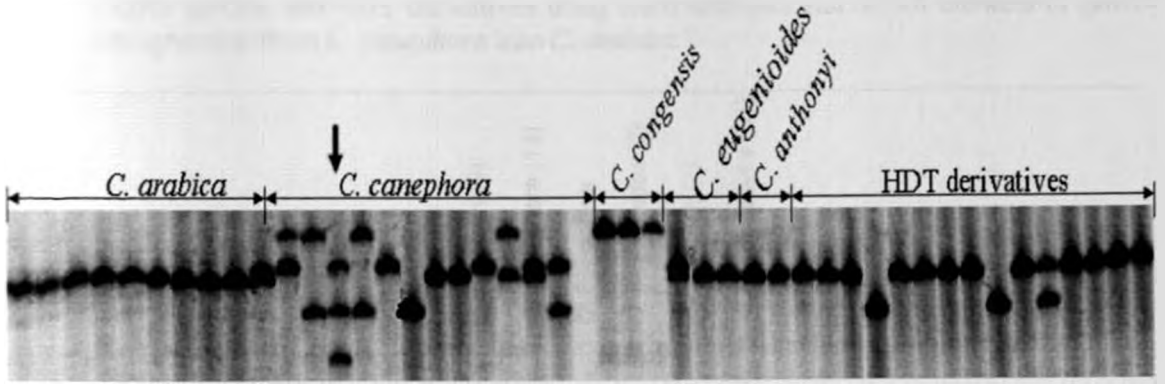
Plate 19. A radiograph of AFLP derived SCAR marker J3 in different *Coffea* species. An accession of *C. canephora* with three alleles is arrowed.

The number of alleles shared between the un-introgressed *C. arabica*, other *Coffea* species and the HDT derivatives were calculated and are presented in Table 12. Only Sat 235 did not have any allele shared between any of the un-introgressed *C. arabica* accessions and the accessions of the canephoroid group (*C. canephora* and *C. congensis*). In some cases, alleles similar to the marker alleles for introgression were observed in some accessions of the un-introgressed accessions of *C. arabica,* as can be seen with Sat 254 in Plate 18 C (the lowest allele). It was observed that one accession of *C. arabica* cv Caturra (Cenicafé) had markers of introgressed fragment T1 including Sat 32 and SCAR J3, which indicated the possibility that this accession was either contaminated or mislabelled/misidentified either in the field or laboratory. In all cases, the genotypes of the HDT derivatives could be constituted by a combination of alleles observed in *C. arabica* and the canephoroid group. The alleles of HDT shared with the eugenioid group (*C. eugenioides* and *C. anthonyi*) were all observed in the un-introgressed *C. arabica* accessions. In HDT derivatives, only one of their alleles was replaced by the introgressed allele even where there more than one allele was amplified per accession of the un-introgressed *C. arabica*. In cases such as with Sat 262 (Plate 18 B) where one of the alleles appeared to be of the eugenioid sub-genome (E^a), this allele was not replaced by the introgressed allele.

Table 12. Tabulation of the number of alleles amplified and shared between accessions of five *Coffea* species and HDT derivatives using microsatellites and SCAR markers of genetic introgression from *C. canephora* into *C. arabica*.

	Total alleles	1. C. arabica (19)	2. C. canephora (13)	3. C. congensis	4. C. eugenioides (3)	5. C. anthonyi (2)	6. HDT derivatives (51)	A. 1 x 2	B. 1 x 3	C. 1 x 4	D. 1 x 5	E. 1 x 6	F. 2/3 x 6	G. 4/5 x 6
Sat 32 **(T1)**	10	5	6	2	3	1	4	2	2	2	1	3	2	2
F1 **(T1**, AFLP-24)	3	2	2	1	1	1	2	1	1	1	1	2	1	1
J3 **(T1**, AFLP-8)	5	1	5	1	3	1	2	1	0	1	0	1	2	2
Sat 262 **(T2)**	11	2	9	3	1	0	3	1	0	1	0	2	2	1
Sat 207 **(T2)**	9	2	7	3	1	1	3	1	0	1	1	2	2	1
Sat 235 **(T2)**	14	6	8	2	0	0	4	0	0	0	0	2	2	0
AA4 **(T2**, AFLP-33)	3	3	3	2	3	2	3	3	2	3	2	3	3	3
N2-1R **(T2**, AFLP-93)	4	4	3	3	4	4	4	3	3	4	4	4	3	4
S3 **(T3**, AFLP-12)	2	2	2	2	2	2	2	2	2	2	2	2	2	2
U2 **(T3**, AFLP-12)	2	2	2	2	1	1	2	2	2	1	1	2	2	1
Sat 11 **(T3)**	4	1	3	3	1	0	2	1	1	0	0	1	2	0
Sat 255 **(T3)**	3	1	3	3	0	0	2	1	1	0	0	1	2	0
G3 **(T5**, AFLP-25)	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Sat 27	6	3	4	2	3	0	2	2	1	3	0	2	2	2
Sat 225	14	5	10	2	2	1	6	2	1	0	0	2	4	1
Sat 227	17	6	11	3	0	0	3	1	2	0	0	2	1	0
Sat 237	9	3	5	2	2	1	3	1	2	1	0	3	2	1
Sat 240	16	6	11	4	2	2	4	2	0	2	1	3	2	0
Sat 254	9	5	9	3	0	0	3	5	1	0	0	3	3	0
Sat 268	12	3	10	3	4	2	3	2	1	1	1	3	2	2
Sat 280	15	8	7	2	3	2	6	2	1	1	2	4	4	2

Legend:
1. Columns A to E show the number of alleles that are common between *C. arabica*, the other species and HDT derivatives.
2. The number of accessions analysed per species/category are in brackets
3. Columns F and G show the number of alleles shared between HDT derivatives and canephoroid species (*C. canephora* and *C. congensis*); and HDT derivatives and eugenioid species (*C. eugenioides* and *C. anthonyi*) respectively.
4. Mapped markers are ordered as mapped and the introgression fragments are indicated in bold.
5. In case of SCAR markers, the parental AFLP markers are indicated as in Appendix 2.

Microsatellites were of higher potential as breeding tools both between and within the *Coffea* species than the SCARs. This is can be observed in Table 12 especially in regard to *C. arabica* by comparing the number of alleles in *C. arabica* and the number of alleles shared with other species. For example although Sat 207, Sat 237 and Sat 254 have nine alleles each; Sat 254 has less selection potential between *C. arabica* and *C. canephora* because it has only four unshared alleles between these species while Sat 207 and Sat 237 have seven each. The SCAR markers were more polymorphic between the diploid canephoroid and eugenioid groups due to group specific alleles but both were present in *C. arabica*. The potential for the use of these microsatellites as breeding tools for resistance to CBD and CLR from varieties Rume Sudan (resistant) and K7 (tolerant) was assessed by identifying polymorphic microsatellites between the accessions of these varieties. Sat 225 was observed to be particularly polymorphic between these varieties (Figure 7; Plate 20). Cultivar Caturra had high polymorphism with the other accessions implying that it is a more suitable mapping parent within *C. arabica*.

It was noted that utility of these microsatellites would better be exploited by high performance techniques such as LICOR which gave better separations than even radioactive PCR electrophoresis in denaturing poly-acrylamide gel. This aspect can be observed by comparing Plate 21 with Plate 18 D although the results of Plate 21 could be improved by longer migration and length of time of exposure of the gel to the film.
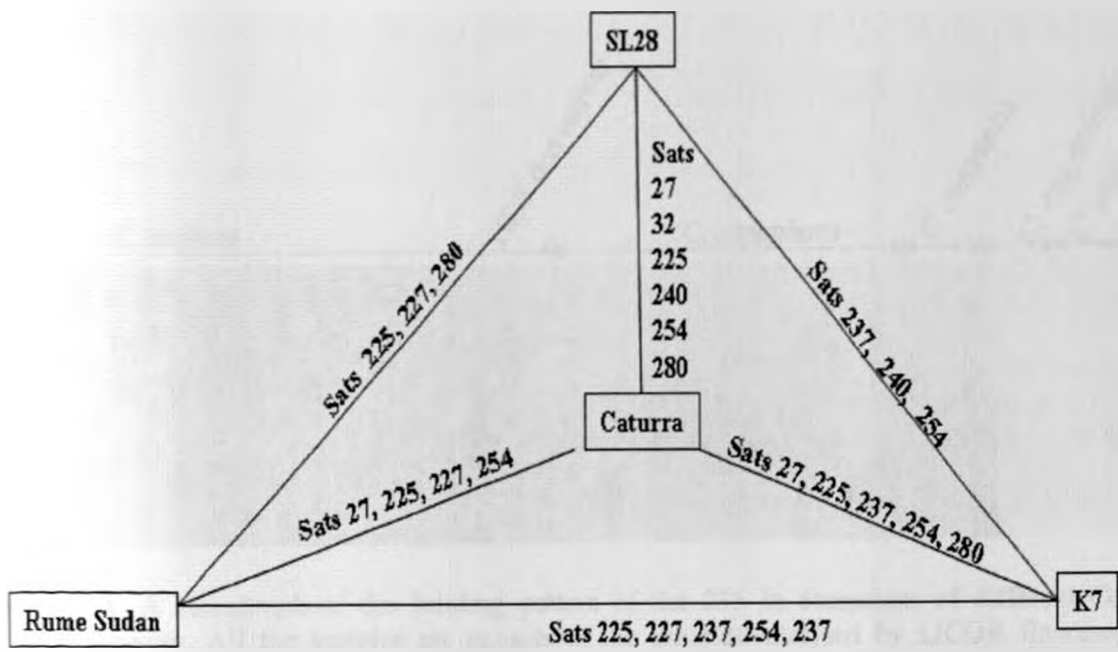
Figure 7. Diagrammatic presentation of microsatellites those were polymorphic between two CBD and CLR susceptible cultivars (SL8 and Caturra) and two donor varieties of resistance (Rume Sudan) and tolerance (K7) to the two diseases.
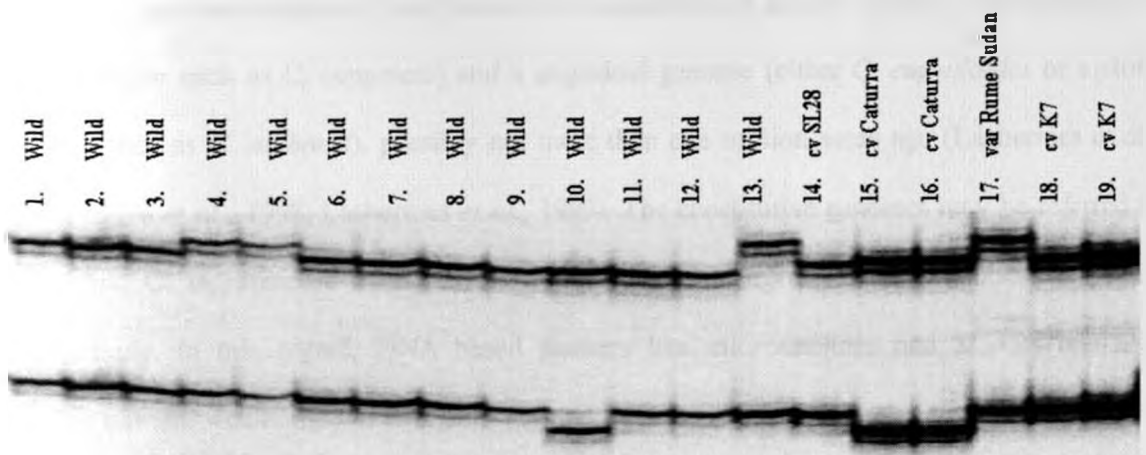
Plate 20. The amplification pattern of Sat 225 in nineteen *C. arabica* accessions including variety Rume Sudan and cv K7 that are used as donors of resistance and tolerance respectively to both CBD and CLR in Kenya . The details of the accessions and serial numbers are as in Table 10.
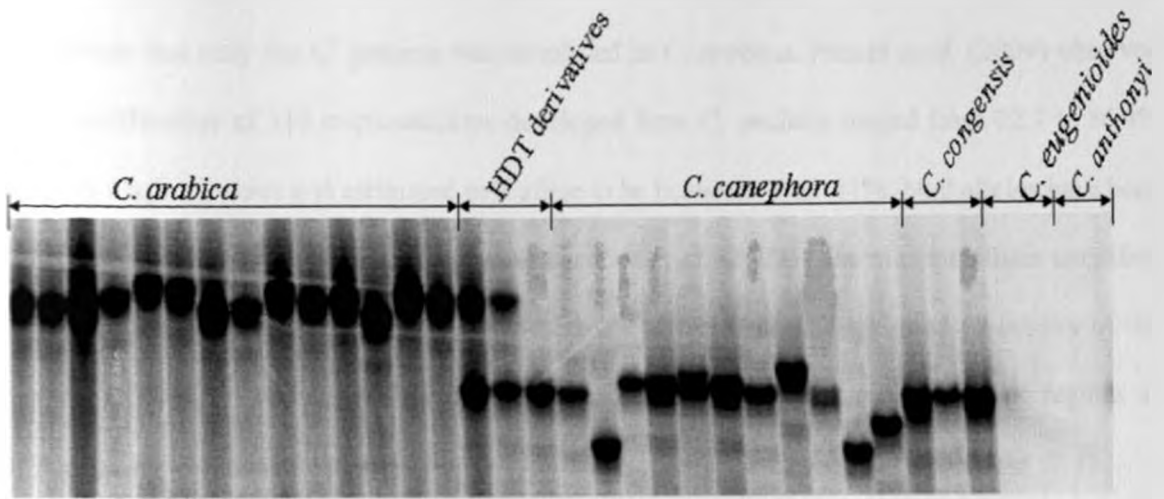
Plate 21. A radiograph of the banding pattern of Sat 235 in accessions of different *Coffea* species. All the samples are subsets of the samples analysed by LICOR fluorescence methodology and results are presented in Plate 18 D. The differences in clarity can be seen especially in the *C. arabica* samples in the two systems.

