

**GENETIC CHARACTERISATION OF *COLLETOTRICHUM*
SUBLINEOLUM THE CAUSATIVE AGENT OF SORGHUM
ANTHRACNOSE**

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Bsc. Agric. (Hons) Muk**

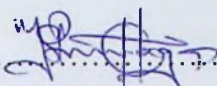
**A THESIS SUBMITTED TO THE SCHOOL OF GRADUATE STUDIES IN
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DECLARATION

I declare that the work in this thesis is my own and has not been submitted for the award of a degree in any other university.

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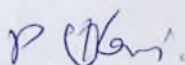


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DEDICATION

To my mother Mrs. Proscovia Nnansubuga Ssemazzi and my late father Mr. Ssemazzi Mayega Jones

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Every beginning always has to come to an ending. It has been two years of hectic struggle, acquiring new knowledge and experience in the field of plant pathology and biotechnology. This places me in a position to pay back to those people who mattered a lot throughout these two years but the only coin I can pay to them, is in the currency of gratitude to all. Therefore I wish to express gratitude to my principal supervisor Dr. Patrick Okori and Dr. Richard Edema for seeing me fit and providing me with this necessary chance to work with them. Dr. Geoffrey Tusiime, and Dr. Ssetumba Mukasa for showing me the correct path to victory by giving me good advises and courages when things were not working as expected. Also special thanks go the management of Fiduga Limited for all the support they gave me.

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SUMMARY

Sorghum is one of the important food crops in Uganda and other East African countries. Its production is curtailed largely by abiotic and biotic constraints. In Uganda and many other countries of East and Southern Africa, sorghum anthracnose is a key threat to production, yet the pathogen is highly variable, thus rapidly breaking down resistance of its host. In Uganda and East Africa in general no population studies of *Colletotrichum sublineolum* have been reported hence there is limited information on nature of epidemics and pathogen. In this present study, the general objective was to characterise sorghum anthracnose and its causative agent *Colletotrichum sublineolum* derived from various agro-ecologies of Uganda. A survey was conducted in 9 agro-ecologies of Uganda where incidence and severity was recorded and diseased leaf samples were taken for laboratory analysis. In the laboratory, DNA of isolates was amplified with three short, 17–21-mer, tandemly-repeated ISSR primers (CCA, CGA, ACA) with two (CCA) or three degenerated bases at their 5'-ends from Cape Town South Africa. The resulting DNA products were separated by agarose gel electrophoresis. The genomic data were scored for absence/presence of bands, respectively. Analysis of Variance (ANOVA) results showed that there was variation in incidence and severity sorghum anthracnose disease. Both incidences varied significantly ($P < 0.05$) between agro-ecologies reflecting the effect of different cropping pattern on sorghum anthracnose epiphytotics. Districts within agro-ecologies showed a significant variation in both incidence ($P < 0.05$) and severity ($P < 0.05$) of sorghum anthracnose. This might be attributed to the different crop management practices carried out by the farmers within an agro-ecology. Similar observations were

made for fields within the districts with significant differences occurring at $P < 0.05$ for both incidence and severity.

The technique of Inter-Simple Sequence Repeats (ISSR) produced 37 polymorphic loci and neighbour-joining analysis of ISSR data on 124 isolates revealed two major groups. Analysis of molecular variation (AMOVA) of ISSR data on the basis agro-ecologies revealed the presence of a population structure, ($\Phi F_{ST} = 0.08$ $P = 0.05$) leading to acceptance of the null hypothesis stating a presence of population differentiation between the agro-ecologies in Uganda. Gene flow between agro-ecologies was 5.75 calculated from ΦF_{ST} . The results of the AMOVA analysis also revealed that allelic variation (92.5%) was shared between populations. Average gene diversity over all loci ranged from 0.192 to 0.335 showing high diversity within population rather than between populations. The number of polymorphic loci were similar for the population studied.

Variation in the prevalence, incidence and severity of the anthracnose epidemic in the sorghum growing agro-ecologies of Uganda suggest that sorghum anthracnose is a major threat to production in virtually all agro-ecologies in Uganda. Moreover the high severities suggest cultivation of very susceptible varieties. The cultivation of resistant varieties will support management of the disease.

CHAPTER ONE

INTRODUCTION

1.1 Distribution and importance of sorghum

Sorghum (*Sorghum bicolor* (L.) Moench) is one of the most important food crops of the world. It is cultivated in over 100 countries in the tropical, sub tropical and temperate regions of the world where it is a major staple for millions of people and provides income through local and international trade. Sorghum has a distinct advantage compared to other major cereals, being drought resistant. Consequently, sorghum plays a crucial role in the world food economy as it contributes to rural household food security (ICRISAT, 2001). Sorghum is ranked the fifth most important cereal after wheat, maize, rice and barley (FAO, 2003). However, in terms of tonnage, sorghum is Africa's second most important cereal. The continent produces about 20 million tonnes of sorghum per annum and about one-third of the world crop (FAO, 2003). The United States is the largest producer and exporter of sorghum, accounting for 25% of world production and 75% of world sorghum exports (USDA, 2006). In Africa, the 10 largest sorghum producers include: Nigeria, Sudan, Ethiopia, Burkina Faso, Niger, Tanzania, Mali, Chad, Egypt and Uganda. These countries also contain some of the centers of diversity for sorghum (Poehlman and Sleper, 1995).

