CHARACTERIZATION OF THE GENETIC DIVERSITY AND PATHOGENECITY OF C*olletotrichum kahawae,* USING RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD) ANALYSIS.

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DECLARATION

This Thesis is my original work, except where due reference is made in the text, and has not been presented for the Award of a degree in this or any other University.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

DEDICATION

This work is dedicated to my mother Esther Aketch, my father Jonna Owaa, my husband Robert and children, Bob, Crystal and Diana Scofield whose love and support made me move on.

ACRONYMS AND ABBREVIATIONS

AFLP	Amplified Fragment Length Polymorphism
ANOVA	Analysis of Variance
CBD	Coffee Berry Disease
CCA	*Colletotrichum Coffeanum Acutatum*
CCM	*Colletotrichum Coffeanum* Mycelia
CCP	*Colletotrichum Coffeanum* Pink
CRF	Coffee Research Foundation
CTAB	Cetylmethylammonium bromide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphates
IEC	Isoelectric Focussing Electrophoresis
EDTA	Ethylenediaminetetra acetic acid
IPM	Integrated Pest Management
MEA	Malt Extract Agar
PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
SRSF	Simple Repetitive Sequence Fingerprinting
TE	Tris EDTA buffer
TAE	Tris-ammonium EDTA buffer
VNTR	Variable Number of Tandem Repeat

ABSTRACT

Coffee is a small shrub which is a source of a stimulating beverage. It ranks as one of the world's major commodity crops and is the major export product of many countries including Kenya. Economic production of Arabica coffee in Kenya is greatly hindered by the Coffee Berry Disease (CBD) caused by *Colletotrichum kahawae*. Since the release of the resistant Ruiru 11 in 1985, efforts have been devoted to the improvement of the genetic base of resistance, but this has faced the problem of possible pathogen variation. A good understanding of CBD pathogen diversity could lead to the development of cultivars with sufficient disease resistance. This study was aimed at determining the genetic and virulence diversity of *Colletotrichum kahawae*, and their correlation with coffee growing regions and interaction with coffee varieties. Infected berries were obtained from three coffee growing regions in Kenya (Western, Central and Eastern regions). Arabica varieties in these regions include SL 28, SL 34, K7, and the resistant Ruiru 11. At each location, diseased berries were randomly sampled from susceptible and resistant plants in marginal and upper coffee zones. In locations where both resistant and susceptible varieties were grown, a larger proportion of the berries were obtained from the resistant varieties to increase the chances of obtaining different pathotypes. A total of 34 single conidia isolates were obtained and subjected to variation analysis using DNA banding patterns. The analysis revealed 5 distinct groups of isolates of which one representative isolate was selected and used to innoculate coffee differentials with known resistance / susceptible reactions. The varieties included Rume Sudan, Catimor, K7 and SL 28 (susceptible). Seedlings were individually scored for disease symptoms developed and mean grade of infection computed. The mean grade data was then used to perform Analysis of Variance (ANOVA) using the random effects model. The results were used to determine the correlation between molecular polymorphism and diversity in virulence. The variety x isolate interaction effects although significant ($p<0.05$) did not conclusively reveal the existence of races because the isolate effect was not significant. The virulence tests revealed that variation was due to main effects of varieties. Rume Sudan was highly resistant with a mean grade of 4.75. The variety is known to carry two resistant genes on R- and k- loci. Catimor (mean grade = 7.66) showed medium resistance. It is also known to carry resistant genes on the dominant T-locus. K-7, with recessive gene on k- locus, showed medium resistance with a mean grade of 9.97. SL 28, with no known resistant genes was highly susceptible with a mean grade of 11.75. The growing regions had no influence on the genetic and virulence diversity since *C. kahawae* isolates from all regions were pathogenic on the coffee cultivars tested. All the isolates were significantly ($p<0.05$) more aggressive on coffee cultivar SL28, followed by K7 and Catimor in that order. Rume Sudan showed high resistance to all the isolates tested irrespective of the region. It is concluded that variation in *Colletotrichum kahawae* population is largely due to differences in aggressiveness of the isolates. There is no conclusive evidence of the existence of races. More biochemical and molecular markers for Pathogenisity should be screened on large sample of isolates across coffee growing regions using large number of primers.

CHAPTER ONE
INTRODUCTION

1.1 *Coffea* Species

Coffea is a large genus containing about 100 species of flowering plants in the family *Rubiaceae* with over 6000 species (Wrigley, 1988). Coffee is a woody perennial evergreen dicotyledon. They are shrubs or small trees native to subtropical Africa, from Congo basin to the highlands of Ethiopia and Southern Asia. Seeds of several species are the source of the beverage coffee. The seeds are called "beans" in coffee trade. Coffee 'beans' are widely cultivated in tropical countries in plantations for both local consumption and export to temperate countries. Coffee ranks as one of the world's major commodity crops and is the major export product of many countries. Two main species have become significant in world trade; *Coffea arabica* which forms the major proportion of world trade (80%) and C*offea canephera* (Robusta coffee) which forms the remainder (Wrigley, 1988; Biratu *et al*., 1995).

Two other species C*offea liberica* and C*offea excelsa,* which contribute less than 1%, produce inferior beverage and are only grown in some African countries, primarily for local use (Wrigley, 1988; Biratu, 1995). Whereas robusta coffee plants are more robust (strong) than the Arabica plants, it produces an inferior tasting beverage with higher caffeine content. Caffeine content in coffee is a natural defense repelling many predators that would otherwise feed on the seeds. Both Robusta and Arabica coffee plants can grow to a height of 10 meters if not pruned; but a reasonable height of 3 – 3.5 is maintained for easy harvesting. C*offea arabica* plant is self-pollinating, limiting the genetic variability of the species while the robusta coffee plant is cross-pollinating. *C. arabica* is the only tetraploid of the genus *coffea* with (2n=44) (Omondi, 1998). It therefore does not hybridize with other species without special measures taken. The basic chromosome number of coffee is 11 (Bouharmont, 1963).

C. canephora is similar to *C. arabica* in many ways although the former is diploid (2n = 22). It is a larger tree, with larger leaves and the laminae between veins are more convex than in Arabica. It is self sterile and cross-pollinates easily. There are two types of *C. canephora*; (1) Erecta type which is an upright tree, (2) Nganda type which is a spreading form. Robusta has higher caffeine content (2-3%) compared to Arabica (1-1.5%) and the beans are usually smaller, having lower quality due to lack of flavor and acidity than Arabica seeds. Most *C. canephora* are resistant to coffee rust caused by *Hemileia vastatrix* (Omondi, 1998). *C. canephora* is found throughout the rain forest of the Congo River basin and on the higher land to the North-East as far as the shores of lake Victoria in Uganda up to about 1500m altitude. It also occurs in the coastal rain forest from the Congo area as far as west of Ivory Coast.

1.2 Origin and distribution of coffee

C. arabica originated in the highlands of South-West Ethiopia (Ferwerda, 1976). In this region there are a number of variants some of which have been considered to be subspecies from Boma plateau in the Sudan where it still grows wild (Sylvain, 1958; Meyer, 1969). Some indigenous populations have also been identified around Mount Imatong in Sudan and Marsabit in Kenya (Berthaud and Charrier, 1988). *C. arabica* was mainly used by the local inhabitants of the Ethiopian highlands but its commercial use started when variety typica was introduced into the Yemen by Arab traders. From Yemen it spread to Asia, Latin America and Africa. Coffee from Yemen gave rise to two botanical types (Krug *et al.*, 1939). *C. Arabica var. typica crammer* was introduced to Latin America and Asia. It has bronze – tipped young leaves and pendulous fruit bearing branches. *C. Arabica* variety bourbon was introduced to S. America and E. Africa through the islands of Reunion, formerly Bourbon. It has young green leaves and fruit bearing branches bent down only at the tips (Lashermes *et al.*, 1996). Coffee in Kenya was introduced by Scottish Missionaries in 1893 and planted in Bura in Coast province (Wrigley, 1988). Climate at Bura was unsuitable and cultivation of Coffee was transferred to Kibwezi in Makueni District, Eastern Province and Kikuyu in Central province and later established in areas around Mt. Kenya, West of the Rift, Taita hills in Coast Province and Abardare Regions.

1.3 Economic importance of coffee

Coffee is the world's second most traded commodity after fossil oil (Clarke, 1985). It is the most important agricultural commodity upon which the economy of more than 50 producer countries depend on (Rodrigues *et al.*,1975) and a foreign currency earner for many countries. For instance, in Uganda and Burundi coffee exports contribute about 80% of total hard currency revenue (Anon, 1994). It plays a key role in creating employment for many people in the countries where it is grown. In Kenya, coffee is third most important agricultural product after tea and horticulture contributing up to 25% of the total foreign exchange earnings (Opile, 1993). About 30% out of the 70% of Kenya's workforce in agriculture are employed by coffee industry.

1.4 Production and market

Brazil dominates the world production and has a major influence on the world coffee industry. Production amounted to approximately 21% of the worlds output in 1992 with Columbia, second largest producer recording an output of 16%, most of which is Arabica. Indonesia is the largest single producer of Robusta coffee in the world followed by Ivory Coast (Omondi, 1998). Highland areas in Kenya, Tanzania, Uganda, Angola, Burundi, Cameroon, Ethiopia, Malawi, Rwanda, Zambia and Zimbabwe also produce Arabica coffee. Robusta coffee is produced commercially at lower altitudes in Western and Central Africa (Wrigley, 1988). Ivory Coast is the largest African producer with 4.1% of world production.

A high proportion of world's coffee is imported by developed countries. Consumption in the producing countries and other developing states is small with the largest individual importer being U.S.A, which imports 23% of the world's total production. European Economic Community as a whole imports 39% of the world's total production. Germany imports most Coffee into the European market followed by France and U.K

1.5 Coffee growing conditions

There are two optimal growing climates for Arabica coffee namely; (1) the subtropical regions at high latitudes of 16 – 24^0 , (2) the equatorial regions at latitude lower than 10^0 and altitudes of 1060 – 1890m. In the subtropical regions rainy and dry seasons must be well defined and altitude must be between 540 – 1080m. These conditions result in one coffee growing season and one maturation season, usually in the coldest part of autumn. Mexico, Jamaica, Brazil and Zimbabwe are examples of areas with these conditions. The equatorial regions are characterized by frequent rainfall causing almost continuous flowering which results in two coffee harvesting seasons. The period of highest rainfall determines the main harvesting period, while the period of least rainfall determine the second harvest season. In such regions with frequent rainfall, mechanical drying is preferred. Examples of countries that have this climate include Kenya, Colombia and Ethiopia. Both species *(C. arabica and C. canephora)* require soils that are deep, well drained, loamy, slightly acidic (pH 5.0-6.0), rich in humus and exchangeable bases, particularly potassium. Phosphorus is essential during flowering (Wrigley, 1988 and Coste, 1992).

1.6 Challenges facing coffee production in Kenya

Kenya produces less than 1% of the world's coffee (Njoroge, 1992). Despite Kenyan coffee fetching premium prices in the world market due to its fine quality (Omondi, 1998; Karanja, 1996), coffee yield has remained low with small holders recording an average of 534 Kg of clean coffee per ha (2.8 Kg of cherry per tree) and plantations recording an average of 1064 Kg of clean coffee per ha (5.6 Kg of cherry per tree) compared to yields of 3.5 tones per ha (18.4 Kg of cherry per tree) achieved in some estates. This has been due to pests and diseases which need intensive fungicide sprays at susceptible stages (Griffiths *et al.*, 1971). The sprays are costly and not affordable to most coffee farmers. The main diseases attacking coffee in Kenya are coffee berry disease (CBD) caused by *Colletotrichum kahawae*, coffee leaf rust (*Hemileia vestatrix Berker*) and bacterial blight of coffee *(Pseudomonus syringae PV.* Garcase Van hall, Waller *et al.*, 1993). Coffee berry disease is a major limitation for arabica coffee cultivation in Africa and for which genetic control is only partially effective (Manuel *et al.*, 2010). More emphasis is being placed on integrated pest management (IPM) which uses self-renewing forms of pest control such as biological control (Omondi, 1998).

An Arabica coffee variety, Ruiru 11 that was developed at the Kenya's Coffee Research Station, Ruiru and released to growers in 1985 is resistant to both CBD and Coffee Leaf Rust (Omondi, 1998). It is also high yielding, has good quality and compact growth which is favorable for high density planting. Farmers who grow Ruiru 11 save up to 30% of the production costs that go to CBD control alone (Nyoro and Prey, 1986). Crop loses can reach more than 50% if no control measures are applied (Loureiro *et al.*, 2007). The resistance of Ruiru 11 to CBD faces the problem of possible pathogen variability (Omondi, 1998). Virulence tests using isolates from Ethiopia (Van der Graaft, 1978) and Kenya (Masaba and Van der Vossen, 1980), revealed variation in aggressiveness but no races were detected.

Omondi (1998), observed that CBD could be isolated from resistant varieties, while (Rodrigues *et al.*, 1991) found that Angolan and Malawian strains of *C. kahawae* possessed characteristics different from the Kenyan isolates. These isolates were highly aggressive on the Catimor variety, some of which were used as the mother parents in the production of the disease resistant hybrid variety Ruiru 11.