5.5.5 DISCUSSION

C. arabica genome originated from the union of a canephoroid genome (either *C. canephora* or a close relative such as *C. congensis*) and a eugenioid genome (either *C. eugenioides* or a close relative such as *C. anthonyi*), possibly not more than one million years ago (Lashermes *et al.*, 1995; Raina *et al.*, 1998; Lashermes *et al.*, 1999). The constitutive genomes may be referred to as E^a and C^a in reference to the chromosome sets from eugenioid and canephoroid species respectively. In this regard, DNA based markers like microsatellites and SCARs regularly display patterns distinct to the two genomes and the *C. arabica* pattern as a juxtaposition of the parental diploids. This was observed in this study and also by Poncet *et al.* (2005). The microsatellites used in this study were selected to identify *C. canephora* chromosomal fragments introgressed into *C. arabica* and therefore a bias towards the C^a genome was expected. This may explain why no apparent null alleles were observed in *C. canephora* and *C. congensis* and these species had higher polymorphism. Hale *et al.* (2005) explained that amplification and variability of microsatellites is higher in focal species that in related species. Null alleles were observed in

eugenioid species in cases where only one allele was amplified in *C. arabica*, prompting the hypothesis that only the C^a genome was amplified in *C. arabica*. Poncet *et al.* (2004) observed that amplification of 110 microsatellites developed from *C. arabica* ranged from 72.7 to 86.4% in other *Coffea* species and estimated null allele to be between -9 to -11%. Null alleles have been observed in other plants such as *Zea* spp (Matsuoka *et al.*, 2002). The microsatellites amplified in this study were not sequenced and therefore the alleles recorded included variability of the number of repeat motifs as well as insertions and deletions (indels) in the flanking regions as explained by Matsuoka *et al.* (2002).

Amplification of intersecting alleles in the different *Coffea* species demonstrated co-evolution within the genus. On the other hand, amplification of alleles specific to C^a with close relationship to alleles of *C. canephora* and *C. congensis* on one hand and alleles specific to E^a with close relationship to alleles of *C. eugenioides* and *C. anthonyi* or null alleles on the other hand reflects differential evolutionary pathways. This specificity was observed with both microsatellites and SCARs. One microsatellite (Sat 235) amplified alleles that were not common between un-introgressed *C. arabica* and canephoroid species, indicating possible unique origin of the C^a allele and differential evolution thereafter. This type of a marker has high potential of selecting introgressed genotypes, breeding of Arabusta hybrids and detection of contaminations or adulteration even in coffee trade. SCARs had low polymorphism that agreed with observations made in section 5.1 and there were no null alleles observed with SCAR amplifications. Using nine *Coffea* species Poncet *et al.* (2005) observed high transferability of SCARs derived from AFLP with a minimum of 58% and the SCARs could be used as anchor markers for the genus. The most polymorphic SCAR in this study was J3 with five alleles in *C. canephora*. One accession of this species had three alleles that indicated that it is duplicated. This agrees with the results reported in section 5.1 where two loci were observed in *C. canephora* double haploid

(DH) population duplicated in two different chromosomes of the *C. canephora* genome namely chromosomes two and eight. However, only one allele was observed to be introgressed into the HDT derivatives.

The diversity of the different species and groups of *C. arabica* namely wild, cultivated and HDT derivatives agreed with results of other workers whereby diploid species are more diverse followed by wild *C. arabica* and the least diverse are the cultivated varieties of *C. arabica* (Lashermes *et al.*, 1996; Anthony *et al.*, 2002a; Moncada and McCouch, 2004). HDT derivatives are of a narrow genetic base since they originated from a subset of cultivated *C. arabica* and *C. canephora*. In all cases, there was a canephoroid allele similar to the one introgressed into HDT derivatives for both microsatellite and SCAR markers. It was also observed that only one type of allele was substituted in *C. arabica* by the introgression. The substituted allele was always the one appearing to be C^a, an argument that could be extended even in cases of null E^a alleles. This would indicate non-random introgression in regard to the two sets of chromosomes in the tetraploid *C. arabica*. It was interesting to observe that microsatellite markers of *C. canephora* chromosomal fragment T3 had low polymorphism. This conservative characteristic was observed earlier with AFLP derived SCARs in Section 5.1 and it was speculated that this region might be rich in functional sequences. Microsatellites may also have putative functional roles including maintenance of the structure of a DNA fragment (Li *et al.*, 2002). In such a case, their evolution may be restricted to enhance conservation. Very low diversity was observed in *C. eugenioides* and *C. anthonyi*. This may in part be due to the fact that the samples analysed were collected from small geographical locations. For example, the samples of *C. eugenioides* were from western Kenya although the species occurs widely area in East and Central Africa (Lashermes *et al.*, 1999). More expeditions to collect more germplasm of this species and others need to be undertaken.

Several microsatellites were identified that were polymorphic between cv SL28, cv Caturra, cv K7 and variety Rume Sudan. The last two which are used in breeding programmes as donors of tolerance and resistance respectively to CBD and CLR. Based on the microsatellites and accessions tested, there was higher polymorphism between cv Caturra and the donor varieties than between cv SL28 and the donor varieties. This could mean that it would be easier to map a population between cv Caturra and the donor varieties than between cv SL28 and the donor varieties. However, it would be better in Kenya to use cv SL28 for direct comparison of standard Kenyan commercial traits such as quality and also take advantage of the breeding materials already established. It should also be noted that the efficiency of detecting and analysing the polymorphism would require high performance methodologies such as LICOR. In this study, use of LICOR system gave better resolutions, was faster and less dangerous than radioactivity. However, this methodology requires higher capital installations and is very sensitive thus requiring excellent workmanship. The pattern of radiograph of Sat 235 presented in this section could have been improved by altering the concentration of samples, distance of migration and length of exposure time. Moreover, the observed polymorphisms are only indicative and the actual polymorphism in a particular mapping population can vary.

In conclusion, this part of the study facilitated the identification of microsatellite and SCAR markers for breeding especially in relation to canephoroid *Coffea* species. They would be useful in mining useful genes from these species and introgressing them into *C. arabica*. Some polymorphic microsatellites that could be used in breeding between *C. arabica* varieties were also identified. It appeared that introgression into *C. arabica* was preferential to the related chromosomes and it would be interesting to find out if introgression from the *C. eugenioides* behaves the same. This would facilitate double introgression on the two sub-genomes even at the same locus.

CHAPTER 6.   GENERAL DISCUSSION

Despite its variable morphological and agronomic traits, *Coffea arabica* is characterised by very low genetic diversity at molecular level as observed in various phases of this study and also by other workers using various DNA marker systems (Orozco-Castillo *et al.*, 1994, Lashermes *et al.*, 1999, Steiger *et al.*, 2002, Anthony *et al.*, 2002). However, there is clustering of some accessions with various degrees of similarity related to lineage or geographical origin. Breeders have exploited the differences in certain traits to develop improved cultivars by both selection and cross breeding. The current variations are due to various mutations or enlargement of genetic base by collection of more wild accession from the centre of diversity in Ethiopia. However from a molecular point of view, these mutations can be very difficult to reveal if they are single point or involve very small DNA regions (Krug and Carvalho, 1951; Jones, 1956). Low diversity in adopted cultivars has also been observed in other crops like soybean (Maughan *et al.*, 1996; Yang *et al.*, 2000). This makes it difficult to construct genetic maps using such cultivars but it has the advantage of analysis of agronomic traits and genetic fragments that are introgressed from alien sources are easily detected.