Besides its importance as a source of income, sorghum has a number of desirable attributes to the farmers. It is a versatile crop, with varieties adapted to a wide range of climates, soils, and moisture conditions, making its cultivation possible even in some of the world's most austere environments (Frederiksen, 1991). Elsewhere, sorghum stems

are often used for a wide range of purposes including; construction of walls, fences and thatches, and production of brooms, mats, baskets, fish-traps and sun shades. They are also used as fuel and as a soil organic additive to improve its fertility. Some varieties of sorghum can be “malted” to produce a nutritious drink for infants and for use in bakery products (FAO, 2004). Malted sorghum can also be used in small-scale traditional beer production, an important income-earning activity for village women. Indeed the crop’s importance is underpinned by the millions of smallholder farming communities in developing countries of Africa and Asia.

Despite the diverse importance of sorghum and the role it plays in food security, a number of constraints compound its production. Among the several constraints, pests and diseases are the most important biotic constraint to sorghum production. Indeed, sorghum is distinguished among other cereals by its unusually broad range of diseases (Frederiksen, 1991). The crop is normally attacked by more than one pathogen during its growth cycle (Frederiksen, 1991). One of the most important diseases of sorghum is anthracnose caused by *Colletotrichum sublineolum*.

1.2 Constraints to production of sorghum

Although sorghum is relatively tolerant to adverse growing conditions, production is currently declining in East Africa. Also, compared to yields in the United States, grain yields are very low in Eastern Africa and well below the genetic potential. The total production of sorghum in the world in 1990 was 58 million tonnes, a decrease from 60 million tonnes in 1989 and 62 million tonnes in 1988 (FAO, 1991). A decrease in yield from 1,340 kg/ha in 1989 to 1,312 kg/ha in 1990 was reported, while the area remained

around 44 million hectares in both years. Esele (1995) attributed the decline in production to a number of constraints such as low yielding varieties, poor production practices, infertile soils, drought, labour shortage, poor marketing systems prevalence of weeds, and pests including birds and diseases. In Uganda, total production has declined from 443,000 metric tones in 2003 to 420,000 metric tones in 2005 (FAO STAT, 2005). The decline in production is attributed to a number of biotic and abiotic stresses.

Most important biotic stresses include diseases, pests, and weeds. The major pests are; sorghum shoot fly, birds, grasshoppers and sorghum stem borer. Birds are perhaps one of the most important pests of sorghum not only in Africa but also in other parts of the world. They are capable of inflicting heavy losses and causing economic damage. In Africa the most notorious species is *Quelea quelea* and is found in the Sahel region, right from Senegal in West Africa through to Sudan, Uganda, Kenya, Southern Tanzania, Malawi, Zambia and South Africa (Bruggers and Elliot, 1989; Mundy and Jarvis, 1989). Also stem borers are important throughout semi-arid regions where sorghum is cultivated. The most important anthropod stem borer species include; *Chilo partellus*, *Sesamia calamitis* and *Buseola sorghida*. *Chilo partellus* is mainly found in the semi arid areas of East Africa and India, while *Sesamia calamitis* and *Buseola sorghida* are distributed throughout sorghum growing areas in Africa (Songa *et al.*, 2001).

Another important environmental related issue is infestation by *Striga*, a parasitic weed (Ramaiah *et al.*, 1983). The tiny brown *Striga* seeds can remain viable in the soil for 15 to 20 years in the absence of a suitable plant host (Ramaiah *et al.*, 1983). Infestation of continuously cultivated fields has become a major constraint to sorghum production in

many parts of Africa (Ramaiah *et al.*, 1983). Heavy infestation can leave the land unfit for cropping and fields have been abandoned in the worst affected areas. *Striga* affects an estimated 8 million hectares in Africa about 40 % of the total sorghum area with annual yield losses estimated at over US\$ 90 million (Ramaiah *et al.*, 1983).

The most prevalent diseases include; Maize Dwarf Mosaic disease caused by maize Dwarf Mosaic Virus (MDMV), sorghum downy mildew (*Peronosclerospora sorghi*), head smut (*Sporisorium reilianum*), anthracnose (*Colletotrichum sublineolum*), that damages leaves, stems, and panicles of grain sorghum plants. Yield losses are proportional to duration and severity of disease, but losses of 30 – 50% have been reported in the United States (Ali and Warren, 1992) and yield losses of 8 – 67% have been reported from West Africa (Thomas, 1992).

Abiotic stresses cause extensive losses to agricultural production worldwide (Bray *et al.*, 2000). Most abiotic stresses are attributed to climatic and edaphic factors such as unreliable rainfall, high temperatures and soil fertility related aspects. Most of the agriculture practiced in sub-Saharan Africa is rain fed, and hence greatly affected by drought which is common in Southern and Eastern Africa (Pingali, 2001). Individually, abiotic stresses such as drought, salinity or heat have been the subject of intense research (Bray *et al.*, 2000). However, in the field, crops and other plants are routinely subjected to a combination of different abiotic stresses (Moffat, 2002).