1.7 Problem Statement and Justification

Economic production of Arabica coffee in Kenya is greatly hindered by coffee berry disease. It is believed that breeding for resistance to CDB may provide a sustainable long-term management of the disease (Omondi, 1999). Since the release of resistant Ruiru 11 in 1985 efforts have been devoted to the improvement of the genetic base of resistance, but this has faced the problem of possible pathogen variation (Omondi, 1999). A good understanding of CBD pathogen's genetic diversity could lead to development of cultivars with sufficient disease resistance. According to Nyoro and Sprey (1986), CBD control requires intensive spray programmes which account for up to 30% of the total cost of the production in the estate sector. Small scale farmers are not able to carry out the recommended intensive spray programmes and sometimes end up applying one or two fungicide sprays per year thus inducing higher levels of CBD than what could occur in their total absence (Griffiths, 1972). CBD fungus was also reported to have developed resistance to some of the recommended systematic fungicides only after 2-3 years of continuous spraying (Cook, 1975; Okioga, 1976). Host – plant resistance is therefore relatively cheap, biologically safe and self sustaining (Hogenboom, 1993). This study was aimed at documenting the diversity of the CBD pathogen population, demonstrating pathogen diversity in correlation with location and coffee varieties and proposing the deployment of resistance genes in coffee varieties and coffee growing regions.

1.8 Hypotheses

- There is no genetic diversity in *C. kahawae* species.
- There is no virulence diversity among *C. kahawae* species.
- The pathogen population is uniform across regions and host varieties.

1.9 General Objective

To determine the diversity of *C. kahawae,* and possible correlation between pathogen diversity and localities of origin of the isolates and host varieties.

1.9.1 Specific Objectives

- To determine the genetic diversity of *C. kahawae* using Random Amplified Polymorphic DNA (RAPD).
- To determine virulence diversity by inoculation of hypocotyls seedlings of coffee varieties with known resistance/susceptibility
- To determine the correlation between pathogen diversity and growing regions as well as host varieties.

CHAPTER TWO
LITERATURE REVIEW

2.1 *Colletotrichum* species of *Coffea arabica*

Several species and strains of C*olletotrichum* have been isolated from coffee but only one of them is capable of causing CBD or green berry anthracnose. CBD was originally thought to be caused by a virulent form of *C. coffeanum* but studies by Waller *et al.*, (1993), revealed that it is a separate species; *C. kahawae*, related to the group of species *C. gloesporioides*. Studies by Sreenivasaprasad *et al.*, (1993), using RFLP found a close association between the two species. *C. coffeanum* that causes leaf spots and twig symptoms on *C. arabica* in Brazil was described by Noak (1901). The name was also adopted by Mc Donald (1926), for the disease of immature fruits in coffee plantations in Western Kenya, around Mt. Elgon. The Brazilian strain was not pathogenic on green coffee berries (Omondi, 1998). Rayner (1941), distinguished the E. African strain causing CBD as *C. coffeanum var.virulans* which is believed to have arisen due to mutations from mild parasitic form like the one causing brown blight (Nutman and Roberts, 1960 b) and was occurring on *C. eugenioides*, a diploid coffee species found in high altitudes forests of Western Kenya and Eastern Uganda (Robinson, 1976; Mogaka, 1975).

C. kahawae attacks young, expanding berries between 8 and 20 weeks old and ripe berries. No other strains are pathogenic on green berries and many occur epiphytically in the bark (Stephen and Rebecca, 1991). The different strains of C*olletotrichum* are distinguished on the basis of conidia shapes and colony characteristics while growing on agar. The CBD strains have a green to dark mycelium after 2-3 days on all media used while saprophytic forms exhibit white mycelium (Stephen and Rebecca, 1991).

Gibbs (1969), differentiated *Colletotrichum* species into four strains based on colony characteristics of a single conidial cultures of isolates from host tissue grown on 2% Malt Extract Agar (MEA), as follows: (a) *Colletotrichum coffeanum* (*Var. virulans*) with slow growth, profuse grayish-black mycelia and conidia borne directly on hyphae; (b) *Colletotrichum coffeanum* pink type (CCP) with slow growth, profuse pink areal mycelia, conidia borne directly on hyphae; (c) *Colletotrichum coffeanum* mycelia type (CCM) with fast growing profuse pace aerial mycelia and conidia borne directly on hyphae and (d) *Colletotrichum coffeanum acutatum* (CCA) with moderately fast growth, sparse pale aerial mycelia, conidia produced in acervuli.

Hindorf (1970), used discriminant analysis on a range of morphological characteristics and retained the name *C. caffeanum* for the CBD strain. He later along with other colleagues (Hindorf *et al.*, 1997), confirmed that *C. kahawae* correctly identifies the pathogen causing CBD. In a comparative study on a range of *Colletotrichum* isolates using morphological, pathological and biochemical criteria, Waller *et al.*, (1993), differentiated the CDB pathogen from other similar *colletotrichum* strains which occur on coffee and suggested that it belongs to a distinct species which was then named *C. kahawae* species. Genus C*olletotrichum* in which the species belong is classified into *Eumycota*, a major subdivision of the *deutromycotina*, in the class *coelomycetes*, order *melanciniales* and family *melanconiaceae* (Biratu *et al.*, 1995).

2.2 Distribution of *Colletotrichum kahawae* species

Coffee Berry Disease was first reported in Kenya in 1922 (Mc Donald, 1926). It has since then been reported in most Arabica coffee producing countries in Africa such as Angola in 1930, Congo in 1937, Cameroon in 1957, Uganda in 1959, Tanzania in 1964, Ethiopia in

1971 (Van der Graaff, 1981; Van der Vossen, 1985) and Malawi in 1985 (Lutzeyer *et al.*, 1993). The disease is not known outside Africa, although a leaf sport and ripe berry anthracnose caused by related *Colletotrichum* species has been reported in Guatemala and Brazil (Stephen and Rebecca 1991).

2.3 Symptoms of *Colletotrichum kahawae* species

Although the pathogen is capable of infecting leaves, stem, bark, flowers and twigs of the coffee plant, the species of c*olletotrichum* that can infect immature or green berries is the causal organism of CBD (Stephen and Rebecca, 1991). Direct loss occurs as a result of flower and young fruit infection. Flowers are susceptible at all developmental stages from the pale green unopened spike. Immature fruit or berries are most susceptible during their expansion phase which occurs from 4 -14 weeks after flowering. Earlier berry stages or pin head beans are fairly resistant, becoming susceptible again as the berry begins to ripen. Symptoms on green berries appear as small, dark, sunken lesions typical of anthracnose which may spread to cover the whole berry (Omondi, 1998). The pathogen sporulates rapidly and is evidenced by a pale pink crust on lesion surface. If infection occurs early and climatic conditions favor disease development, berry development is arrested, resulting in dry, black, mummified berries of no commercial value (Fig.1). When the berry ripens and anthracnose fully develops, the bean can become infected and seed borne. Scab lesions can also occur. In this case the pathogen grows sparsely and sporulates poorly or not at all. Scab lesions can also be observed when infections occur during the less susceptible stages of berry development and on resistant coffee varieties as a defense reaction (Mc Donald, 1932; Bock, 1956; Mulinge, 1970; Masaba and Van der Vossen, 1982; Masaba, 1991).

Fig 1: Infected coffee berries

2.4 Damage and losses

Where the virulent strains of CBD occur, serious losses have been reported. The Losses of up to 75% were reported in Kenya when the disease first occurred. In other areas, losses of up to 80% are not unusual. More conservative estimates of losses occurring in Kenya are reported to be 20%. Successful fungicide control programs frequently double or triple yields (Griffiths, 1971).

2.5 Epidemiology

Medium and long distance dispersal or spread is aided by vectors such as man (coffee pickers), birds, wind and possibly insects. Conidia produced in acervuli on developing bark of young twigs and on diseased berries provide the initial inoculum for a CBD epidemic (Nutman and Roberts, 1967; Wrigley, 1988). Omondi (1998), observed that infection process is favoured by availability of liquid water and suitable temperature. He also observed that splashes of rain disperse conidia to new infection sites. Spores germinate to produce germ tubes and appressoria from which infection pegs emerge to penetrate the surface cuticle of the berry (Firman and Waller, 1977). Typical black sunken anthracnose lesions of CBD initially appear about 1 week after inoculation and most by 2 weeks for berries in the ages between 5 – 14 weeks after flowering (Bock, 1956). Infection and disease development is influenced by several factors including temperature, moisture, plant parts and other microorganisms. The optimum temperature for the germination of spores in presence of water is about 22°C but requires the presence of nutrients, such as dilute sugar from coffee berries (Bock, 1956; Nutman and Roberts, 1960a). Optimum temperature for germination could be increased to 27°C in the presence of nutrients (Nutman and Roberts, 1960a). The maximum temperatures for germination are 30°C (35°C with nutrients) and a minimum of 10°C (Bock, 1956). This information is based on both laboratory and field observation. At optimum temperatures, spores can germinate and develop appressoria within four hours (Firman and Waller, 1977). Bock (1956), reported that under field conditions, typical black sunken lesions start to appear after one week but are fully developed after 2weeks. Mulinge (1970), reported an incubation period of between 2-4 weeks. On older and more resistant berries, lesions take longer to appear. Certain tissues of coffee appear to be extremely susceptible to CBD, with flowers, young berries and fully ripe fruit being particularly susceptible. The disease has negligible effect on berries which are more than 5 months old until during ripening when the berries become susceptible to infection again (Masaba *et al.*, 1982).

Although the incidence of CBD is high on coffee grown in the cooler high altitude zones, Waller (1971), observed that the influence of climatic conditions on lesion growth, spore production and host resistance is not adequately known and that periods suitable for infection were so numerous that even at low altitude and in dry year, the low level of CBD in these regions could not be explained by the lack of potential infection periods. Mwang'ombe *et al.*, (1991), reported that the optimum temperature for appressoria and lesion formation is between 15-25°C, which coincides with the range of temperate in the cool high altitude coffee growing zones and in the low altitude zone during the cold and wet months. Rainfall in the afternoon provides the conditions for the release of spores, their distribution and the droplets of water on the berries are unlikely to dry out for at least five hours, which enables the spores to germinate and infect the green berries (Wrigley, 1988).

2.6 Variability of *Colletotrichum kahawae*

C. arabica has its primary center of genetic diversity in Ethiopia, but the disease was not detected in Ethiopia until 1971, suggesting that the pathogen had not co-evolved with the host into a diversity of forms and therefore the rapid spread of the disease may have been due to narrow genetic base of the cultivated *Coffea arabica* (Omondi, 1998). Pathogen diversity may have also been limited by lack of a perfect state which promotes sexual recombination (Omondi, 1998).

Virulence tests with isolates of *C. kahawae* also confirmed that the CBD pathogen exhibited variation for aggressiveness but no races were detected (Van der Graaff, 1978; Masaba and Van der Vossen, 1980). In the contrary (Rodrigues *et al.*, 1992), observed that variation for virulence could be emerging. The possible mechanisms which may be responsible for the apparent physiological specialization of the pathogen include; mutations, recombination and migration (Leung *et al.*, 1993; Burdon, 1993). Variants in asexual reproducing population can emerge as a result of several mutations, one at a time which develops into co-adaptive gene complexes (Crow and Kimura, 1965). Burdon (1993), reported that in a span of 10 years eleven new races of *puccinia striiformis* in wheat were recorded in Australia. These were different from the pre-existing race at a single virulence locus. Luig and Watson (1970), also reported a proliferation of the Australian *P. graminis. sp. Tritici* in 15 years following the appearance of race 21 in 1954 which could be traced back to the process of spontaneous mutation. In Brazil, it was demonstrated that within 10 years of introduction of *Hemileia vastatrix* causing coffee leaf rust, 10 different races were detected in the breeding plots of the Instituto Agronomico in Campinas (Eskes, 1981). Burdon (1993), noted that the interactions between the ploidy level of the pathogen, the rate of mutation and the size of the population determines how rapidly variants emerge. The CBD pathogen is believed to be a product of mutation from a mild parasitic form of C*olletotrichum* species originally found in *C.*

eugenioides (Nutman and Roberts, 1960(b)). Pathogen variants may also be generated by exchange of whole nuclei or cytoplasm (heterokaryosis) (Omondi, 1998).

Variation can also emerge as a result of migration or gene flow followed by mutation or recombination (Omondi, 1998). The rapid spread of CBD pathogen from Western Kenya where it was first detected to other parts of the country and to other African coffee growing countries is a demonstration that there has been considerable migration (Omondi, 1998). The migration followed by mutation or recombination could have developed new variants. Studies carried out at CRF in Ruiru, Kenya revealed that isolates vary in aggressiveness, but they do not vary in virulence (Wrigley, 1988). Races tend to develop if the resistance is race-specific and monogenic and if the corresponding resistance is widespread. Studies have also shown that, a large part of the variation in the pathogen population was due to aggressiveness and that the differential effects were too small to suggest conclusively that races exist (Omondi *et al.*, 1999).