Generally, once conditions of a protocol for AFLP have been established, the results exhibit high repeatability of up 99% (Steiger *et al.*, 2002). In this study it was possible to regenerate AFLP patterns that were obtained by other workers in the same laboratory such as Lashermes *et al.* (2000a), Noir *et al.* (2003) and Ansaldi (2003). However, the repeatability may be lost due some change of PCR conditions. Some of the factors that affect repeatability include human factor (workmanship), gel resolutions, protocol and condition of reagents and machines. In this study, a change of the model of thermocyclers was observed to affect the AFLP patterns obtained, and it can be expected that even one machine may give different performance over time as it ages and therefore affect amplification profiles. Sequence-specific primers such as microsatellites and

CARs are less sensitive to these parameters, but they are relatively few for dense mapping specially in species such as *C. arabica*, which is less studied and is of low genetic diversity. The AFLP technique reveals more polymorphism per reaction by revealing more loci per primer combination but it rarely exhibits multiple alleles per locus as is the case for SSR (Vos *et al.*, 1995; Rafalski *et al.*, 1996; Garcia *et al.*, 2004). The high polymorphism revealed by AFLP is also in a way compensates for the high cost of using AFLP when costed on the basis of the number of markers realised per reaction. In this study, the number of microsatellite alleles per locus per accession in *C. arabica* varied from 2 to 3 while the number of loci varied from 1 to 2. Some of the alleles were always present and not polymorphic, and thus not scored for analysis.

The interpretation of the genetic control of a trait may be affected by the evaluation procedure and genetic constitution of the materials used. In the case of CBD, several researchers have advanced different arguments on different classical screening methods (macro-symptoms) in regard to their reliability, data interpretations, co-relationships, utilities, and genetic control (Van der Vossen *et al.*, 1976; Van der Graaff, 1978 and 1982; van der Vossen and Walyaro, 1980; Dancer, 1986; Owuor and Agwanda, 1990; Gichuru, this thesis). The introduction of more technical screening methods like tissue culture (Nyange *et al.*, 1995 and 1997), DNA molecular markers (Agwanda *et al.*, 1997; Gichuru, this thesis) and numerous biochemical or histochemical factors (Gichuru, 1996, 1997; Gichuru and King'ori, 1999; Gichuru *et al.*, 1999; Gichuru, 2001, Rodrigues *et al.*, 1999; Silva *et al.*, 2006) does not seem to provide outright solutions to the matter, but points out at the depth of intricate factors involved. However, this should be viewed as a challenge to understand the disease in details and finally utilise all the information in optimising the management of the disease in a holistic way in the background of agronomic, biotic and abiotic factors. This should be supported by similar studies on the pathogen.

This study encompassed macro-symptomatic assessment and molecular analysis of CBD in order to identify, characterise and map resistance to the disease derived from *C. canephora* and introgressed into *C. arabica* via HDT. The first step (Section 5.1) involved molecular analysis of cv Catimor which are selected and fixed for resistance to CBD. This was to enable identification of *C. canephora* derived chromosomal fragments or markers that are present in these breeding lines and their progenies ($BC_1 F_1$, $BC1 F_2$). These markers or fragments would then constitute an inventory of candidate markers or carriers of resistance to both CBD and CLR. This was accomplished by AFLP analysis for markers of introgression and collaboration of the result to those of Lashermes *et al*. (2000a), Noir *et al*. (2003) and Ansaldi (2003) that were generated in the same laboratory (IRD, Montpellier, France). Out of the overall HDT derived markers, it was anticipated that potential markers for genes of resistance to CBD and CLR (at least the genes related to races of CLR present in Kenya) are present in all the cv Catimor and $BC_1 F_1$ plants analysed in this study because they were all resistant to these diseases. Consequently, the identified *C. canephora* chromosomal fragments fitting this criterion whether mapped or not constituted potential candidate markers of the introgressed resistance genes.

Two mapped introgressed *C. canephora* chromosomal fragments T2 and T3 were identified as the most probable candidates while fragment T4 was less likely due to its absence in some resistant trees. Two fragments T1 and T5 were ruled out as candidates because they were absent in the cv Catimor lines analysed. It was not possible to regenerate the RAPD markers of CBD resistance identified by Agwanda *et al*. (1997). However, the product amplified by primers designed from the sequence of the RAPD marker $M20_{830}$ (Agwanda *et al*., 1997) consisted of two bands in *C. arabica*, one of which was the introgressed allele due to its presence in introgressed accessions. When the marker was scored alongside the data of Ansaldi (2003), it was evident that it was linked to the fragment T2, but it could not be clearly mapped due to poor

amplifications in many samples. This phase of study set a firm starting point in the search for CBD resistance gene in a F_2 population that was being bred at the time when these studies were being carried out. This phase also enhanced the information available on the *C. canephora* chromosomal fragments especially in cv Catimor in Kenya, which is useful for any subsequent studies aimed at their utilization and conservation.

A second step (Section 5.2) involved the establishment of two F_2 populations from crosses between cvs SL28 and Catimor, which was a prerequisite for identification and mapping of markers. It was demonstrated that the populations were segregating in resistance to CBD by hypocotyls inoculations. The hypocotyls inoculation results of the two populations exhibited high similarity between and within the populations. However there was a tendency for the first seed lots of the two populations to have slightly more sensitive plants that indicated the possibility of the action of non-genetic factors. The sensibility of the method to environmental conditions especially temperatures has been observed for a long time (van der Vossen and Waweru, 1976; Masaba and van der Vossen, 1982). This may be the cause of the much debate on scaling and data interpretation, but the method is valuable especially in screening populations to obtain resistant plants or using the averaged results to determine the phenotype of the mother plant or line (Van der Vossen, *et al.*, 1976; van der Graaff, 1978, 1982; Dancer, 1986; Owour and Agwanda, 1990). In this study a cut off between presence and absence of resistance was made at Class 10, but only seedlings in Classes 1 to 4 were retained as resistant sub-populations of the two populations so as to have direct comparison with routine breeding programmes at CRF. The preserved plants from the inoculated halves of the F_2 populations constituted Group 1 plants from each of the populations (Population D and Population E). However this method eliminated susceptible plants from the inoculated sub-populations. Molecular analysis using microsatellite markers revealed that two *C. canephora* chromosomal fragments (T2 and T3) were

present and segregating in the two F_2 populations, and they were considered to be the most probable candidates for disease resistance in support of the results of the first stage.

In the third step (Section 5.3), a suitable method to screen the F_2 population for CBD resistance at an early stage, while allowing availability of DNA from the entire population and enhancing the survival of susceptible plants was developed and used to screen the F_2 populations. This was necessary to overcome the problem of elimination of susceptible plants by the hypocotyls inoculation method (van der Vossen *et al.*, 1976) while saving the time that would be necessary if the plants were to be screened at maturity. Inoculation of shoot tips of seedlings at about one year was considered to be a suitable screening method allowing early screening without high loss of susceptible plants. The method was used for inoculation of the Group 1 and the un-inoculated (Group 2) sub-populations of the F_2 populations. The methodology was modified by provision of suitable environmental conditions that were anticipated to enhance infection beyond the shoot tips irrespective of the stage of growth of the tips. This facilitated the screening of the entire population because the seedlings did not need to be selected as described by van der Vossen *et al.* (1976). The results enabled adequate separation of resistant and susceptible plants for identification of molecular markers linked to the resistance, but it was expected that some plants would be misclassified into the wrong phenotypes and an intermediate genetically ambiguous category was observed. However subsequent molecular studies were expected to identify misclassified plants and Group 1 plants from both populations that were obtained in the second step of this study were considered as confirmatory for maker identification.

The fourth step of this study (Section 5.4) involved molecular studies to identify markers of resistance to CBD and infer its mode of gene action. Firstly, microsatellites were analysed to identify candidates markers in the populations established and characterised in preceding

sections whereby Group 1 plants were used to confirm linkage to CBD resistance and Group 2 plants were used to assess segregation behaviour of the markers in the populations. Sat 207 which is mapped onto *C. canephora* chromosomal fragment T2 was found to be linked to CBD resistance and the introgressed allele of fragment T3 was highly present due to segregation distortion. AFLP analysis was carried out to confirm these results further as well as to establish the limits of the location of the resistance gene. Fragment T2 was concluded to be linked to the resistance and the gene was localised within a fragment measuring 26.9 cM. In addition, Sat 235 was also found to be tightly linked to the resistance and mapped onto the T2 fragment. As expected, misclassified plants were identified in both resistant and susceptible plants of Group 2 but none were observed in Group 1 category. The high complementation of the two phenotypic screening methods and molecular studies was evident. Analysis of a SCAR (SRAPD-M20$_{830}$) designed from the sequence of a RAPD marker for CBD resistance derived from HDT (Agwanda *et al.*, 1997) demonstrated that it is linked to fragment T2, which further validated the results. One AFLP marker of the resistance was converted into a dominant SCAR marker (AGC-CTG-c-$_{AA4}$).

The segregation behaviour of the microsatellite markers indicated that both heterozygous and homozygous plants were similarly resistant to CBD and it was therefore concluded that the resistance was major. However the possibility of effect of the dosage of the gene cannot be ruled out especially in regard to results obtained by hypocotyls inoculation test. The locus was designated *Ck-1* (the first mapped locus for resistance to *Colletotrichum kahawae*) and is likely to be synonymous to the T-locus described by van der Vossen and Walyaro (1980). Surveillance for the markers in diverse derivatives of HDT with both field and laboratory resistance to CBD demonstrated the reliability of the markers. However, further studies are required to refine the

map further. Additionally, the studies of this phase lead to speculation that three *C. canephora* chromosomal fragments T2, T3 and T4 may be involved in resistance to CLR.

In the final part of the study, the diversity of microsatellite markers and SCARs derived from AFLP markers of introgression of *C. canephora* genomic fragments into *C. arabica* facilitated the identification of microsatellite and SCAR markers for breeding especially in relation to canephoroid *Coffea* species. They would be useful in mining useful genes from these species and introgressing them into *C. arabica*. Some polymorphic microsatellites that could be used in breeding between *C. arabica* varieties were also identified. It appeared that introgression into *C. arabica* was preferential to the related chromosomes in *C. arabica* (C^a sub-genome). Species specific and non-specific evolutionary patterns of the microsatellites were also demonstrated. Microsatellites that are polymorphic between the susceptible cultivars SL28 and Caturra against K7 and Rume Sudan that are donors of resistance and tolerance respectively to CBD and CLR were identified.