Ruiru 11, which combines resistance to CBD and leaf rust with high yield, fine quality and compact growth, was a major break through in management of CBD but it still faces the threat of possible pathogen variation (Omondi *et al.,* 1999). In a study with isolates from Angola, Malawi and Kenya, Rodrigues *et al.,* (1991), found that Angolan and Malawian strains of *C. kahawae* possessed different characteristics to those of the Kenyan strain. Isolates from Angola and Malawi were highly aggressive on the cultivar ‘Catimor’, which was used in Kenya as a mother parent in the production of the disease resistant hybrid variety, Ruiru 11. Rodrigues *et al.,* (1992), concluded that 5 physiological races of the CBD pathogen might exist among the Angolan, Malawian and Kenyan isolates.

Omondi *et al.*, (1999) observed a small differential interaction between isolates and the varieties indicating that the Kenyan population of *C. kahawae* has not developed into physiological races, and that the apparent absence of races was probably due to the fact that the pathogen co-evolved with genetically narrow-based host species. It was concluded that variation for virulence within *C. kahawae* is unlikely to be due to the existence of races but was predominantly due to differences in aggressiveness in combination with other environmental factors (Omondi 1998)

Bridge *et al.*, (2008), used Restriction Fragment Length Polymorphism (RFLP), Variable Number of Tandem Repeat (VNTR) and Amplified Fragment Length Polymorphism (AFLP) in which *C. kahawae* showed minimal genetic variability across the African countries. Analysis with AFLP failed to detect variability within 12 isolates from Kenya and other African countries. Further analysis of *C. kahawae* isolates with VNTR Primers, Simple Repetitive Sequence Fingerprinting (SRSF) Primers and AFLP showed almost no variability within the main groups, indicating a virtually clone population.

2.7 Management of CBD

2.7.1 Non-chemical control

Differences in host susceptibility to CBD are known. Cultivars considered to be resistant are; Ruiru 11, Geisha, Geisha 10, Blue mountain, Rume Sudan, Progenies of Hibrido Timor, and K7 (Stephen and Rebecca, 1991). Some of the susceptible cultivars are: Harar, SL selections and Bourbon. Inoculation of seedlings and subsequent analysis of hypocotyls infection is the method for selecting resistant varieties. Host-plant resistance is a means of controlling pests and diseases that is relatively inexpensive, biologically safe and therefore self sustaining (Hogenboom, 1993). The mechanism of resistance to CBD was found to be based on formation of cork barriers which was reported to be stable against changes in pathogen populations (Masaba and Van der Vossen, 1982). High levels of caffeine content have also been associated with resistance to CBD (Biratu *et al*., 1995).

Ruiru 11 which is resistant to CBD was developed in Kenya from single crosses which were combined into multiple crosses to have resistance from different sources in a single plant (Omondi, 1998). This was followed by backcrossing to restore the superior yield and quality of the traditional varieties that were otherwise susceptible (Omondi, 1998). The variety is therefore a genetically heterogeneous population of hybrids that are morphologically similar, especially for compact growth and disease resistance (Omondi, 1998). Some strains of *C. kahawae* have been isolated from Ruiru 11, thus investigation into the possible physiological specialization of the fungus and pyramiding of resistance genes is necessary (Omondi, 1998).

2.7.2 Chemical control

Rayner (1952), observed that some degree of control of CBD in Kenya could be achieved by spraying fungicides. Griffiths *et al.*, (1971), observed that early season spraying gave good results in those years when flowering was early and the rains ended early. However in a normal season the sprays failed to protect the crop and the disease became worse. It was recommended that, if the climatic and cropping conditions are favorable for CBD infection, fungicide sprays should be aimed at continuously protecting the developing crop. This intensive spray programme was found to be very expensive accounting for up to 30% of the total cost of production in the estate sector (Nyoro and Sprey, 1986). Copper fungicides are the most effective and these also control leaf rust and reduce the intensity of bacterial blights of coffee (Stephen and Rebecca, 1991). Other fungicides include; captatol, chlorothalonil, benomyl, thiophanate-methyl, thiobendazole and dithianon. Fungicide tolerance has been noted with cabendazim, cypendazols and to lesser extent benomyl (Stephen and Rebecca, 1991), thus their use was withdrawn in Kenya in the late 1970s. Cultivars with sufficient disease resistance make spraying unnecessary and save time and money for the coffee grower thus saving the scarce foreign exchange spent on importing fungicides.

2.7.3 Biological control of CBD

Masaba (1991), observed that CBD was less severe on coffee farms which have never been sprayed with fungicides. Bock (1963), also observed that inadequate fungicide sprays or improper timing of sprays also result in higher levels of disease than where the sprays have not been applied. This indicated that the sprays had substantial quantitative and qualitative effect on the non-target micro flora some of which are antagonistic to the CBD pathogen (Masaba, 1991). Intergration of biological control of CBD with other methods of control such as host plant resistance may be possible (Omondi, 1998).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Sampling isolates of *C. kahawae.*

Infected berries were obtained from areas with CBD epidemics. The areas included coffee growing regions to the East and West of Rift Valley (Table1). The locations were representative of the agroecological zones where Arabica coffee is grown in Kenya. Arabica varieties in these regions include Rume Sudan, Catimor, K7 and SL28 (susceptible). Diseased berries were sampled from susceptible and resistant plants from which 34 single conidia isolates were derived. In locations where both resistant and susceptible varieties were grown, a larger proportion of the berries were obtained from the resistant varieties to increase the chances of obtaining different pathotypes.

3.2 *C. arabica* varieties used in virulence test.

1. Rume Sudan
    - Introduced to Kenya from Sudan
    - Resistant to CBD at the dominant R- and the recessive k- loci.
2. Catimor
    - Introduced to Kenya from Portugal in 1960
    - Resistant to CBD at the dominant T- locus.
    - Also resistant to leaf rust
3. K 7
    - Selection from Kenya
    - Resistant to CBD at the recessive k- locus
    - Has partial resistance to leaf rust
4. SL 28
    - Selected from the National Agricultural Laboratories formerly Scott Laboratories in Kenya.
    - Highly susceptible to CBD and leaf rust
    - High yielding, adaptable and good quality.

Table 1: Source of *C. kahawae* isolates used in PCR.

Serial No.	**MOL. LAB No.**	**SOURCE**	**DATE OF ISOLATION**
1	C1	Central	4.10.08
2	C2	Western	,,
3	C3	Eastern	,,
4	C4	Eastern	,,
5	C5	Western	22.12.08
6	C7	Western	,,
7	C8	Central	,,
8	C9	Eastern	,,
9	C10	Eastern	,,
10	C11	Western	,,
11	C12	Central	,,
12	C13	Central	,,
13	C14	Central	,,
14	C15	Western	,,
15	C16	Central	,,
16	C17	CRF Koru (Western)	,,
17	C18	CRF Koru ,,	23.3.09
18	C19	CRF Koru ,,	,,
19	C20	CRF Koru ,,	,,
20	C21	CRF Koru ,,	,,
21	C22	Central	,,
22	C23	Central	,,
23	C24	Western	,,
24	C25	Western	,,
25	C26	Central	,,
26	C27	Western	,,
27	C28	Central	,,
28	C29	Eastern	,,
29	C30	,,	,,
30	C31	,,	,,
31	C32	Western	,,
32	C33	,,	,,
33	C34	,,	,,
34	C35	,,	,,

3.3 Isolation of *Colletotrichum kahawae* in the laboratory

To obtain monoconidial isolates, berries were washed individually in running distilled water and incubated at 24°C for 48 hrs in Petri dishes containing moist cellulose wadding to promote sporulation. A pure culture from each berry was obtained by inoculating Malt Extract Agar (MEA) and subsequently sub-culturing from regions where culture characteristics corresponded with those documented for *C. kahawae*. The resulting cultures were suspended in distilled water and standardized to a concentration of $2x10^6$ conidia/ml by haemocytometer counting and serial dilution. The suspensions were spread on pure agar in thin lines for conidia to germinate. After 24 hrs, single germinating conidia of each isolate were re-isolated and sub cultured on 3.4 MEA (oxoid) containing 0.04% streptomycin. Each isolate was inoculated in 3 replicates and incubated at room temperature in an orbital shaker for 10 days. The resulting mycelial balls were collected on a mesh sieve and washed in deioniszed distilled water. The mycelia balls were blotted dry between several layers of toweling paper. The dry mycelia were lyophilized for two days at -20°C and stored at the same temperature until required.

3.4 DNA analysis by RAPD

DNA was extracted from lyophilized mycelia of 34 isolates (Appendix1, plate 2) using the method of Diniz *et al.,* (2005). Lyophilized mycelia were ground with metal beads in a grinding mill. Lyses and extraction buffer were added and wetted correctly. Tubes with the contents were kept in a water bath at 62°C for 20 – 30 minutes while mixing the contents well at regular intervals. One ml of chloroform and Isoamyl alcohol (24/1) was added to each tube and mixed vigorously. Tubes were centrifuged at 13000 rpm for 5 minutes. The supernatant was transferred to 2 ml tube to which 20 µ1 of RNAse (10 mg/ml) was added and incubated at 37°C for 30 minutes. Equal volume of isopropyl alcohol was added to the solution and centrifuge at 13000 rpm for 5 minutes. The supernatant was discarded. Pellets were washed with 200 µ1 of 70% ethyl alcohol and centrifuged at 13000 rpm for 1 minute. The supernatant was discarded with automatic pipette taking care not to touch the pellets with pipette extremity. The pellets were dried in the speed vacuum for 15 minutes then dissolved in 20 – 25 µlof TE. DNA quality and quantity were tested (Appendix 2) by agarose gel using a known standard (lambda 3).

Polymerase chain reaction (PCR) was conducted using 9 primers (Table 2) in 25 µl volume containing ddH$_2$O (13.05µl), Buffer (10x) (2.50µl), Mg++ (25mM) (2.5µl), dNTPs (500mM) (3.75µl), Primers (x20) (1.00µl), Taq (5 U/ µI) (0.20µl), DNA (1ng/ µl) (2.0µl). Reaction was performed in a programmable thermocycler (Appendix 1 plate 3). Amplification conditions were set with an initial denaturation temperature at 94°C for 5 minutes followed by 45 cycles of annealing for 1 minute at 35°C, extension for 3 minutes at 72°C and denaturation for I minute at 94°C. The final step consisted of 1 minute at annealing temperature followed by 6 minutes at 72°C to produce fully double stranded fragments. The PCR products were separated on a 1.5% agarose gel in TAE (Tris-Ammonium EDTA buffer). The gel was stained in ethidium bromide, visualized and photographed under UV light 260 nm. The DNA marker used was 100 bp ladder. RAPDs generated by single primer PCR were used to compare relationship among 34 isolates. For each isolate, a data record was constructed in which each band of a particular molecular weight, as generated by each primer, was represented as either being present, “1” or absent ” 0”. Binary matrix was constructed combining all the data records for each isolate - primer combination which yielded reproducible bands.

3.4.1 Analysis with re-extracted DNA.

DNA was re-extracted using the method of Diniz *et al*., (2005) from 5 isolates which showed polymorphism to test for consistency and subsequently used to inoculate different coffee varieties. The isolates were C3, C11, C14, C23 and C27. A second PCR was conducted using Primers N-18, L-18, I-20 and J-19. Primers for the second PCR were chosen based on the unique bands observed with the 5 isolates in the first experiment. The non-pathogenic, CCM and CCP, species were also run through electrophoresis in the same gel with DNA of first and second extractions from the pathogenic species to determine genetic differences and as a confirmatory test for the variations observed among the five 5 isolates. For each isolate, a data record was constructed in which each band of a particular molecular weight, as generated by each primer, was represented as either being present, “1” or absent ” 0”. The SPSS program was used to construct a Euclidian distant matrix and to perform an Average Linkage Hierarchical Cluster Analysis for the 5 isolates both from first and second DNA extractions.

Table 2: Sequence of the primers used for RAPD analysis of *C. kahawae*

Primer	**Primer sequence**
N – 18	5'-GGTGAGGTCA-3'
X – 20	5'-CCCAGCTAGA-3'
I – 20	5'-AAAGTGCGGG-3'
Y – 15	5'-AGTCGCCCTT-3'
L – 18	5'-ACCACCCACC-3'
M – 4	5'-GGCGGTTGTC-3'
I – 7	5'-CAGCGACAAG-3'
J – 19	5'-GGACACCACT-3'
Y – 10	5'-CAAACGTGGG-3'

3.5 Inoculations

Seeds from the four *C. arabica* varieties were obtained and germinated to obtain the hypocotyls for inoculations (Appendix Plate 5). For each of the 5 isolates, 100 seeds of each variety were sown in 3 replications in moist sterilized sand in plastic boxes with closely fitting transparent lids. The boxes were kept at room temperature in the laboratory and watered regularly at intervals of 2 days. After 6 weeks when the hypocotyls stems were 3-5cm long and cotyledons still enclosed in the testa, the seedlings were inoculated using the method of Van der Vossen *et al.,* (1977).

Conidia suspension was prepared from 10 days old monoconidial cultures by flooding the Petri dishes with 5ml of distilled water and scrapping the conidia bearing mycelia off the agar medium while gently mixing. The suspension was filtered using a Hessian cloth and standardized to 2×10^6 conidia/ml by haemocytometer counting followed by serial dilution. Viability of conidia was tested by placing a drop of inoculum on a cavity slide and counting from 100 conidia, the proportion that germinated after 4 hours. Inocular with conidial germination in excess of 80% was used for virulence tests. Hypocotyl seedlings were sprayed with enough inoculum to cover the whole surface of each seedling using a hand sprayer fitted with an atomizing nozzle. For each replicate, control plants were sprayed with distilled water. Infection was induced by maintaining the lids of the boxes closed to increase humidity and covering the boxes with black polythene sheets to provide darkness (Appendix1, plate 6). A double inoculation was performed after 48 hrs. The same conditions were maintained for a period of 4 days followed by an incubation period at a lower temperature of 19-20°C with polythene sheets and lids removed from the boxes to allow for normal humidity and light. The boxes were arranged in the incubation room in a completely randomized design. At the end of the incubation period lasting about 3 weeks and determined by the full expression of the disease on susceptible SL28 variety, the seedlings were individually scored (Appendix 1, Plate 10 and Plate 11) for disease symptoms developed on the hypocotyls stem using the scale of Van der vossen *et al*., (1977)

3.5.1 Coffee berry disease scoring scale (Van der vossen *et al.,* 1977)

Grade	**Symptoms**
1	No visible symptoms
2	A few scab lesions
3	Small scab lesions
4	Scab and brown lesions
5	Scab and brown lessons and a few small black lesions
6	Brown and narrow black lesions
7	Narrow black lesions, some more than 1 cm long

8	Black lesions becoming wider and starting to coalesce
9	Large coalescing black lesions but not complete girdling
10	Large coalescing black lesions and complete girdling of the stem
11	Most of the stem affected, more than 1/3 of the stem shriveled and seedling dead.
12	Whole stem affected and shriveled seedling dead.

The mean grade of infection was computed for each box. The mean grade data was then used to perform an Analysis of Variance (ANOVA) according to random effects model (Steel and Torrie, 1981).

Mean grade of infection (G) was computed for each box as follows;

$$\mathbf{G} = \frac{1}{N}\sum_{i=1}^{12} in_i$$

Where i is the disease grade, n_i is the number of seedling in the grade i and N is the total number of seedlings scored. The laboratory data on the *C. kahawae* virulence/aggressiveness on various coffee varieties was analyzed using MINITAB version 13.0 Computer package.

CHAPTER FOUR

RESULTS AND DISCUSSIONS

4.1: Genetic diversity of *C. kahawae* using Random Amplified Polymorphic DNA (RAPD).

Random Amplified Polymorphic DNA (RAPD) was used to determine genetic diversity of *C. kahawae* used in this study. A total of 9 primers assayed showed amplification with the 34 isolates. A total of 1272 amplification products were generated by the 9 primers. Primer I-7 recorded 240 bands (Table 3) but did not amplify C10. C4 and C29 were not loaded on the gel since they evaporated during PCR (Fig. 2a and 2b). The primer also recorded the highest average number of bands per isolate (7.5). Other primers that also generated amplification products among the 34 isolates were, Primer N – 18 which distinguished total of 172 bands with a mean of 5.06, primer L – 18 (125 bands), primer J – 19 (162 bands), primer M – 4 (139 bands), primer X – 20 (119 bands), primer Y – 15 (150 bands), primer Y-10 (125 bands) and primer I-20 (40 bands) as shown in Table 3 and Fig. 2a to Fig. 6b. The average number of DNA bands per primer for the 34 isolates, excluding I-7 which tested 32 isolates (Tables 3 and 4) was 141.3. Primer L-18 revealed an the average of 3.67, primer I-7 (7.5), primer X-20 (3.5) , Primer Y-10 (3.67), primer J-19 (4.76), primer I-20 (1.25), primer M-4 (4.09),primer N-18 (5.06) and primer Y-15 (4.4).

Table 3: Number of distinct bands in 34 isolates using different primers.

	Primers used									
Isolates	L -18	I - 7	X-20	Y-10	J - 19	I - 20	M - 4	N-18	Y-15	Ave.bands
C1	4	8	5	3	5	1	4	5	5	4.44
C2	4	8	4	4	4	1	4	5	5	4.33
C3	5	7	4	4	6	2	6	7	5	5.11
C4	4	0	5	3	5	1	4	5	5	4.44
C5	4	8	4	4	5	1	4	7	5	4.67
C7	4	8	6	4	5	1	4	5	5	4.67
C8	4	8	4	4	5	1	4	5	4	4.33
C9	4	8	4	4	5	2	4	5	4	4.44
C10	7	0	5	4	6	2	7	7	8	5.11
C11	4	8	4	4	5	1	4	5	4	4.33
C12	4	8	5	4	5	1	4	5	4	4.44
C13	6	9	5	4	4	5	4	5	8	5.56
C14	3	8	6	6	5	1	4	6	4	4.78
C15	4	8	5	4	5	1	4	6	4	4.56
C16	4	8	2	4	5	1	4	5	4	4.11
C17	3	8	2	4	2	1	4	4	4	3.56
C18	1	8	1	1	4	1	4	4	4	3.11
C19	3	8	1	3	5	1	4	5	4	3.78
C20	4	8	1	4	4	1	2	5	5	3.78
C21	4	9	4	4	5	1	4	6	5	4.67
C22	4	8	5	5	5	1	4	5	6	4.78
C23	4	8	4	5	5	1	4	5	7	4.78
C24	4	8	4	5	5	1	4	5	4	4.44
C25	1	7	3	4	6	1	4	5	4	3.89
C27	4	8	0	1	5	1	5	5	2	3.44
C28	4	8	4	3	6	1	5	5	3	4.33
C29	0	0	0	0	5	1	5	5	4	2.22
C30	4	8	4	5	5	1	5	5	4	4.56
C31	4	8	7	5	5	1	5	5	4	4.89
C32	4	8	1	5	5	1	5	5	4	4.22
C33	4	8	4	5	5	1	5	5	4	4.56
C34	4	8	3	2	5	1	0	5	4	3.56
C35	4	8	3	4	5	1	5	5	4	4.33
Total bands	125	240	119	125	162	40	139	172	150	
Ave. bands	3.67	7.5	3.5	3.67	4.76	1.25	4.09	5.06	4.4	

Table 4: Average number of bands produced using 9 primers.

.

Primer	**Average bands**
N – 18	5.06
X – 20	3.50
I – 20	1.25
Y - 15	4.4
L – 18	3.67
M – 4	4.09
I - 7	7.5
J – 19	4.76
Y - 10	3.67

Primer I-7

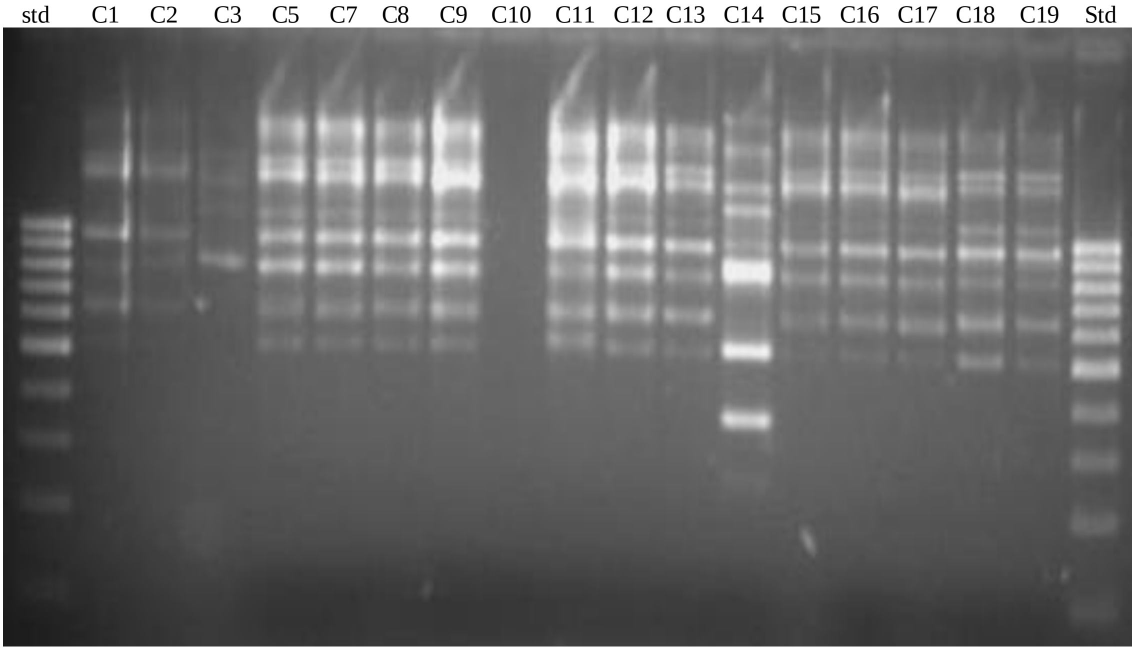

Fig.2a: RAPD profiles generated by primer I-7 on a first set of 17 isolates

Primer I –7.

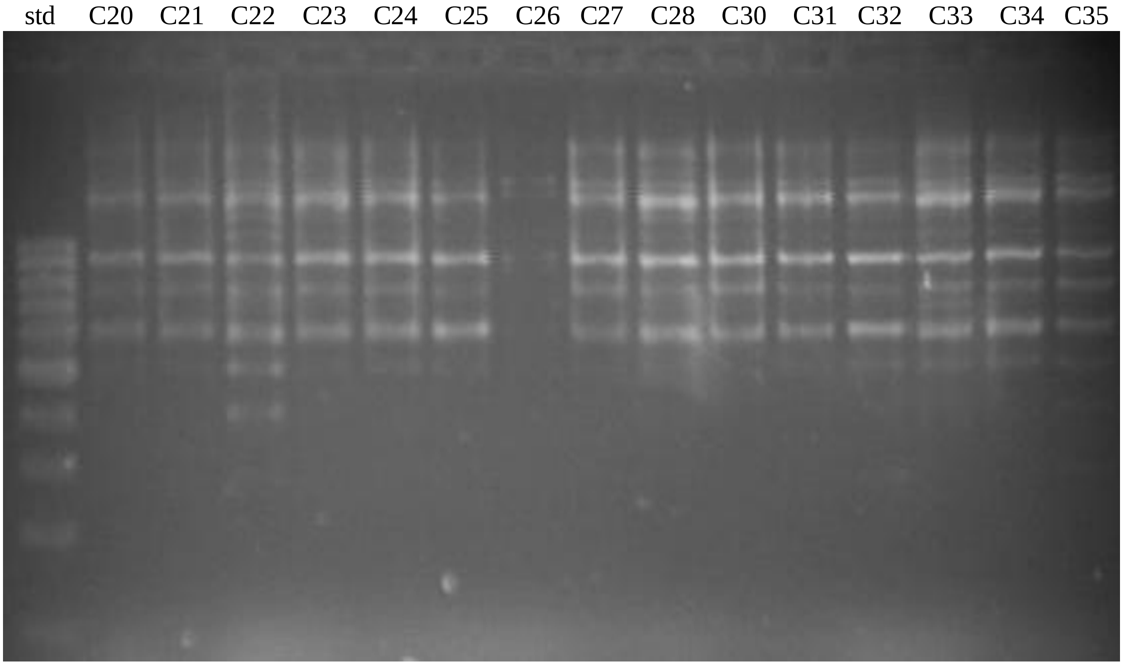

Fig.2b: RAPD profiles generated by Primer I – 7 on a second set of 15 isolates

In Fig. 2a to Fig.6b, C1-C35 represents *C. kahawae* isolates, Std. represents 100bp ladder marker (Lambda). CCM and CCP represent non - pathogenic sps. of *Colletotrichum.*

Primer I-20

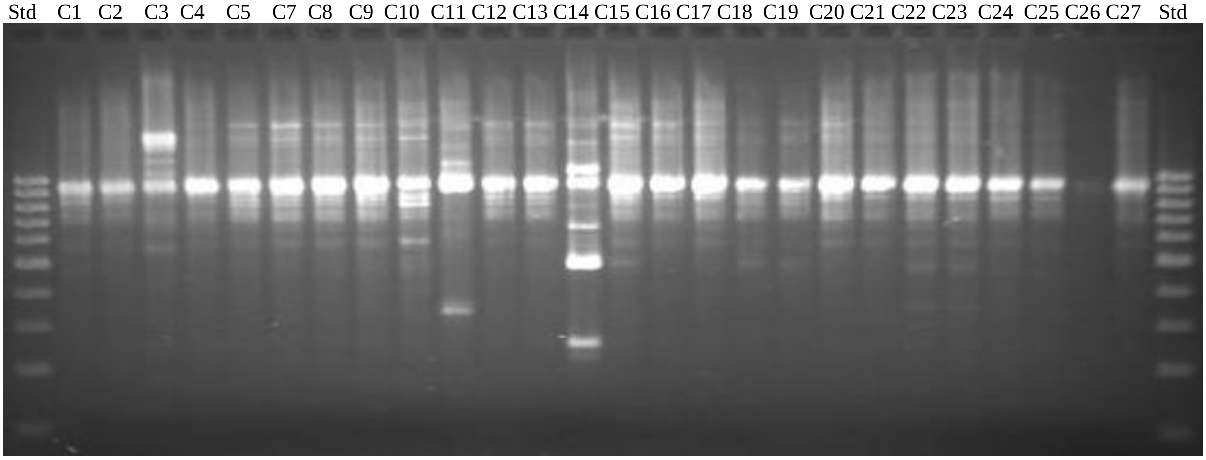

Fig. 3a: RAPD profiles generated by Primer I-20 on a first set of 26 isolates.