CHAPTER 7.   CONCLUSIONS AND RECOMMENDATIONS

This study focussed on chromosomal fragments of *C. canephora* that are introgressed into *C. arabica* genome and their effects on disease resistance particularly CBD. In view of all the analysis done in this study, it is concluded that a major CBD resistance gene hereby designated *Ck-1* that is located on the introgressed fragment T2 was identified and plant genotypes were established for its further fine mapping and/or breeding. Though not conclusive, two *C. canephora* fragments (T2 and T4) were highlighted as priority candidates for CLR resistance in the derivatives of HDT in Kenya. Another prominent fragment T3 maybe of some function in these plant materials and it is of interest to study it. Recommendations from this study include:

i. To confirm the disease reaction phenotypes of the plants that are recombinant in regard to Sat 235 after they start bearing by field inoculation, natural infection and analysis of their progenies.

ii. To adapt microsatellites 207 and 235, and the SCAR maker AGC-CTG-c-$_{AA4}$ to low technology use such as in CRF laboratory under local conditions and using locally available reagents so that they can be more widely used for MAS.

iii. To utilize the differentially recombinant plants established in the field to develop elite breeding parents, single line varieties and to study the influence of different introgression fragments in the genotypes in regard to agronomic traits.

iv. To screen large numbers of segregating progenies of HDT derivatives and HDT accessions by hypocotyls inoculation method, followed by analysis of the resistant plants by methodologies of recommendation (ii) to obtain more recombinant plants for future fine mapping.

v. To collaborate with other laboratories such as IRD, France and assess more markers those become available for coffee genomics in recombinant plants.

vi.   With further collaborations, endeavour to walk on the chromosome with the final objective of cloning the gene and using it for transformation.

vii. To design studies for identifying markers for resistance to CLR using the plants with different introgression fragments established in this study.

viii. To develop markers of disease resistance from other donors such as varieties Rume Sudan and K7 with the identified polymorphic microsatellites as starting point.

8. REFERENCES

Adams, K. L. and Wendel, J. F. (2005). Polyploidy and genome evolution in plants. Current Opinion in Plant Biology **8**: 153-151.

Aga, E., Bryngelsson, T., Bekele, E. and Salomon, B. (2003). Genetic diversity of forest arabica coffee (*Coffea arabica* L.) in Ethiopia as revealed by random amplified polymorphic DNA (RAPD). Hereditas **138**:36-46.

Agwanda, C. O., Lashermes, P., Trouslot, P., Combes, M C and Carrier, A. (1997). Identification of RAPD markers for resistance to Coffee Berry Disease, *Colletotrichum kahawae* in Arabica coffee. Euphytica **97**: 241-248

Ansaldi, C. (2003). Analyses moléculaires de l'introgression génétique chez le caféier (*Coffea arabica* L.). DESS Bioingénierie, Université Paul, Toulose, France. 31p

Anthony, F., Combes, M. C., Astrorga C., Bertrand, B., Graziosi, G. and Lashermes, P. (2002a). The origin of cultivated *Coffea arabica* revealed by AFLP and SSR markers. Theoretical and Applied Genetics **104**: 894-900

Anthony, F., Quiros, O., Topart, P., Bertrand, B. and Lashermes, P. (2002b). Detection by simple sequence repeat markers of introgression from *Coffea canephora* in *Coffea arabica* cultivars. Plant Breeding **121**: 542-544.

Anthony, F. and 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.

Anthony, F., Noirot, M., Couturon, P. and Stoffellen, P. (2006). New coffee (*Coffea* L.) species from Cameroon bring original characters from breeding. 21st ASIC Conference, 11-15 September, Montpellier, France.

Bertrand, B., Guyot, B., Anthony, F. and Lashermes, P. (2003). Impact of the *Coffea canephora* genome introgression on beverage quality of *C. arabica*. Theoretical and Applied Genetics **107**: 387-384.

Bettencourt, A. J. (1973). Consideraçoes gerais sobre o "HDT". Instituto Agronomico de Campinas, Circular No. 31, Campinas, Brazil.

Bettencourt, A. J. (1983) Caracteristicas agronomicas de seleccoes derivadas de cruzamentos entre Hibrido de Timor e as variedades Caturra, Villa Sarchi e Catuai. *In* Simposio sobre Ferrugens do Cafeeiro, Oeiras, Portugal, pp 353-373.

Blears, M. J., De Grandis, S. A., Lee, H., Trevors, J. T. (1998). Amplified Fragment Length Polymorphism (AFLP): a review of the procedure and its applications. Journal of Industrial Microbiology and Biotechnology **21**: 99-114.

Botstein, D., White, R. I., Skolnick, M. H. and Davis, R. W. (1980). Construction of genetic in man using restriction fragment length polymorphisms. American Journal of Human Genetics **32**: 314-331.

Boukar, O., Kong, L., Singh, B. B., Murdock, L. and Ohm, H. W. (2004). AFLP and AFLP-derived SCAR markers associated with *Striga gesnerioides* resistance in cowpea. Crop Science **44**: 1 259-1264.

Carvalho, A. (1952). Taxonomia de *Coffea arabica* L. Caracters morfologicos do haploids. Bragantia **12**: 201-212.

Caicedo, A. L., Schaal, B. A. and Kunkel, B. N. (1999). Diversity and molecular evolution of the RPS2 resistance gene in *Arabidopsis thaliana*. Proceedings of National Academy of Sciences of USA **96**: 302-306.

Charrier, A. and Berthaud, J. (1985). Botanical classification of coffee. *In*: Coffee botany, biochemistry and production of beans and beverage. (Eds. Clifford, M. N. and Wilson, K. C.). Croom Helm, London pp 13-47.

Chen, Z.. Guimaraes, L. G. and Rodrigues Jr, C. J. (2004). Pectate lyase inhibitors may condition non-pathogenicity of *Colletotrichum gloeosporioides* to green coffee berries. Actas do 4° Congresso da Sociedade Protuguesa de Fitopatologia pp 111-115.

Chen, Z., Nunes, M. A., Silva, M. C. and Rodrigues Jr, C. J (2004). Appressorium turgor pressure of *Colletotrichum kahawae* might have a role in cuticle penetration. Mycologia **96**: 1199-1208.

Chongo, G.. Gossen, B. D. and Bernier, C. C. (2002). Infection by *Colletotrichum truncatum* in resistant and susceptible lentil genotypes. Canadian Journal of Plant Pathology **24**: 81-85.

Combes. M. C., Andrzejewski, S., Anthony, F., Bertrand, B.. Rowelli, P., Graziosi, G. and Lashermes, P. (2000). Characterization of microsatellite loci in *Coffea arabica* and coffee species. Molecular Ecology **9**: 1178-1180.

Coulibaly, I., Revol, B., Noirot, M., Poncet, V., Loriex, M., Carasco-Lacombe, C., Minier, J., Dafour, M. and Hamon, P. (2003). AFLP and SSR polymorphism in a *Coffea* interspecific backcross progeny [(*C. heterocalyx* x *C. canephora*) x *C. canephora*]. Theoretical and Applied Genetics **107**: 1148-1155.

Crochemore, M. L., Nunes, L. M., Andrade, G. A., Molinari, H. B. C. and Vasconcellos, M. E. (2004). Variety identification of coffee seeds by RAPD techniques. Brazilian Archives of Biology and Technology **47**: 7-11

Cros. J.. Combes, M-C., Chabrillange, N., Duperray, C., Monnot des Angles, A. and Hamon, S. (1995). Nuclear content in the subgenus *Coffea* (Rubiaceae): inter- and intra-specific variation in African species. Canadian Journal of Botany **73**: 14-20

Dancer, J. (1986). The evaluation of arabica coffee cultivars for resistance to coffee berry disease: some comments. Euphytica **35**: 125-126.

Diniz, L. E. C., Sakiyama, N. S., Lashermes, P., Caixeta, E. T., Oliveiera, Zambolin, E. M., Loureiro, M. E., Pereira, A. A. and Zambolin, L. (2005). Analysis of AFLP markers associated to the *Mex*-1 locus in Icatu progenies. Crop Breeding and Applied Biotechnology **5**: 387-393.

Esquerré-Tugayé, M. T, Mazau, D., Barthe, J. P., Lafitte, C. and Touzé, A. (1992). Mechanisms of resistance to *Colletotrichum* species. *In*: Bailey, J. A. and Jerger, M. J. (Eds), *Colletotrichum*; Biology, pathology and control. CABI Wallingford. UK. pp 121-133.

Farooq, S. and Azam, F. (2002). Molecular markers in plant breeding-II. Some pre-requisites for use. Pakistan Journal of Biological Science **5**: 1141-1147.

Flor, H. H. (1971). Current status of the gene-for-gene concept. Annual Review of Phytopathology **9**: 275-296.

Garcia, A. A. F., Benchimol, L. L., Barbosa, A. M. M. , Geraldi, I. O., Souza Jr., C. L., and Souza, A. P. (2004). Comparison of RAPD, RFLP, AFLP and SSR markers for diversity studies in tropical maize inbred lines. Genetic and Molecular Biology **27**: 579-588.

Gichuru, E. K. (1993) Isozyme patterns and enzyme activities of *Coffea arabica* L. cultivars resistant and susceptible to coffee berry disease (*Colletotrichum kahawae* Waller and Bridge: syn; *Colletotrichum coffeanum* Noack). MSc. Thesis, University of Nairobi, 181 p.

Gichuru, E. K., Mwang'ombe A W. And Gupta, V. K. (1996). Peroxidase activity in *Coffea arabica* cultivars resistant and susceptible to coffee berry disease. African Crop Science Journal **4**: 223-232.

Gichuru, E. K. (1997). Resistance mechanisms in arabica coffee to coffee berry disease (*Colletotrichum kahawae* Sp. Nov.)- A review. Kenya Coffee **62**: 2441-2444.

Gichuru, E. K. and King'ori, P. N. (1999). Proteolytic enzyme activity in *Coffea arabica* varieties varying in resistance to coffee berry disease. ASIC, 2-6 August, Helsinki, Finland. Paper No. A 12 7

Gichuru, E. K., King'ori, P. N. and Masaba, D. M. (1999). Histochemical differences during infection on *Coffea arabica* by *Colletotrichum kahawae* isolates. ASIC proceedings, 2-6 August, Helsinki, Finland. Paper No. P 717.