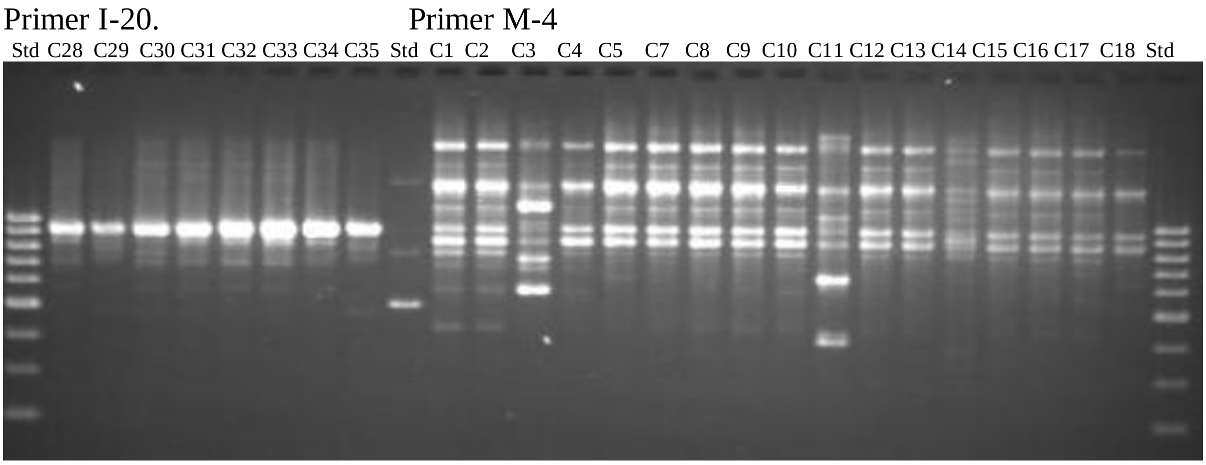

Fig. 3b: RAPD profiles generated by Primer I – 20 on a second set of 8 isolates and primer M-4 on a first set of 17 isolates.

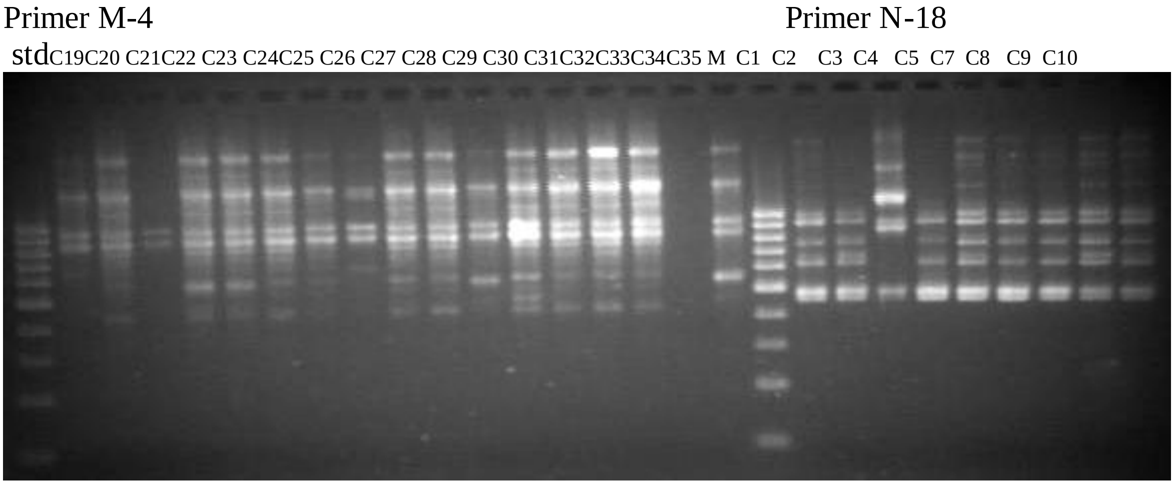

Fig. 4a:RAPD generated by primer M-4 on a second set of 17 isolates and N-18 on a first set of 9 isolates.

Primer N-18

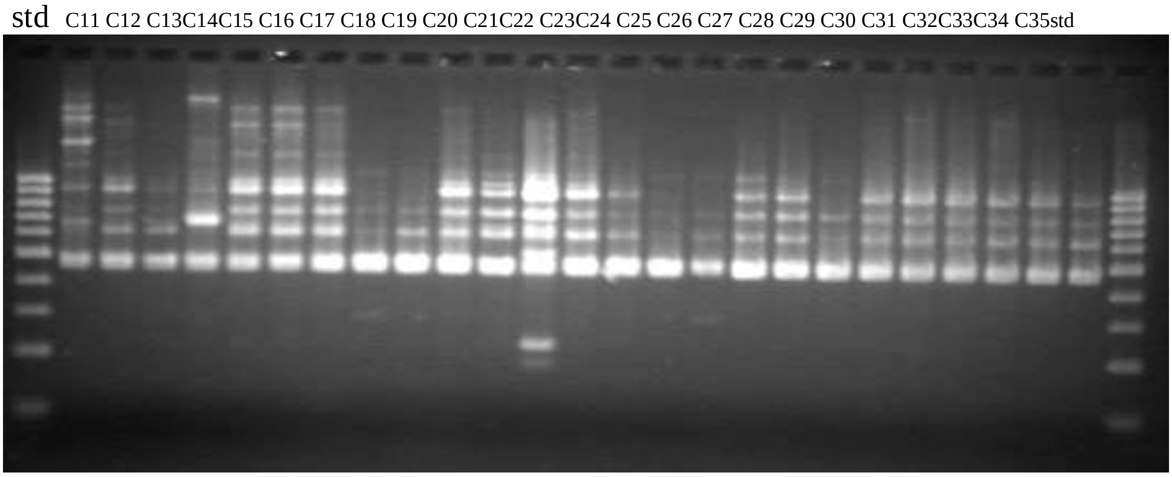

Fig. 4b: RAPD profiles generated by Primer N – 18 on a second set of 25 isolates.

Primer J-19.

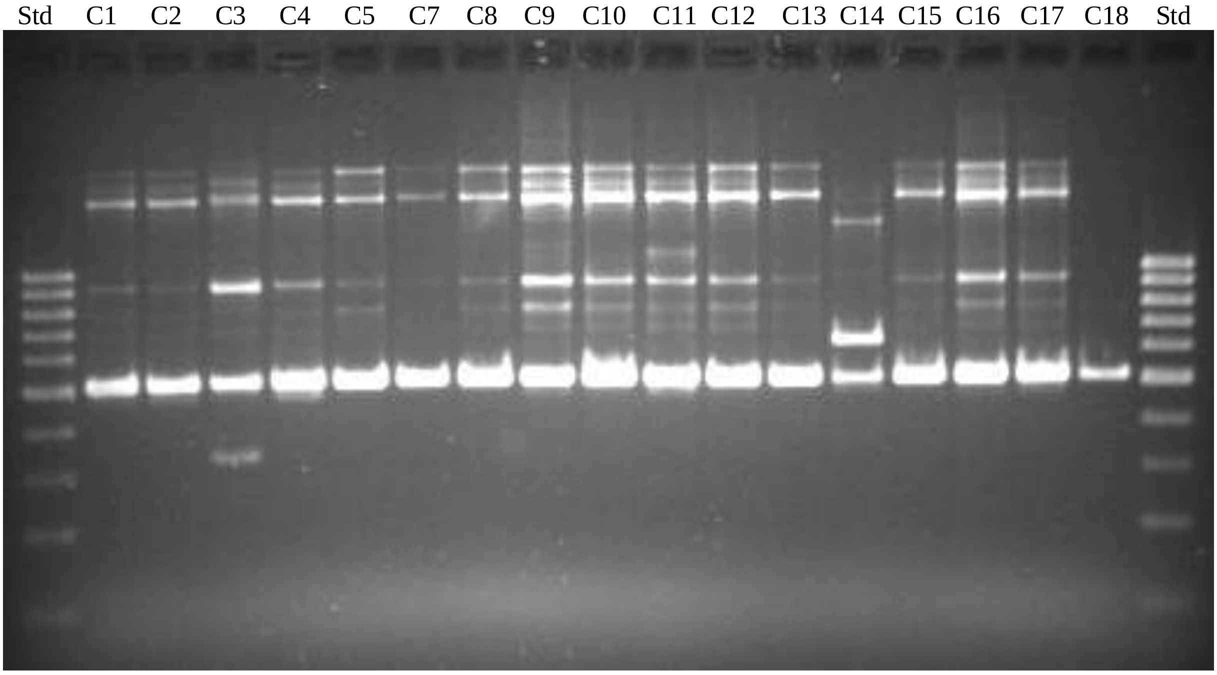

Fig. 5a: RAPD profiles generated by Primer J – 19 on a first set of 17 isolates.

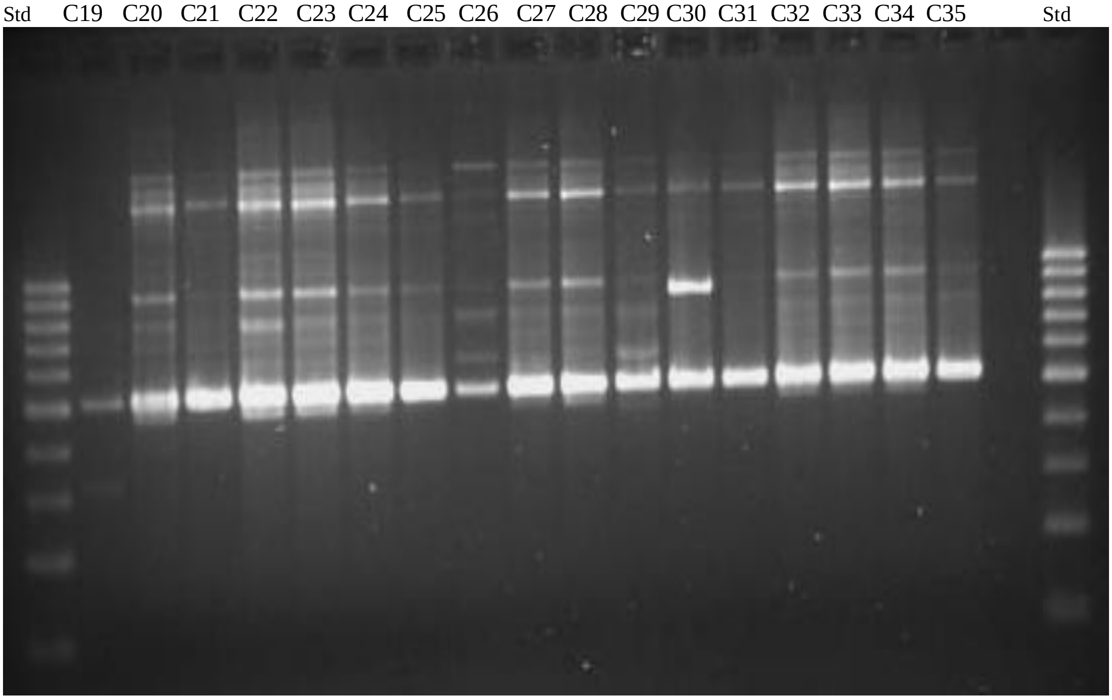

Fig. 5b: RAPD profiles generated by Primer J – 19 on a second set of 17 isolates.

Primer L-18.

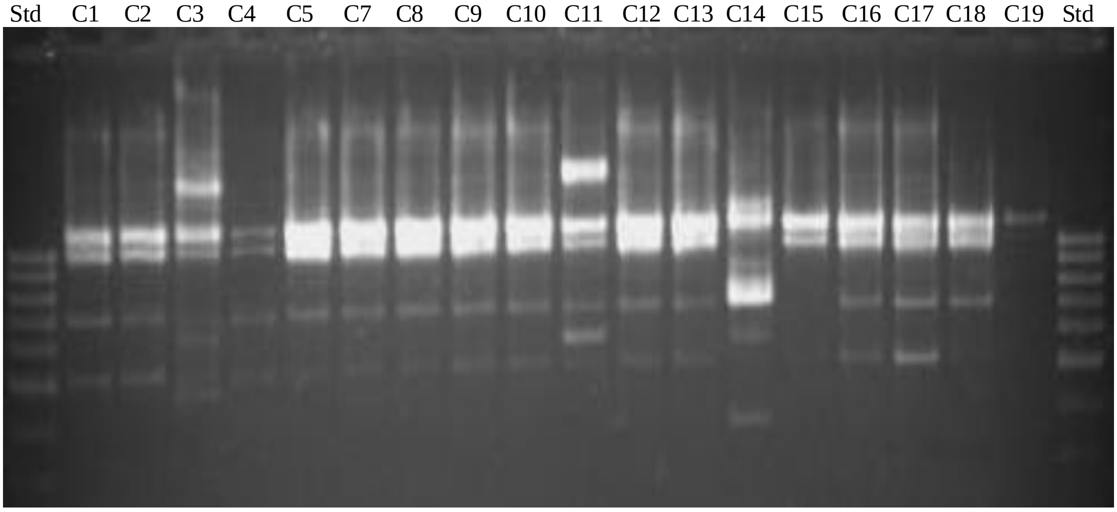

Fig. 6a: RAPD profiles generated by Primer L-18 on a first set of 18 isolates.

Primer L-18.

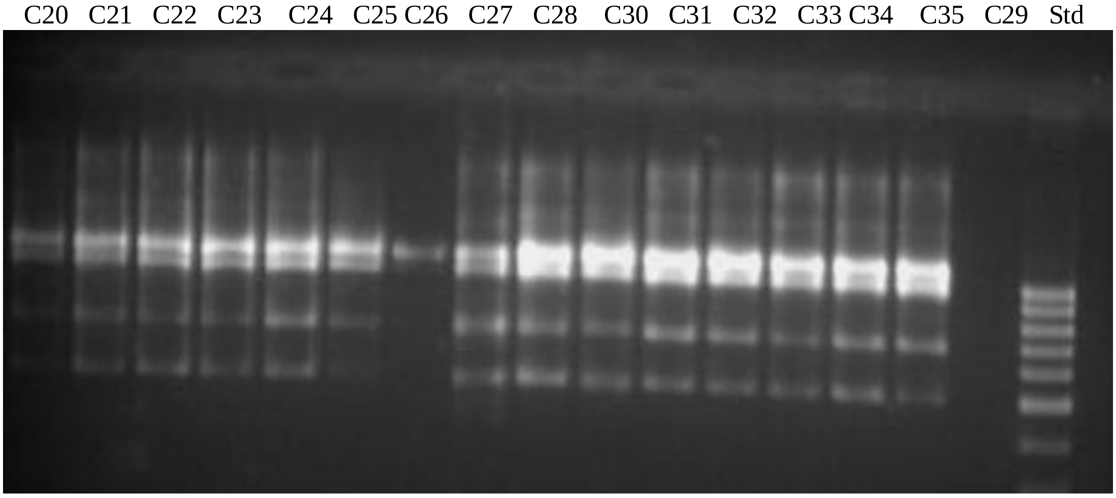

Fig. 6b: RAPD profiles generated by Primer L – 18 on a second set of 16 isolates.

Although primer I-7 detected the highest number of bands as shown in table 3, it only detected 2 unique bands (0.8% polymorphism) with isolates C1, and C22 (Table 5a and 5b) . Other primers detected unique bands with all other isolates. Primer I – 20 was able to detect 4 unique bands (10% polymorphism) with isolates C3, C10, C11 and C14 (Table 5a and 5b). Primer M-4 depicted polymorphism with C3, C11, C21 and C34 (2.9%). Primer N-18 depicted polymorphism with isolate C3, C5, C11, C14 and C22 (2.9%). Primer Y-15 depicted polymorphism with isolates C11, C14, C23, C24, C27 and C28 (4%). Primer L-18 was able to differentiate isolates C3, C11, C14 and C18 (3.2%), while primer J-19 differentiated isolates C 3, C5, C11, C14.C18 and C19 (3.7%). Primer X-20 and Y-10 depicted the highest number of polymorphism among isolates except with isolates C3, C11 and C14 (Table 5a). Primer X-20 recorded 3.4% polymorphism while primerY-10 indicated 7.2% polymorphism (Table 5b). Isolate C14 recorded the highest number of unique bands (7) followed by C11 (6), C3 (5), C23 (3) and C27 (3), making them be chosen for inoculation experiments. Other isolates were polymorphic on 1or 2 bands while others lacked polymorphism. Other isolates C1, C2, C9 C12, C13, C17, C20, C25, C26, C29, C30 and C35 showed uniform banding patterns indicating genetic uniformity among them. The average percentage polymorphic band per primer was 3.5 indicating low genetic variation among *C. kahawae* species studied (Table 5b).

Table 5a: Polymorphism depicted by RAPD primers

	Primers									
Isolates	**I-7**	**N-18**	**Y-15**	**L-18**	**I-20**	**J-19**	**M-4**	**X-20**	**Y-10**	Unique bands
C3		X		X	X	X	X			5
C4								X		1
C5		X								1
C7						X				1
C8								X	X	2
C10					X					1
C11		X	X	X	X	X	X			6
C14	X	X	X	X	X	X			X	7
C15								X	X	2
C16								X	X	2
C18				X		X				2
C19						X				1
C21							X			1
C22	X	X								2
C23			X					X	X	3
C24			X							1
C27			X					X	X	3
C28			X							1
C31								X	X	2
C32								X	X	2
C33								X	X	2
C34							X			1
Unique bands/primer	2	5	6	4	4	6	4	9	9	49

Table 5b: Percentage polymorphism by 9 primers.

Primer	**Total No. of Bands**	**Polymorphism**	**% polymorphism**
L – 18	125	4	3.2
I – 7	240	2	0.8
X – 20	119	4	3.4
Y – 10	125	9	7.2
J – 19	162	6	3.7
I – 20	40	4	10.0
M - 4	139	4	2.9
N – 18	172	5	2.9
Y – 15	150	6	4.0
Total	1272	44	3.7

The hierarchical cluster analysis was used to group the isolates (Fig.7). 5 distinct groups were identified at dissimilarity distances of 5.4 (C14), 4.2 (C11), 3.8 (C3), 3.4 (C27) and 2.5 (C23 chosen to represent the others in the group). The 5 isolates were subsequently used for inoculation experiments.

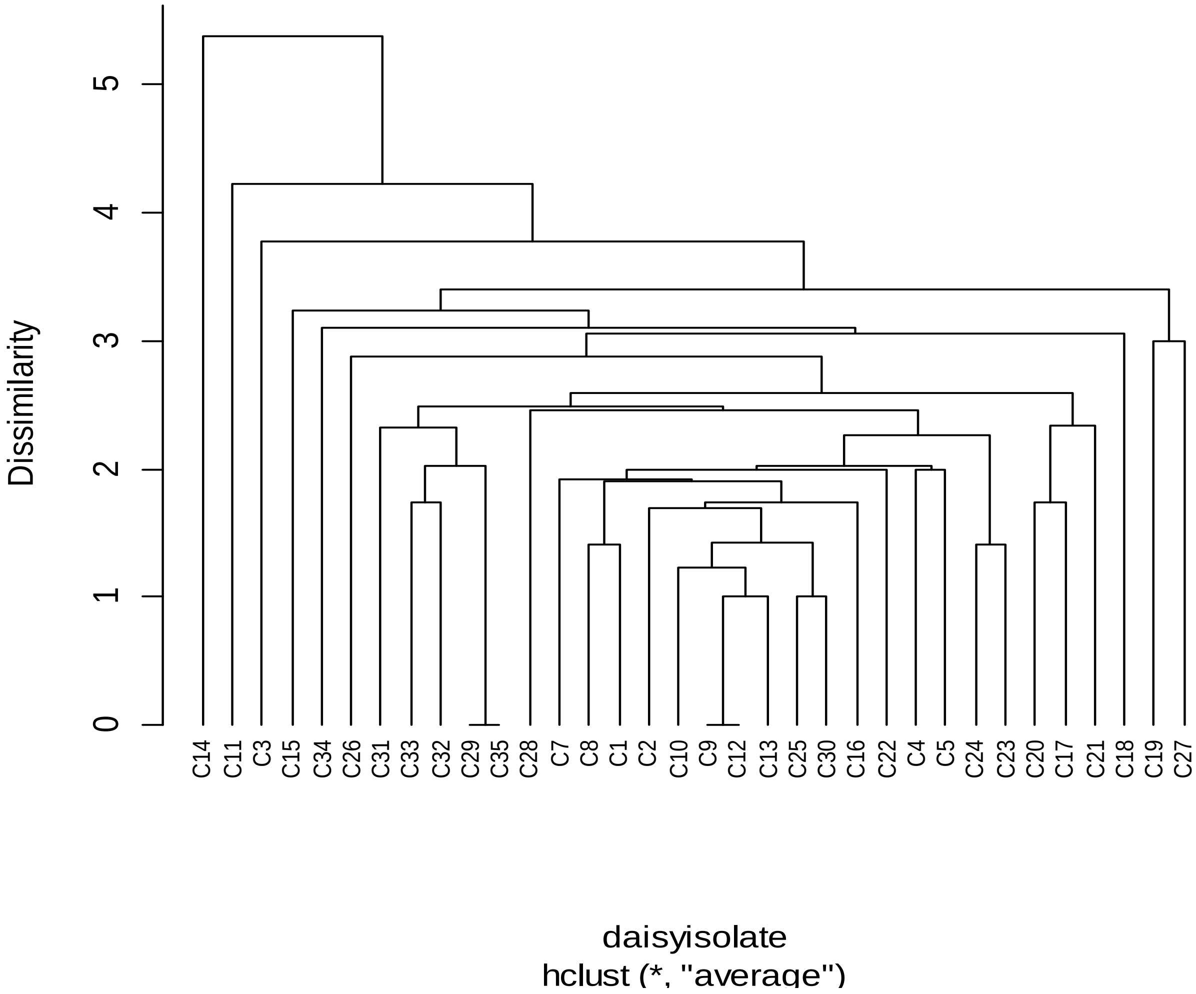

Figure: 7: Dendrogram derived from distant matrix based on RAPD profiles of 34 isolates and 9 primers.

4.1.0 RAPD analysis of the re-cultured DNA

A second PCR was done using isolates representing different clusters in the dendrogram (Fig.7). The isolates chosen were C3, C11, C14, C23 and C27. In the second PCR experiments, Primers N – 18, L – 18, I – 20 and J – 19 were used. Primers were chosen based on unique bands noted with the isolates C3, C11 and C14, isolates with highest number of polymorphic bands as indicated in Table 5a. Most amplification products were similar among isolates whose DNA were re-extracted and a second PCR conducted. Re-extracted DNA for isolate C14 showed different banding patterns with primers N-18, L-18, and J-19 in the second PCR as compared with DNA of C14 used in first PCR (Fig.8 and Fig. 9). However most of the DNA used in the first PCR indicated minimal variations in banding patterns from the re-extracted DNA of the same isolates when loaded on same gel. It was noted that CCM, the non pathogenic *Colletotrichum sp.*, did not amplify with primers N-18, I – 20, J – 19 and L – 18. CCM showed 1 band with N – 18 and 4 bands with L – 18. Variation between non-pathogenic and pathogenic isolates was detected by primers N-18 and L-18 as shown by Fig. 8 and Fig. 9. Amplifications of the second PCR is shown in Table 6a and Table 6b. A dendrogram was constructed (Fig. 10) combing results from table 6a and 6b. The results indicated that the non-pathogenic sps. of *colletotrichum* (CCM and CCP) are distinctly different from *C. kahawae* (Fig.10). Results from Fig. 10 revealed that C23, C27, C11 are tightly linked indicating that they share some similarity that was detected by DNA analysis. C3 and C14 vary from the first cluster at a Euclidian distance of 7% and 16% respectively. The non-pathogenic CCM is different at the maximum Euclidian distance showing that the species were highly divergent.

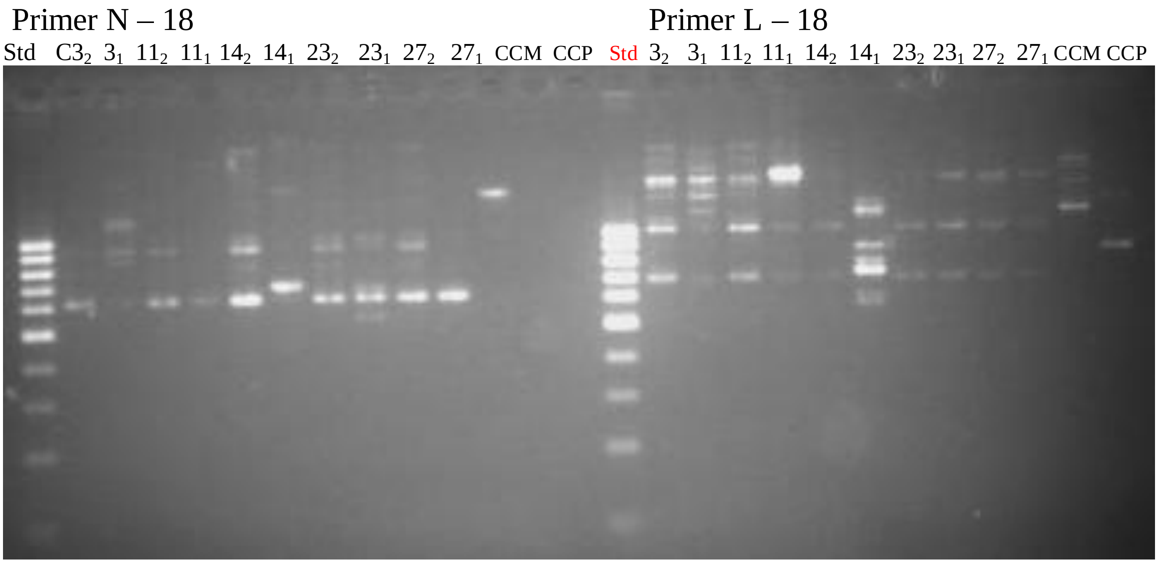

Fig. 8: RAPD Profiles generated by primers N -18 and primer L-18 on selected *C. kahawae* isolates and *Colletotrichum* species, CCM and CCP in second PCR.