Gichuru, E. K., Varzea, V. M. P., Rodrigues Jr., C. J. and Masaba, D. M. (2000). Vegetative compatibility grouping of *Colletotrichum kahawae* in Kenya. Journal of Phytopathology **148**: 233-237.

Gichuru, E. K. (2001). Microscopic variations in susceptible and resistant infections of *Coffea arabica* by *Colletotrichum kahawae*. ASIC proceedings 14-18 May, Trieste, Italy. Paper No. PA 602.

Gichuru, E. K (2005). Some notes on coffee leaf rust (*Hemileia vastatrix*) with regard to changing coffee production conditions in Kenya. 1st International Workshop on Durable Resistance to Coffee Leaf Rust, 26-28 September, Viçosa, Brazil.

Griffiths E., Gibbs, J. N. and Waller, J. M. (1971). Control of coffee berry disease. Annals of Applied Biology **67**: 45-74.

Grovet, L. and Noyer, J-L. (2003). Biochemical and molecular markers. In P. Hamon, M. Seguin, X. Perrier and J. C. Glaszmann (Eds). Genetic diversity of cultivated tropical plants Scientific Publishers, Inc., USA and CIRAD, France pp1-29.

Hale, M. I., Borland, A. M. and Wolf, K. (2005). High degree of conservation of nuclear microsatellite loci in the genus *Clusia*. Genome **48**: 946-950.

Hammond-Kasack, K. E. and Parker, J. E. (2003). Deciphering plant-pathogen communication: fresh perspectives for molecular resistance breeding. Current Opinion in Biotechnology **14**: 177-193.

Herrera, J-C. Combes, M-C., Anthony, F., Charrier, A. and Lashermes, P. (2002). Introgression into the allotetraploid coffee (*Coffea arabica* L.): segregation and recombination of the *C. canephora* genome in the tetraploid interspecific hybrid (*C. arabica* x *C. canephora*). Theoretical and Applied Genetics **104**: 661-668

Jeuken, M. J. W. and Lindhout, P. (2004). The development of lettuce backcross inbred lines (BILs) for exploitation of the *Lactuca saligna* (wild lettuce) germplasm. Theoretical and Applied Genetics **109**: 394-401.

International Trade Centre, (2002). Coffee: an exporter's guide. Geneva, 310 p

Jones, P. A. (1956). Notes on the varieties of *Coffea arabica* in Kenya. Coffee Board of Kenya, Monthly Bulletin, November

Kairu, G. M. (1998). Research activities in Plant Pathology Section. Kenya Coffee **63**: 2703-2705

Karanja, A. M. (1996). Highlights of coffee production, cost of production and milling in Kenya. Kenya Coffee **61**: 2325-2329.

Kirk, P. M., Cannon, P. F., David, J. C. and Stalpers, J. A. (Eds) (2001). Ainsworth and Bisby's Dictionary of the Fungi (9th Edition). CABI, Wallingford, 624pp

Konieczny, A. and Ausbel, F. M. (1993). A procedure for mapping *Arabidopsis* mutations using co-dominant ecotype-specific PCR based markers. Plant Journal **4**: 403-410.

Krug, C. A. and Carvalho, A. (1951). The genetics of coffee. Advances in Genetics **4**:127-158.

Lashermes, P., Cros, J., Marmey, P. and Charrier, A. (1993). Use of random amplified DNA markers to analyse genetic variability and relationship in *Coffea* species. Genetic Resources and Crop Evolution **40**: 91-99.

Lashermes, P., Couturon, E. and Charrier, A. (1994). Doubled haploids of *Coffea canephora*: development, fertility and agronomic characteristics. Euphytica **74**: 149-157.

Lashermes, P., Combes, M. C., Cros, J., Trouslot, P., Anthony, F. and Charrier, A. (1995). Origin and genetic diversity of *Coffea arabica* L. based on DNA molecular markers. Proceedings of 16th ASIC, Kyoto, Japan pp 528-536.

Lashermes, P., Trouslot, P., Anthony, F., Combes, M. C., and Charrier, A. (1996). Genetic diversity for RAPD markers between cultivated and wild accessions of *Coffea arabica*. Euphytica **87**: 59-64.

Lashermes, P., Combes, M. C., Robert, J., Trouslot, P., Hont, A. D., F Anthony, F. and Charrier, A. (1999). Molecular characterization and origin of the *Coffea arabica* L. genome. Molecular and General Genetics **261**: 259-266

Lashermes, P., Andrzejewski, S., Bertrand, B., Combes, M. C., Dusert, S., Graziosi, G., Trouslot, P. and Anthony, F. (2000a). Molecular analysis of introgressive breeding in coffee (*Coffea arabica* L.) Theoretical and Applied Genetics **100**: 139-146.

Lashermes, P., Paczek, V., Trouslot, P., Combes, M-C. Couturon, E. and Charrier, A. (2000b). Single-locus inheritance in the allotetraploid *Coffea arabica* L. and interspecific hybrid *C. arabica* x *C. canephora*. Journal of Heredity **91**: 81-85.

Lashermes P., Combes, M. C., Prakash, N. S., Trouslot, P., Lorieux, M. and Charrier, A. (2001). Genetic linkage map of *Coffea canephora*: effect of segregation distortion and analysis of recombination rate in male and female meioses. Genome **44**: 589-596.

Li, Y-C., Korol, A. B., Fahima, T., Beiles, A. And Nevo, E. (2002). Microsatellites: genomic distribution, putative functions and mutational mechanisms: a review. Molecular Ecology **11**: 2453-2465.

Lolle, S. J., Victor, J. L., Young, J. M. and Pruitt, R. E. (2005). Genome-wide non-mendelian inheritance of extra-genomic information in *Arabidopsis*. Nature **434**: 505-509.

Masaba, D. M. and van der Vossen, H. A. M (1982). Evidence of cork barrier formation as a resistance mechanism to coffee berry disease (*Colletotrichum coffeanum*) in Arabica coffee. Netherlands Journal of Plant Pathology **88**: 19-22.

Masaba, D. M. and Waller, J. M. (1992). Coffee Berry Disease: The current status. *In*: Bailey, J. A. and Jerger, M. J. (Eds), *Colletotrichum*; Biology, pathology and control. CABI Wallingford. UK. pp 237-249.

Masumbuko, L. I., Bryngelsson, T., Mneney, E. E. And Salomon, B. (2003). Genetic diversity in Tanzania Arabica coffee using random amplified polymorphic DNA (RAPD) markers. Hereditas **139**: 56-63.

Masumbuko, L. I. and Bryngelsson, T. (2005). Inter simple sequence repeat (ISSR) analysis of diploid coffee species and cultivated *Coffea arabica* L. from Tanzania. Genetic Resources and crop Evolution. **00**:1-10

Matsuoka, Y., Mitchell, S. E., Kresovich, S., Goodman, M. and Doebley, J. (2002). Microsatellites in Zea-variability, patterns of mutations, and use for evolutionary studies. Theoretical and Applied Genetics **104**: 436-450.

Maughan, P. J., Saghai-Maroof, M. A., Buss, G. R. and Huestis, G. M. (1996). Amplified fragment length polymorphism (AFLP) in soybean: species diversity, inheritance and near-isogenic line analysis. Theoretical and Applied Genetics **93**: 392-401.

McDowell, J. M. and Woffenden, B. J. (2003). Plant disease resistance genes: recent insights and potential applications. Trends in Biotechnology **21**: 178-183.

Michelmore, R. W. (2003). The impact zone: genomics and breeding for durable disease resistance. Current Opinion in Plant Biology **6**: 397-404.

Moncada, P. and McCouch, S. (2004). Simple sequence repeats diversity in diploid and tetraploid *Coffea* species. Genome **47**: 501-509.

Mueller, U. G. and Wolfenbarger, L. L. (1999). AFLP genotyping and fingerprinting. TREE 14: 389-394.

Mulinge, S.K. (1970). Development of coffee berry disease in relation to the stage of berry growth. Annals of Applied Biology **65**: 269-276.

Nikaido, A., Yoshimaru, H., Tsumura, Y. Suyama, Y., Murai, M. and Nagasaka, K. (1999). Segregation distorting for AFLP markers in *Cryptomeria japonica*. Genes and Genetic Systems **74**: 55-59.

Noir, S., Combes, M. C., Anthony, F. and Lashermes, P. (2001). Origin, diversity and evolution of NBS disease-resistance gene homologues in coffee trees (*Coffea* L.). Molecular and General Genetics **265**: 654-662

Noir, S., Anthony, F., Bertrand, B., Combes, M. C., and Lashermes, P. (2003). Identification of a major gene (Mex-1) from *Coffea canephora* conferring resistance to *Meloidogyne exigua* in *Coffea arabica*. Plant Pathology **52**: 97-103.

Noir, S., Patheyron, S., Combes, M-C., Lashermes, P. and Chalhoub, B. (2004). Construction and characterisation of a BAC library for genome analysis of the allotetraploid coffee species (*Coffea arabica* L.). Theoretical and Applied Genetics **109**: 225-230.

Nutman, F.J. and F.M. Roberts (1960). Investigations on a disease of *Coffea arabica* caused by a form of *Colletotrichum coffeanum*. I. Some factors affecting germination and infection and their relation to disease distribution. Transactions of the British Mycological Society **43**: 643-659.

Nyange, N.E., Williamson, B., McNicol, R. J., Lyon, G. D. and Hackett, C. A. (1995). *In vitro* selection of *Coffea arabica* callus for resistance to partially purified phytotoxic culture filtrates from *Colletotrichum kahawae*. Annals of Applied Biology **127**: 425-439.

Nyange, N.E., Williamson, B., Lyon, G. D., McNicol, R. J., and Conolly, T. (1997). Responses of cells and protoplasts of *Coffea arabica* genotypes to partially purified culture filtrates produced by *Colletotrichum kahawae*. Plant Cell Reports 16: 763-769.

Nyoro, J. K. and Sprey, L. H. (1986). Introducing Ruiru 11 to estates and smallholders. Kenya Coffee 51: 7-28.

Olsen, K. M. and Schaal, B. A. (2003) Microsatellite variation in cassava (*Manihot esculenta*, Euphorbiaceae) and its wild relatives: Further evidence for a southern Amazon origin of domestication. American Journal of Botany 88: 131-142.

Omondi, C. O. (1994). Resistance to Coffee Berry Disease in Arabica Coffee Variety 'Ruiru 11'. Plant Breeding 112: 256-259.

Omondi, C O, Ayiecho, P O, Mwang'ombe, A W and H Hindorf, H (2000). Reaction of some *Coffea arabica* genotypes to strains of *Colletotrichum kahawae*, the causal agent of coffee berry disease. Journal of Phytopathology 148: 61-63.