Note; The subscripts 1and 2 indicate DNA of first extraction and DNA of re-cultured isolates respectively.

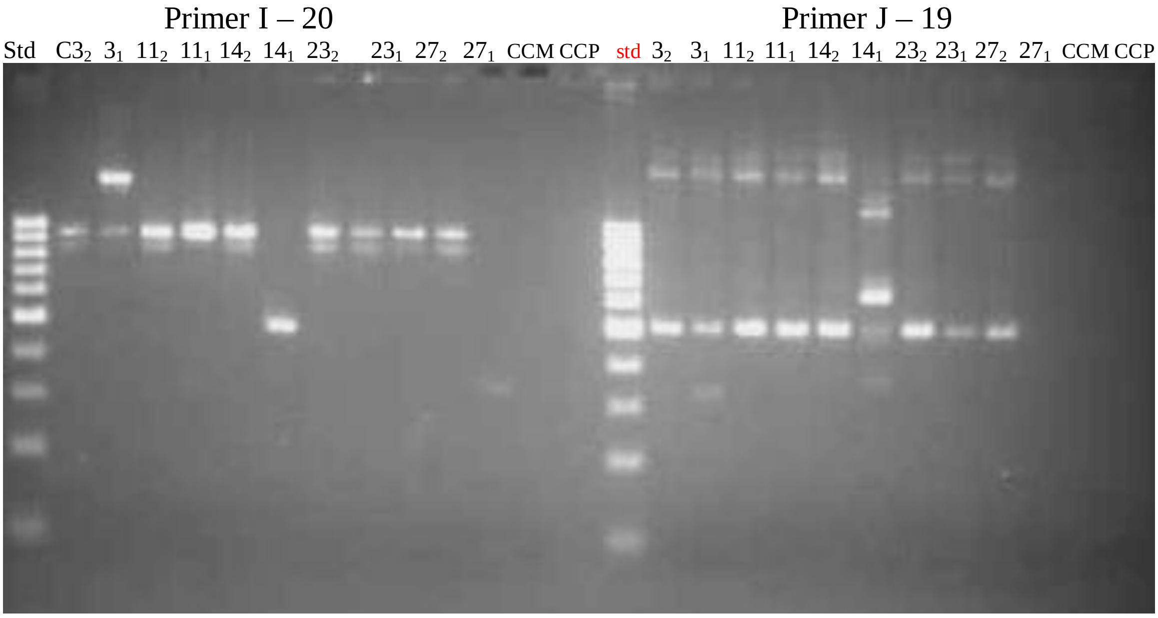

Fig. 9: RAPD Profiles generated by primer I-20 and primer J – 19 on selected *C. kahawae* isolates and *Colletotrichum* species CCM and CCP in second PCR.

Note; The subscripts 1and 2 indicate DNA of first extraction and DNA of re-cultured isolates respectively.

Table 6 a: Amplification products observed from second PCR using Primers N – 18, L-18, I – 20 and J – 19 in combination with DNA from first extraction.

Primer	Bands	C_3	C_{11}	C_{14}	C_{23}	C_{27}	CCM
N - 18	1	0	0	0	0	0	1
	2	1	0	0	0	0	0
	3	1	0	0	1	0	0
	4	0	0	1	0	0	0
	5	1	1	0	1	1	0
L-18	1	0	0	0	0	0	1
	2	1	1	0	1	1	1
	3	1	0	0	0	0	1
	4	1	0	1	0	0	1
	5	1	1	0	1	1	0
	6	0	0	1	0	0	0
	7	1	1	1	0	0	0
I - 20	1	1	0	0	0	0	0
	2	1	1	0	1	1	0
	3	0	0	1	0	0	0
J - 19	1	1	1	0	1	0	0
	2	0	0	1	0	0	0
	3	0	0	1	0	0	0
	4	1	1	0	1	0	0

Table 6 b: Amplification products observed from second PCR using Primers N – 18, L-18, I – 20 and J – 19 in combination with re-extracted DNA of the re-cultured isolates.

Primer	Bands	C_3	C_{11}	C_{14}	C_{23}	C_{27}	CCM
N - 18	1	0	0	0	0	0	1
	2	0	1	1	1	1	0
	3	1	1	1	1	1	0
L-18	1	0	0	0	0	0	0
	2	1	1	0	0	1	0
	3	1	1	1	1	1	0
	4	1	1	1	0	0	0
I - 20	1	1	1	1	1	1	0
	2	1	1	1	1	1	0
J - 19	1	1	1	1	1	1	0
	2	1	1	1	1	1	0

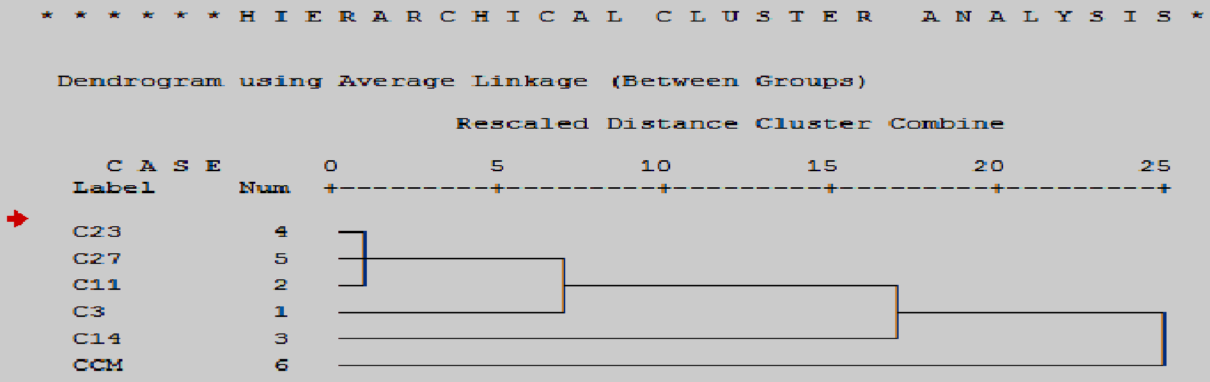

Fig.4.8 Dendrogram derived from the Euclidian Distance matrix based on RAPD profiles.

RAPD analysis therefore detected the large genetic variation between species but variation within *C. kahawae* was relatively of low magnitude as indicated in Fig. 8, Fig. 9 and Fig 10. Some of the uniformity of the DNA bands observed from second PCR agrees with the DNA patterns of *C. kahawae* documented so far. Bridge *et al.*, (2008) analyzed *C. kahawae* using AFLP and failed to detect variability among 12 isolates from Kenya and other African countries. An analysis using VNTR also showed lack of variability within the main groups suggesting limited variation within *C. kahawae* species. The variations observed during amplification of *C. kahawae* in this study could be due to experimental error or transient differences where bands appear to occur and then disappear in the next experiment (Fig.8 and Fig 9). It could also be due to the effect of storage outside the host or due to lack of repeatability of the method used. Primer I-7 failed to amplify DNA of isolate C10. CCM did not amplify with primer N-18, I-20, J-19 and L-18. This could be due to mismatch between the primer and the template DNA (Williams *et al.*, 1990) or total lack of homology between the primer and the annealing sites (Kelly *et al.,* 1994). Omondi (1998), conducted protein electrophoresis and noted that most isolates of *C. kahawae* isolates had similar protein profiles while the non-pathogenic *C. acutatum* had protein profiles distinctly different from *C. kahawae.* Protein profiles of *C. kahawae* were highly identical indicating lack of genetic diversity (lack of races) among *C. kahawae*. Phylogenetic analysis using multy-gene data showed that *C. kahawae* is genetically distinct from other closely related species in the complex (Prihastuti *et al.*, 2009). Grouping of Kenyan *C. kahawae* isolates using vegetative compatibility showed that all the *C. kahawae* isolates belonged to one vegetative compatibility group (Gichuru *et al.*, 1999). Molecular studies by Loureiro *et al.*, (2007), using RAPD techniques did not show polymorphism within the isolates tested. However isoelectric focusing electrophoresis (IFE) and polyacrylamide gel electrophoresis (PAGE) detected some polymorphism (Loureiro *et al.*, 2007). Manuel *et al.,* (2010), used 30 isolates of *C. kahawae* in Angola and detected limited genetic diversity. Variation studies using morphological and pathological criteria as well as random amplification of genomic DNA using 10 primers showed similar trends in the nucleotide sequence (Waller *et al.,* 2009). Bridge *et al.*, (2008), assessed genetic variability using Restriction Fragment Length Polymorphism (RFLP) and Variable Number of Tandem Repeat (VNTR) on *C. kahawae* and observed minimal molecular variability with two isolates from Cameroon showing slightly different banding patterns in RFLP analysis. Amplified Fragment Length Polymorphism (AFLP) failed to detect variability within 12 isolates from Kenya, however the isolates from

Kenya were vegetatively compatible but different from the two from Cameroon and other *colletotrichum* species tested (Bridge *et al.*, 2008).

4.2 Virulence of different isolates of *C. kahawae* on a set of coffee varieties.

C. kahawae isolates C3, C23, C11, C14 and C27 were selected based on their polymorphic reaction when subjected to DNA analysis and used to inoculate seedlings of four coffee cultivars namely; Catimor, Rume Sudan, K7 and SL 28. The varieties were chosen on the basis of their resistance/susceptibility. The hypocotyl inoculation test was done to establish its correlation with products of DNA amplification. Table 7 shows no significant effect of isolates ($P>0.05$) while effect of varieties was highly significant ($P\leq0.01$). The results of mean separation in Table 8 indicate that Rume Sudan was highly resistant; Catimor and K7 were partially resistant while SL28 was susceptible. Rume Sudan carries resistance on the dominant R - and the recessive k -loci. Catimor and K7 carry resistance on the dominant T- and the recessive k - locus respectively. Combination of two or more genes as in the case of Rume Sudan appears to enhance resistance. Although RAPD analysis detected some polymorphism among the five isolates used for inoculation, (Fig. 10), their reaction with the coffee differentials did not show any significant difference. This may be attributed to the fact that the differences detected by RAPD analysis were not related to virulence of the isolates. RAPD could be more useful in studying diversity of populations rather than differences in specific traits.

The variety x isolate interaction effect was significant at ($P\leq0.05$) (Table7). This could be attributed to some isolates being more aggressive on some varieties than others. This is also illustrated in Fig.11 where isolate C23 was more aggressive on Rume Sudan than C3, C11, C14 and C27. On the other hand, it was the least aggressive on Catimor compared to C3, C11, C14 and C27. C3 was less aggressive on K7 than C23, C11, C14 and C27. The interaction effect cannot be attributed to existence of races because the isolate effect was not significant and the reaction with different isolates indicated no cross-over effect (Fig.11). Lack of interaction of cross-over type is an indication that races do not exist. Fig. 11 also indicates that coffee cultivar Rume Sudan was the most resistant cultivar while SL28 was the most susceptible. Catimor and K7 had same level of reaction with isolate C3 while all other isolates were more aggressive on K7 than Catimor.

4.3 Reaction of *C.arabica* varieties when infected with *C. kahawae* isolates

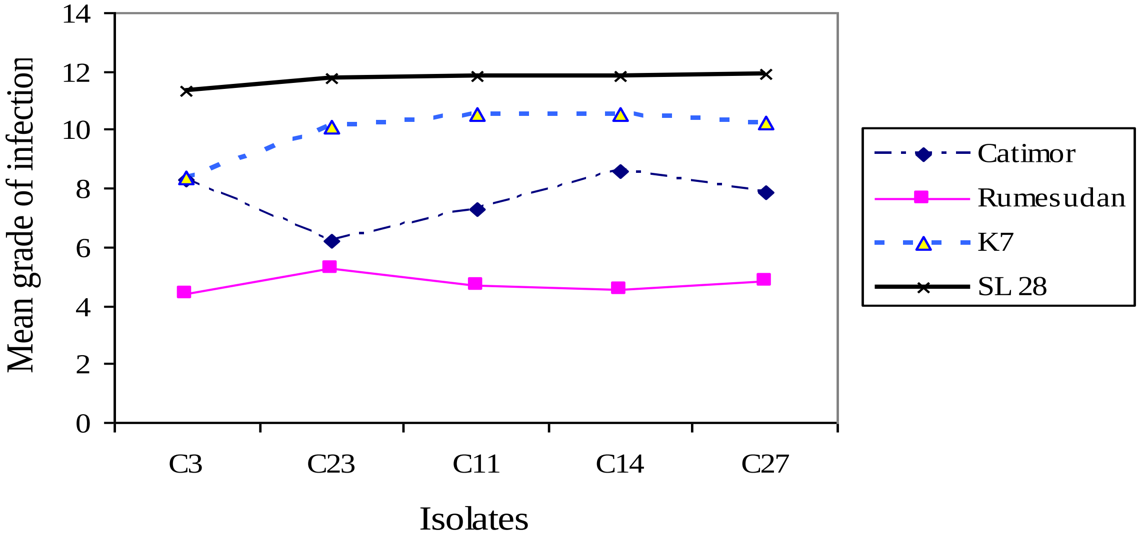

Fig. 11: Reaction of *C.arabica* varieties when infected with *C. kahawae* isolates

Table 7: Two-way ANOVA of infection level recorded on varieties inoculated with different isolates.