Omondi, C. O., Ayiecho, P. O., Mwang'ombe, A. W. and Hindorf, H. (2001). Resistance of *Coffea arabica* cv Ruiru 11 tested with different isolates of *Colletotrichum kahawae*, the causal agent of coffee berry disease. Euphytica 121: 19-24

Orita, M. Iwahana, I, Kanazawa, H, Hayashi, K and Sekiya, T (1989). Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms. Proceedings of National Academy of Sciences of USA 86: 2766-2770.

Orozco-Castillo, C., Charlmers, K. J., Waugh, R. and Powell, W. (1994). Detection of genetic diversity and selective introgression in coffee using RAPD markers. Theoretical and Applied Genetics. 87: 934-940.

Osorio, N. (2002). The global coffee crisis: a threat to sustainable development. ICO, London.

Owuor, J. B. O. and Agwanda, C. O. (1990). Comparison of the sensitivity of the mean and the mode in screening for resistance to coffee berry disease (*Colletotrichum coffeanum* Noack sensu Hindorf) (A short communication). Euphytica **45**: 247- 250.

Paillard, M., Lashermes, P. and Pétiard, V. (1996). Construction of a molecular linkage map in coffee. Theoretical and Applied Genetics **93**: 41-47.

Paran, I. and Michelmore, R. W. (1993). Development of reliable PCR-based markers linked to downy mildew resistance genes in lettuce. Theoretical and Applied Genetics **85**: 984-993.

Pare, S. (2002). Expériences du commerce équitable du café Recherche et caféiculture, (Ed M Harvelaar), Cirad-CP, Montpellier, France pp 22-29.

Parlevliet J. E. (2002). Durability of resistance against, bacterial and viral pathogens; present situation. Euphytica **124**: 147-156.

Pearl, H. M., Nagai, C., Moore, P. M., Steiger, D. L., Osgood, R. V. and Ming, R. (2004). Construction of a genetic map for arabica coffee. Theoretical and Applied Genetics **108**: 829-835.

Pereira, A. A., Sakiyama, N. S., Zambolin, L. Moura, W. M., Zambolin, E. and Caixeta, E. T. (2005). Identification and use of sources of durable resistance to coffee leaf rust in the UFV/EPAMIG breeding programme. *In*: Zambolin, L., Zambolin, E. and Varzea, V. (Eds), Durable resistance to coffee leaf rust. Universidade Federal de Viçosa, Brazil, pp 215-232.

Poncet, V., Hamon, P., Minier, J., Carasco, C., Hamon, S., and Noirot, M. (2004). SSR cross-amplification and variation within coffee trees (*Coffea* spp.). Genome **47**: 1071-1081

Poncet, V., Hamon, P., Sauvage de Saint Marc, M. B., Bernard, T., Hamon, S. and Noirot, M. (2005). Base composition of *Coffea* AFLP sequences and their conservation within the genus. Journal of Heredity **96**: 59-65.

Prakash, N. S., Combes, M. C., Somanna, N. and Lashermes, P. (2002). AFLP analysis of introgression in coffee cultivars (*Coffea arabica* L.) derived from a natural interspecific hybrid. Euphytica **124**: 265-271.

Prakash, N. S., Marques, D. V., Varzea, V. M. P., Silva, M. C., Combes, M. C. and Lashermes, P. (2004). Introgressive molecular analysis of a leaf rust resistance gene from *Coffea liberica* into *C. arabica* L. Theoretical and Applied Genetics **109**: 1311-1317.

Rafalski, J. A., Vogel, J. M., Morgante, M., Powell, W. Andre, C. and Tingey, S. V. (1996). Generating and using DNA markers in Plants. In: Non-mammalian genomic analysis: A practical guide (Biren, B. and Lai, E., Eds), Academic Press Inc. New York p 75-134.

Raina. S. N., Mukai, Y. and Yamamoto, M. (1998). In situ hybridization identifies the diploid progenitors of Coffea arabica (*Rubiaceae*). Theoretical and Applied Genetics **97**: 1204-1209.

Rayner, R.W. (1952). Coffee Berry Disease, a survey of investigations carried up to 1950. East African Agricultural and Forestry Journal **17**: 130-158.

Riesenbierg, L H, Baird, S. J. E. and Gardner, K. A. (2000). Hybridization, introgression and linkage evolution. Plant Molecular Biology **42**: 205-224.

Robinson, J. P. and Harris, S. A. (1999). Which DNA Marker for Which Purpose? Chapter 12 in: Final Compendium of the Research Project Development: optimisation and validation of molecular tools for assessment of biodiversity in forest trees in the European Union DGXII Biotechnology FW IV Research Programme Molecular Tools for Biodiversity. Gillet, E.M. (ed.). URL/http://webdoc.sub.gwdg.de/ebook/y/1999/which marker/index.htm

Rodrigues Jr, C. J., Varzea, V. M. P., Hindorf, H. and Medeiros, E. F. (1991). Strains of *Colletotrichum coffeanum* Noack causing coffee berry disease in Angola and Malawi

with characteristics different to the Kenyan strain. Journal of Phytopathology **131**: 205-209.

Rodrigues Jr, C. J., Varzea, V. M. P. and Medeiros, E. F. (1992). Evidence for the existence of physiological races of *Colletotrichum coffeanum* Noack sensu Hindorf. Kenya Coffee **57**: 1417-1420.

Rodrigues Jr, C. J., Varzea, V. M. P. and Silva, M. C. (1999). Research and creation of coffee varieties with resistance to coffee berry disease. 4th Annual report Project TS3-CT94-0307, CIRAD, France.

Rodrigues Jr. C. J, Varzea V, Silva M. C., Guerra-Guimarães L., Rocheta M., Marques D. V. (2000) Recent advances on coffee leaf rust. *In*: Proceedings of the International Scientific Symposium on Coffee. Bangalore, India, pp.179-193.

Saliba-Colombani, V., Causse, M., Gervais, L. and Philouze, J. (2000). Efficiency of RFLP, RAPD and AFLP markers for the construction of an interspecific map of the tomato genome. Genome **43**: 29-40.

Sambrook, J., Fritsch, E. F. and Manitanis, T. (1989). Molecular cloning techniques: a laboratory manual, 2nd edition, Cold Spring Habour Laboratory Press, New York.

Schmidt, T. and Heslop-Harrison, J. S. (1996). The physical and genomic organisation of microsatellites in sugar beet. Proceedings of the National Academy of Sciences, USA **93**: 8761-8765.

Schneider, K. (2005). Mapping populations and principles of genetic mapping. *In*: The Handbook of Plant Genome Mapping. Genetic and Physical Mapping (Eds. K. Meksem and G. Kahl) Wiley-VCH Gmbh & Co. Weinhem, Germany. Pp 4-21.

Shan, X., Blake, T. K. and Talbert, L. E. (1999). Conversion of AFLP markers to sequence specific PCR markers in barley and wheat. Theoretical and Applied Genetics **98**: 1072-1078.

Silva, M. C., Varzea, V., Guerra-Guimaraes L., Azinheira, H. G., Fernandez, D., Petitot, A-S, Bertrand, B., Lashermes, P. and Nicole, M.(2006). Brazilian Journal of Plant Physiology **18**: 119-147.

Stansfield, W. D. (1986). Theory and problems of genetics. McGraw-Hill Book Company, New York.

Steel, R. G. D. and Torrie, J. H. (1981). Principles and procedures of statistics: A biometric approach (2nd Edition). McGraw-Hill Book Inc. Auckland

Steiger. D. L., Nagai, C., Moore, P. H., Morden, C. W., Osgood, R. V. and Ming, R. (2002). AFLP analysis of genetic diversity within and among *Coffea arabica* cultivars. Theoretical and Applied Genetics. **105**: 209-215.

Tanksley, S. D. and McCouch, S. R. (1997). Seed banks and molecular maps: unlocking genetic potential from the wild. Science **277**: 1063-1066.

Van der Graaff, N. A. (1978). Selection for resistance to coffee berry disease in arabica coffee in Ethiopia. Evaluation of selection methods. Netherlands Journal of Plant Pathology **84**: 205-215.

Van der Graaff, N. A. (1982). The principles of scaling and the inheritance of the coffee berry disease in *Coffea arabica*. Euphytica **31**: 735-740.

Van der Vossen, H. A. M., Cook, R. T. A. and Murakaru, G. N. W. (1976). Breeding for resistance to coffee berry disease caused by *Colletotrichum coffeanum* Noack Sensu Hindorf in *Coffea arabica* L. I. Methods of preselection for resistance. Euphytica **25**: 733-756.