Source	DF	MS	F	P
Isolates	4	1.109	1.63	0.186
Cultivar	3	136.862**	200.87	0.000
Interaction	12	1.507*	2.21	0.030
Error	40	0.681		
Total	59			

*** Significant at $P \leq 0.01$ * Significant at $P \leq 0.05$*

Table 8 indicates that C14 had the highest mean grade of infection (8.88) followed by C27 (8.71), C11 (8.58), C23 (8.35 and C3 (9.11) in the order. It also indicates that coffee cultivar "Rume Sudan" was the most resistant cultivar while SL28 was the most susceptible. Catimor and K7 had intermediate resistance. *C. kahawae* isolates were uniformly more aggressive on the susceptible SL 28. C23 was least aggressive on variety Catimor but most aggressive on Rume Sudan and intermediate on K7. This is an indication that most isolates differ in aggressiveness rather than virulence. These results are in agreement with the findings of Omondi *et al.,* 1999 who performed Pathogenisity tests on 11 genotypes of *Coffea arabica* using single-isolate suspension of *Coletotrichum kahawae* obtained from 90 monconidial isolates and found that a large part of the variation in the pathogen population was due to aggressiveness. They observed that the differential effects were too small to suggest conclusively that races exist. Waller *et al.,* 2009, further observed that isolates of CBD pathogen taken from across its range of distribution in Africa have common morphological, biochemical and pathogenic characteristics distinguishing them from other species of *Colletotrichum.* This could explain the lack of variation observed among isolates.

Table 8: Reaction of some selected coffee differentials with different isolates.

Isolates	**Coffee differentials**				
	Catimor	**Rume Sudan**	**K7**	**SL 28**	**Mean**
C3	8.33b	4.37c	8.40b	11.33a	8.11
C23	6.23b	5.30b	10.13a	11.73a	8.35
C11	7.27c	4.70d	10.53b	11.83a	8.58
C14	8.60b	4.57c	10.57ab	11.80a	8.88
C27	7.87c	4.80d	10.23b	11.93a	8.71
Mean grade	7.66c	4.75d	9.97b	11.73a	

NB: Mean score in the same row denoted by similar letters are not significantly different at P≤ 0.05

4.4 Correlation between pathogen diversity and growing regions

Sampling sites for *C. kahawae* were divided into Western, Central and Eastern regions of Kenya. *C. kahawae* isolate C11 and C27 were sampled from Western region of Kenya. C23 and C14 were sampled from Central region while isolate C3 was sampled from Eastern part of Kenya.

Virulence /aggressiveness levels of the isolates from various regions were tested to establish whether the isolates from particular regions could be more virulent/aggressive on the cultivars. This study established a significant correlation in aggressiveness (Table 9) of isolates from Western to both isolates from Central and from Eastern (r = 0.945 and 0.861 respectively). Isolates from central region similarly had a significant correlation in aggressiveness to isolate from Eastern (r = 0.716).

Table 9. Correlation among regions where isolates were obtained

	Western	**Central**	**Eastern**
Western	1.000	0.945	0.861
Central		1.000	0.716
Eastern			1.000

These findings therefore indicate that the isolates from all the regions had similar reaction on coffee cultivars tested. However, an analysis of their aggressiveness showed that isolates from

Central Kenya (C14 and C23) had the highest mean grade (8.82) followed by isolates from western (C11 and C27) with a mean of 8.63. Isolate C3 from eastern had the lowest mean grade of 8.12, (Table 10). The results are also shown on Fig.12. Table 10 further indicate that Rume Sudan recorded highest resistance (mean grade 4.68) followed by Catimor (mean grade 7.77), K7 (mean 9.71) and SL28 recorded the highest mean grade of 11.66 when inoculated with *C. kahawae* from different regions.

Table 10: Levels of virulence/aggressiveness of *C. kahawae* isolates from different regions

Region	**Coffee cultivars**				
	Catimor	**Rume Sudan**	**K7**	**SL 28**	**Mean**
Western	7.57c	4.75d	10.38b	11.88a	8.63
Central	7.42b	4.93c	10.35a	11.77a	8.82
Eastern	8.33b	4.37c	8.40b	11.33a	8.12
Mean	7.77	4.68	9.71	11.66	

NB: Mean score in the same row denoted by similar letters are not significantly different at $P \leq 0.05$

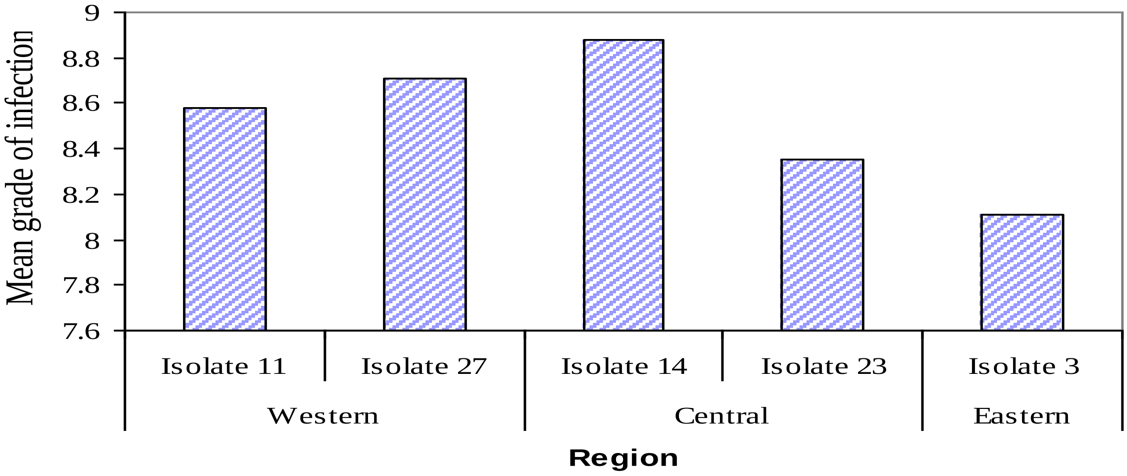

Fig. 12: Levels of virulence/aggressiveness of *C. kahawae isolates* from different regions

Omondi, (1998), observed that the apparent lack of variety specific variation associated with existence of races was probably due to the fact that the pathogen co-evolved with a genetically narrow based host species. Bridge *et al*., (2008) observed that geographic variability within *C. kahawae* isolates is very small leading to genetic uniformity among the isolates. Rodrigues *et al*., (1991), also reported that variation was due to both aggressiveness and some cultural characters such as rates of sporulation and growth.

CHAPTER FIVE

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

Findings from this study show that there is minimal genetic diversity of *C. kahawae*. The DNA banding patterns using RAPD indicate predominant genetic uniformity among *C. kahawae* with minor differences. RAPD analysis using 9 primers was able to distinguish polymorphism at different sites mainly with C3, C11, C14, C23 and C27 but some of the variations were not consistent in the repeat PCR experiments with re-extracted DNA of the same isolates, indicating minimal variation among *C. kahawae.*

There was pathogen diversity detected by RAPD analysis which could not be attributed to differences in virulence or aggressiveness. Virulence tests revealed that the reaction of isolates on the varieties tested was to a large extent uniform indicating that there were no races. However, minor differences were observed in disease score which can be attributed to difference in aggressiveness. Isolate C14 was found to be more aggressive with mean score of 8.88 compared to other isolates. The least aggressive isolate was isolate C3 with a mean score of 8.11.

It was also observed that there was uniformity in pathogen population across the regions where the isolates were collected. Location of origin had little significance on virulence of *C. kahawae* tested. However the results indicate that there are some variations in aggressiveness of the isolates from the specific regions. Isolates from Western recorded a mean of 8.63, Central a mean of 8.62 and Eastern a mean of 8.12 on all coffee varieties tested. There was no evidence indicating variation in virulence across the regions. Resistant varieties are likely to be deployed for wide adaptability without risk of breakdown of resistance.

5.2 Recommendation for further research

1. RAPD analysis appears to be useful in detecting diversity in the pathogen population. Other robust methods of DNA analysis that can detect variation on specific traits need to be tested.
2. There is need to analyze a large sample of isolates across coffee growing regions.
3. There is need to use large number of primers when studying population diversity so that chances of recording polymorphism is enhanced.
4. Due to the nature of coffee varieties used, some of which have duplicate combinations of resistance genes, more biochemical and molecular markers for Pathogenesity should be screened.

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APPENDICES

Appendix 1: Plates

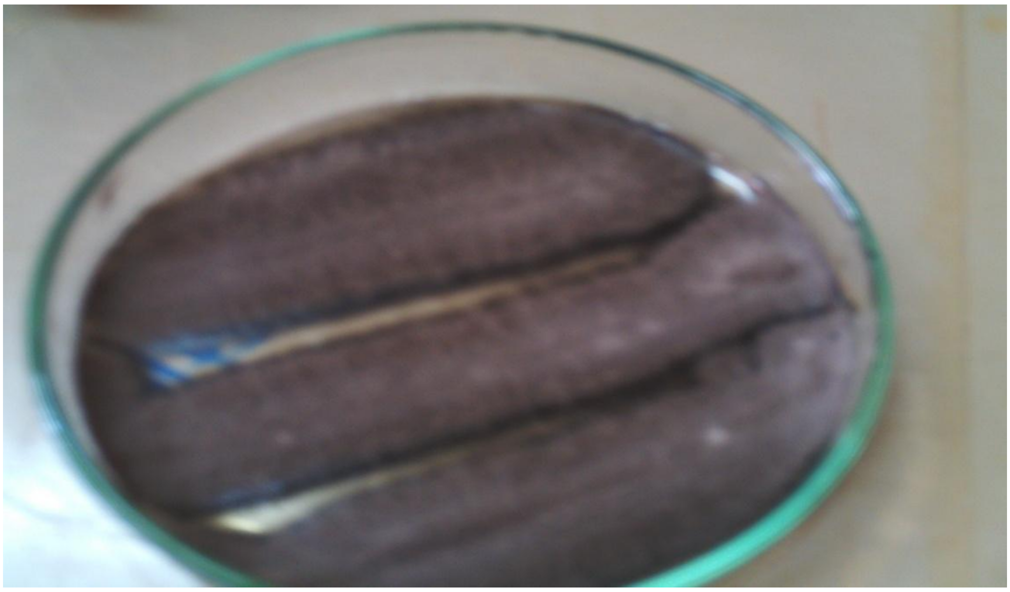


Plate 1: *C. kahawae* cultured in a Petri dish

Plate 2: DNA Extraction

Plate 3: DNA Amplification during PCR.

Plate 4: Coffee in bloom (left) and ripe coffee berries (right).

Plate 5: *Coffea arabica* beans

Plate 6: Coffee seedlings ready for inoculation

Plate7: Preparation of inoculum.

Plate 8: Coffee seedlings in boxes covered with black polythene papers after inoculation.

Plate 9: Seedlings ready for scoring for disease symptoms

Plate 10: Scoring for disease symptoms

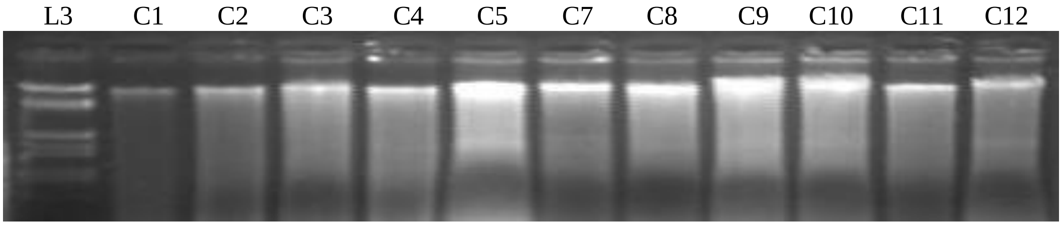
Plate 11 (a): Seedlings arranged in boxes according to the scale of Van der vossen *et al*., (1977).

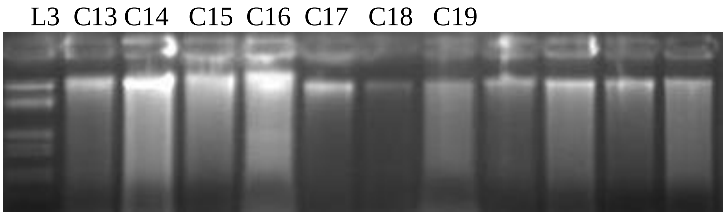
Plate 11(b): Seedlings in grade 12.

Appendix 2: QUANTIFICATION OF DNA OF THE CBD PATHOGEN ISOLATES

Sample composition

DNA	5µl
L/dye	2µls
Ddwater	3µl
	10µl





AMPLIFICATION

Master Mix	1Rxn (µl)
DdH2O	13.05
Buffer (10x)	2.50
DNTPs (500 µM)	3.75
Mg++ (25mM)	2.50
Primer	1.00
Taq (5 U/µl)s	0.20
DNA (1ng /µl)	2.00
Total	**25.00**