Van der Vossen, H. A. M. and Waweru, J. M. (1976). A temperature controlled inoculation room to increase the efficiency of preselection for resistance to coffee berry disease. Kenya Coffee (May): 164-167.
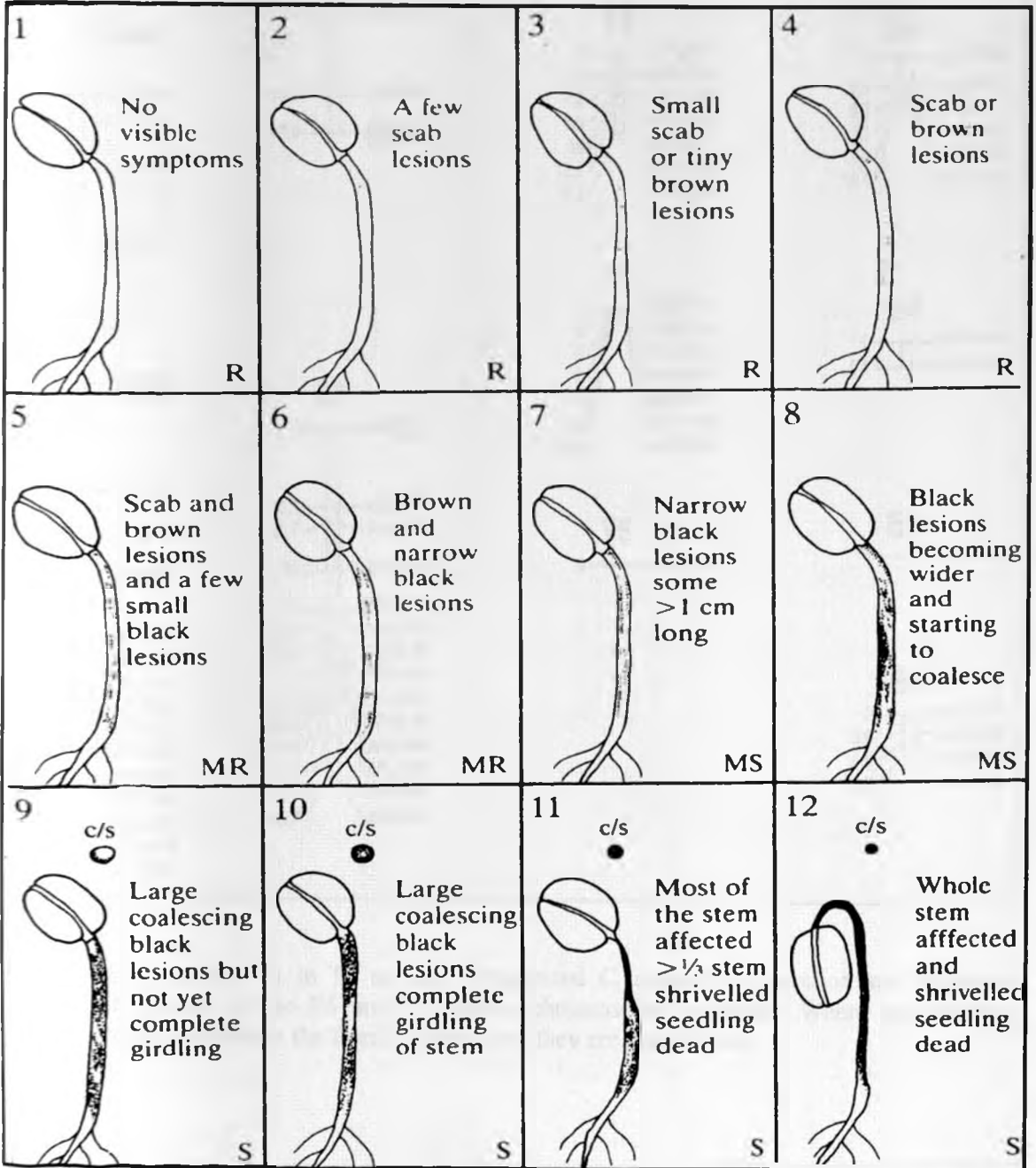
Van der Vossen. H. A. M. and Walyaro, D. J. (1980). Breeding for resistance to coffee berry disease in *Coffea arabica* L. Inheritance of the resistance. Euphytica **29**:777-791.

Van der Vossen. H. A. M. and Walyaro, D. J. (1981). The coffee breeding programme in Kenya: A review of progress made since 1971 and plan of action for the coming years. Kenya Coffee **46**: 113-130.

Van der Vossen, H. A. M. (2006). State-of-Art of developing Arabica coffee cultivars with durable resistance to coffee berry disease (Colletotrichum kahawae).ASIC Proceedings 11-15 September, Montpellier, France pp 794-801.

Varzea, V. M. and Marques, D (2005). Population variability of Hemileia vastatrix vs. coffee durable resistance. *In*: Zambolin, L., Zambolin, E. and Varzea, V. (Eds), Durable resistance to coffee leaf rust. Universidade Federal de Viçosa, Brazil, pp 53-74

Von Korff, M., Wang, H., Léon, J. and Pillen, K. (2004). Development of candidate introgression lines using exotic barley accession (*Hordeum vulgare* ssp *spontaneum*) as donor. Theoretical and Applied Genetics **109**: 1736-1745.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., van der Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M. and M Zabeau (1995). AFLP: a new technique for DNA fingerprinting. Nucleic Acids Research **23**: 4407-4417.

Waller, J. M. (1971). The incidence of climatic conditions favourable to coffee berry disease in Kenya. Experimental Agriculture 7: 303-314.

Waller, J. M., Bridge, P. D.., Black, R. L. and Hakiza, G. (1993). Differentiation of the coffee berry disease pathogen. Mycological Research **97**: 989-994.

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

Walyaro, D. J., van der Vossen, H. A. M., Owuor, J. B. O. and Masaba, D. M. (1984). New varieties to combat old problems in Arabica coffee production in Kenya. *In*: Hawksworth, D. L. (Ed) Advancing agricultural production in Kenya. Commonwealth Agricultural Bureau, Wallingford, UK pp 146-151.

Wang, X., Lai, J., Liu, G. and Chen, F. (2002). Development of a SCAR marker for the *Ph1* locus in common wheat and its application. Crop Science **42**: 1365-1368.

Wang, Y., Reighard, G. L., Layne, D. R., Abbot, A. G. and Huang, H. (2005). Inheritance of AFLP markers and their use for genetic diversity analysis in wild and domesticated pawpaw *Asimina triloba* (L.) Dunal]. Journal of American Society of Horticultural Science **130**: 561-568.

Weiland, J. J. and Yu, M.H. (2003). A cleaved amplified polymorphic sequence (CAPS) marker associated with root knot nematode resistance in sugar beet. Crop Science **43**: 1814-1818.

Welsh, J. and McClelland, M. (1990). Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Research **18**: 7213-7218.

Williams, J. G. K., Kubelik, A. R., Livak, K. J., Rafalski, J. A. and Tingey, S. V. (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Research **18**: 6531-6535.

Wrigley, G. (1988). Coffee. Longhorn Scientific and Technical, New York. 639 p

Yang, W., Weaver, D. B., Nielsen, B. L. and Qiu, J. (2000). A preliminary linkage map of soybean using an intraspecific cross of two cultivars: 'Pekin' and 'Lee'. Soybean Gen. Newsletter **27** (Online Journal) http://www.soygenetics.org/articles/sgn2000-019.htm.

Zamir, D. (2001). Improving plant breeding with exotic genetic libraries. Nature Reviews|Genetics **2**:983-989

Zhang, Y. and Stommel, J. R. (2001). Development of SCAR and CAPS markers linked to the *Beta* gene in tomato. Crop Science **41**: 1602-1608.

9. APPENDICES

Appendix 1. Sketch diagrams of the scoring system (Classes 1 to 12) of coffee seedling hypocotyls after inoculation with *C. kahawae* as described by van der Vossen *et al.* (1976).
(Source: Wrigley, 1988).

1 No visible symptoms R	**2** A few scab lesions R	**3** Small scab or tiny brown lesions R	**4** Scab or brown lesions R
5 Scab and brown lesions and a few small black lesions MR	**6** Brown and narrow black lesions MR	**7** Narrow black lesions some >1 cm long MS	**8** Black lesions becoming wider and starting to coalesce MS
9 c/s Large coalescing black lesions but not yet complete girdling S	**10** c/s Large coalescing black lesions complete girdling of stem S	**11** c/s Most of the stem affected >⅓ stem shrivelled seedling dead S	**12** c/s Whole stem afffected and shrivelled seedling dead S

Key: R: Resistant; MR: Medium resistant; MS: Medium resistant, S: Susceptible
c/s: cross section

Appendix 2: Genetic linkage groups in an introgressed *C. arabica* line, based on analysis of a F_2 population of a cross between cv Sarchimor line T5296 and a wild Ethiopian *C. arabica* collection (ET6) as mapped by Ansaldi (2003).
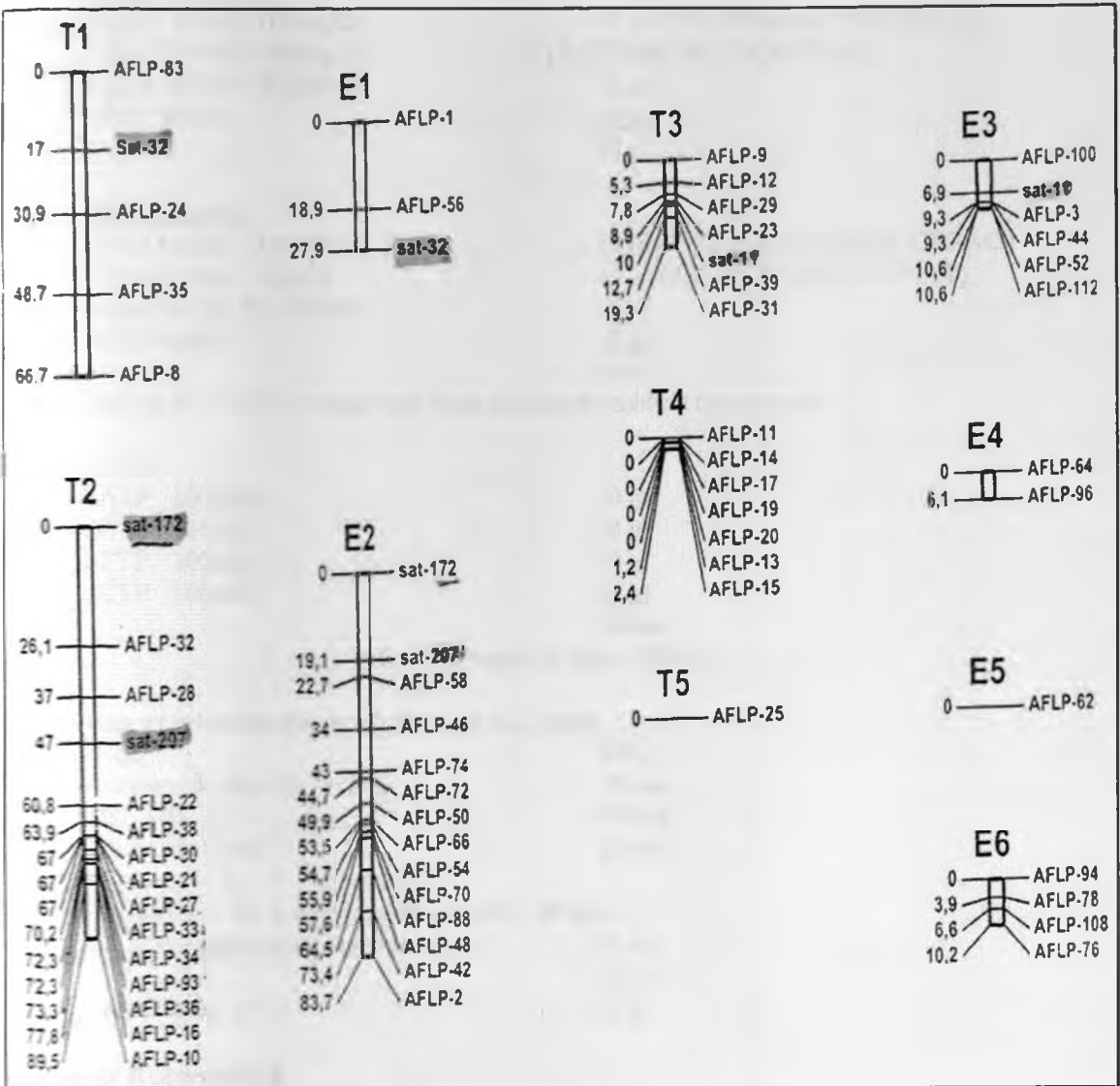
NB.   Linkage groups T1 to T5 are the introgressed *C. canephora* chromosomal fragments while groups E1 to E6 are *C. arabica* chromosome segments. Where the numbers correspond between the T and E fragments, they are homologous.

Adaptors for AFLP

*Eco*RI Adaptor

Oligo 1 *Eco*RI 100 ng/µl	17 µl (CTCGTAGACTGCGTACC)
Oligo 2 *Eco*RI 100 ng/µl	15 µl (CTGACGCATGGTTAA)
Ligase buffer 5X (Gibco)	12 µl
AFLP Water	<u>16 µl</u>
	60 µl

*Mse*I Adaptor

Oligo 1 *Mse*I 1µg/µl	16 µl (5'-GACGATGAGTCCTGAG)
Oligo 2 *Mse*I 1µg/µl	14 µl (5'-TACTCAGGACTCAT)
Ligase buffer 5X (Gibco)	12 µl
AFLP water	<u>18 µl</u>
	60 µl

Heat to 95°C for 1 minute and leave to cool at ambient temperature

dNTPs 5mM

dATP	100mM	50 µl
dGTP	100mM	50 µl
dTTP	100mM	50 µl
dCTP	100mM	<u>50 µl</u>
		200 µl

Add AFLP water to make 1000 µl

Denaturing acrylamide stock solution for 1 L (6%)

Urea	500 g
Acrylamide bis (19:1) 40%	150 ml
TBE 10X	100 ml
Water (distilled)	350 ml

Ingredients for 1 acrylamide gel (33 x 39 cm)

6% acrylamide stock solution	80 ml
10% APS	300 µl
TEMED	30 µl

Denhardt Reagent 50X

Ficoll (type 400)	5 g
Polyvinylpyrrolidone	5 g
Bovin Serum Albumin	5 g
Deionised water	make volume to 500 ml

The solution was filtered and stored at -20°C.

DNA Extraction buffers
(Before extraction, the buffers were kept for 20-30min at 62 °C).

(i) Extraction buffer*

NaCl	8.77 g
Matab	2% (2 g, added just before extraction) (Mixed Alkyltri-methylammonium Bromide)
Sarcosil	3% (9.5 ml of 5% solution) (N-Lauroyl-Sarcosine)
Sodium bisulphite	1% (1 g, added just before extraction)
Tris HCl	0.20M (20 ml of 1 M, pH=8.0)
EDTA	40mM (1.49 g)

* The solution was viscous. It was dissolved at 40°C and stored at 4 °C

(ii) Lysis buffer

Sorbitol	0.35M (6.38 g)
Tris-HCl	0.20M (20 ml of 1 M, pH=8.0)
EDTA	40mM (1.49 g)
PVP	2% (2g) (polyvinyl pyrrolidone, added just before extraction)
	Volume up to 100ml with distilled water

NB: Addition of 0.1% active carbon charcoal helped to reduce off colour of the DNA.

EDTA 0.5M pH 8 at 25°C (1L)

EDTA	186 g
NaOH	20 g

Add distilled water, dissolve, adjust pH and adjust final volume to 2L

Formamide Blue (for loading in denaturing acrylamide gels)

Formamide 98%	49 ml
EDTA 10mM	186 mg
Bromophenol Blue	125 mg
Xylene cyanol	a pinch

KPB pH 6.5, 0.5 mM (Potassium Phosphate Buffer)

Dissolve enough weights of KH_2PO4 and K_2HPO4 to make 0.5M of each in deionized water and adjust pH to 6.5

LB medium (Luria-Bertani)

LB premix	25 g
Water	1 L

To make LB plates, add 15g per litre to the mix above, autoclave and pour into 9 cm plates

LICOR loading buffer 2X

For 50 ml of the stain

49 ml	98% of Formamide
1 ml	EDTA 0.5M, pH 8 (10mM EDTA)
A pinch	Bromophenol blue

Loading blue for Agarose

10X (100mls)	
Glycerol	30% (30 ml)
Bromophenol Blue	0.25% (250 mg)
Xylene cyanol	0.25% (250 mg)
EDTA	10mM (20 ml of 0.5M pH 8.0)

Either of the dyes can be omitted to have a single dye loading solution

SSC 20X (Saline Sodium Citrate)

NaCl	175.3 g
NaAct	88.2 g

Dissolve in about 800 ml deionized water and adjust pH to 7.0 with 1N HCl
Adjust volume to 1 L

SSR dNTPs

dATP	1.25 µl*
dCTP	25 µl
dGTP	25 µl
dTTP	25 µl
AFLP water	423.7 µl
Total volume	500 µl

*this nucleotide is added in small amount because more of it is added as the radioactive nucleotide (αdATP P^{33})

TAE 50X (1L)

Tris	242 g
Glacial acetic acid	57.1 ml
EDTA 0.5M pH 8	100 ml
Make volume to 1 L	

TBE 10X (2L) (Tris Boric acid EDTA)

Tris	216 g
Boric acid	110 g
EDTA 0.5M pH 8	80 ml
Distilled water	top up to 2 L

TE (Tris –EDTA buffer)

Tris HCl 1M pH=8	1 ml
EDTA 0.5 M pH=8	200 µl
Make volume to 100 ml	

Appendix 4: DNA Cloning Protocol
Adopted from manufacturer's manual (Invitrogen Life technologies)

(i) Remove the required number of vials of the competent cell from the -80°C freezer and place them in ice to thaw. Never at room temperature and do not shake them.
(ii) Prepare the cloning/ligation reaction as follows:-

- Fresh PCR product   0.5-4 µl (estimate according to intensity of bands in the agarose gel)
- Salt solution   1 µl
- Sterile (AFLP) water   add to make volume to 5 µl (depends on the volume of PCR added)
- TOPO® vector   1 µl
                              Total volume is 6µl

NB: store all reagents at -20°C when finished but salt solution and water can be stored at room temperature. The cloning reaction mix may be stored overnight at -20 °C

(iii) Mix the reaction mix gently (by gentle stir) and incubate for 5 MINUTES at room temperature (22-23 °C)
(iv) Place the reaction on ice and proceed to transforming competent cells (which have been thawing in ice).
(v) Equilibrate a water bath at 42 °C and warm the vial of SOC medium (provided with kit) to room temperature
(vi) Add 2 µl of the cloning reaction into vials of One Shot® Chemically competent E coli and mix gently. Do not mix by pipetting up and down (Stir slightly)
(vii) Incubate the mix on ice for 5-30 min (15)
(viii) Heat shock the cells for 30 sec in the water bath at 42 °C and immediately transfer back into ice
(ix) Add 250µl of the room temperature SOC medium
(x) Cap tubes lightly and shake them horizontally (200rpm) at 37 °C for 1 hr (do not exceed)
(xi) While this is on-going, prepare the plates as follows:
(a) Warm pre-selection plates for 30 min (LB medium containing 50-100 µg/ml ampicillin)
(b) Spread 40 µl of 40mg/ml X-gal in DMF (dimethylformamide) on each plate and incubate at 37 °C until ready to use
(xii) Spread 10-50µl from each transformation on a pre-warmed selective plate. To ensure good spreading of small volumes, add 20 µl of SOC medium
(xiii) Incubate the plates at 37 °C overnight.
(xiv) Pick 5-10 white or light blue colonies for analysis of positive clones

Appendix 5: Extracting plasmid DNA from transformed bacteria
Adopted from manufacturer's manual (QIAprep® spin miniprep kit using Micro-centrifuge)

(i) Transfer 1800 µl (2x900 µl) of the culture broth into 2ml tubes. Centrifuge at 8000 rpm for 5 min to obtain bacterial clot. Discard the supernatant and add the same quantity again and repeat the centrifugation

(ii) Re-suspend the pelleted bacterial cells in 250 µl buffer P1 (stored in refrigerator). Ensure that RNase A has been added to buffer P1. Vortex to mix completely so that no clumps are visible.

(iii) Add 250µl of buffer P2 and immediately gently invert the tubes 4-6 times. Do not vortex. You can invert more times if necessary. Do not allow the lysis reaction to proceed for more than 5min.

(iv) Add 350µL of buffer N3 and invert the tube immediately but gently 4-6 times. To avoid localized precipitation, mix the solution gently but thoroughly immediately after addition of buffer. NB. The solution should become cloudy.

(v) Centrifuge for 10 min at 13,000 rpm (~17,900xg) in a conventional table-top micro-centrifuge. A compact white pellet will form

(vi) Apply the supernatants from step (v) to the QIAprep spin column by decanting or pipetting and centrifuge for 30-60 sec. and discard the flow through.

Optional:- Wash the QIAprep spin column by adding 0.5 ml buffer PB and centrifuging for 30-60 s and discard the flow through.

(vii) Wash QIAprep spin column by adding 0.75 ml Buffer PE and centrifuging for 30-60 s

(viii) Discard the flow through, and centrifuge for an additional 1min to remove residual wash buffer. Note: the residual wash buffer will not be completely removed unless the flow-through is removed. Residual ethanol in the buffer will inhibit subsequent enzymatic reactions.

(ix) Place the QIAprep column in a clean 1.5 ml micro-centrifuge tube. To elute DNA, add 50 µl Buffer EB (10mM Tris-HCl, pH8.5) or water to the centre of each QIAprep spin column, let stand for 5 min, and centrifuge for 1 min. The pure plasmid DNA is now in the flow-through.

Appendix 6: Labelling of hybridization probes
Adopted from manufacturer's manual (Megaprime™ DNA labelling Systems: RPN 1604-Amersham Biosciences)

1. Dissolve the DNA to be labelled to a concentration of 25 ng/µl in distilled water
2. Thaw the required solutions of Megaprime system except the enzyme from -20 °C to room temperature, and return them immediately after use
3. Place 25ng of template DNA into reaction tube (0.5 ml) and 5µl of the primers followed by enough water to make the volume 50 µl. Denature by heating to 95-100 °C for 5minutes in a boiling water-bath or incubator
4. Spin briefly in a micro-centrifuge to bring the contents to the bottom of the tube
5. Keeping the tubes at room temperature, add 4µl of each of the unlabelled nucleotide, 5 µl of reaction buffer (RPN 1604/5) followed by 5µl of the radio-labelled dNTP (dCTP) and enzyme (2 µl)
6. Mix gently by pipetting up and down
7. Incubate at 37 °C for 10 min
8. Denature the labelled DNA by heating to 95-100 °C for 5 min and chill on ice until when added into the hybridization tubes