1.3 Justification of the study

Sorghum is an important staple food crop in tropical and sub-tropical agro-ecologies of sub-Saharan Africa, it is genetically adapted to harsh drought-prone environments and is

capable of producing grain and fodder where other crops can not survive (Frederiksen, 1991). However, the crop is distinguished among other cereals by its unusually broad range of diseases. Sorghum is normally attacked by more than one pathogen during its growth cycle and thus diseases are the most important constraint to sorghum production world-wide (Frederiksen, 1991).

Sorghum anthracnose caused by *Colletotrichum sublineolum* is one of the most devastating diseases affecting cultivated sorghum (Sherriff *et al.*, 1995; Thakur and Mathur, 2000). The disease is known to occur in epidemic proportions in many parts of the world, causing severe losses depending on the crop stage, cultivar susceptibility and to a great extent, the conduciveness of the environment. The severity and significance of damages caused by this pathogen necessitates development of strategies to control and manage the disease so as to reduce crop losses and avert epidemics.



Figure 1. Typical anthracnose symptoms showing on infected sorghum leaf.

Host resistance has been used in the management of several diseases and thus breeding sorghum varieties with genetic resistance to sorghum anthracnose is an effective and economical strategy for control of the disease. *Colletotrichum sublineolum* is a highly variable pathogen that rapidly breaks down resistance (Thakur, 1995). Changes in the virulence pattern of pathogen populations have been reported from several parts of the world.

The first report on existence of races of *C. sublineolum* was in 1967 (Thakur and Mathur, 2000). From 1967 to 1991, 44 *Colletotrichum spp* races or pathotypes have been reported, but because of the use of different sets of host differentials and the use of different rating scales, a global picture of the pathogenic races is not clear (Thakur, 2004). The appearance of different pathogenic races could be explained by the long-time association of the pathogen with sorghum (Ali and Warren, 1987). Different symptoms produced on sorghum genotypes and differences in cultural characteristics suggested physiological variation within pathogen populations (Pande *et al.*, 1991). Increased variability of *C. sublineolum* has been attributed to the parasexual cycle (Souza-Paccola *et al.*, 2003). A gene-for-gene interaction between sorghum genotypes and pathogenic races has not been demonstrated (Rosewich *et al.*, 1998), although it has been considered that the pathogen evolved alongside the traditional sorghum landraces, conceivably suggesting a gene-for-gene interaction (Ngugi *et al.*, 2002).

The rapid resistance break-down poses a great challenge to sorghum breeding. Therefore one way to reduce rapid resistance breakdown is to slow down the rate of evolution of novel genotypes through use of techniques that do not create very high selection pressure

on the pathogen (McDonald and Linde, 2002). Since some resistance genes are effective only against particular pest or pathogen sub-populations, it is important to understand the structure of pathogen populations in order to determine the best strategy for deployment of resistance and/or incorporate the non-matching resistance genes to the existing pathogen. Consequently, information on pathogen population, diversity, and virulence will be very important for a successful sorghum-breeding program. Unfortunately, there is limited information regarding *Colletotrichum sublineolum* populations in Uganda, yet the disease continues to impede production both locally and elsewhere.

1.4 Study goal

The goal of this study is to characterise sorghum anthracnose and its causative agent *Colletotrichum sublineolum* derived from various agro-ecologies of Uganda.

1.4.1 Study objectives

1. To determine the incidence and severity of *Colletotrichum sublineolum* among the sorghum growing districts of Uganda.
2. To assess the genetic variability of *Colletotrichum sublineolum* populations in Uganda.

1.5 Hypothesis

1. Sorghum anthracnose epiphytotics are influenced by genetic variability of *Colletotrichum sublineolum*, sorghum genotype selection pressure and crop culture.
2. A population sub-structure of *Colletotrichum sublineolum* exists in Uganda.

CHAPTER TWO

LITERATURE REVIEW

2.1 Sorghum anthracnose occurrence and importance

The cause of anthracnose in cereals, including maize and sorghum, had been ascribed to be *Colletotrichum graminicola* (Holiday, 1980). However recent rDNA sequence analysis has demonstrated that isolates from maize and sorghum are different species. Isolates from maize are now regarded as *C. graminicola*, and those from sorghum as *C. sublineolum* P. Henn. Apud Kabát & Bub (Sherriff *et al.*, 1995; Wharton and Julian, 1996). *Colletotrichum sublineolum* poses a serious threat to grain sorghum production and profitability (Sherriff *et al.*, 1995; Thakur and Mathur, 2000). The disease was first observed in Togo, West Africa in 1902 and is currently found in many sorghum growing regions of the world. (Thakur and Mathur, 2000). In the United States, the disease was first observed in Texas in 1912 (Harris *et al.*, 1964). Anthracnose is considered one of the most destructive diseases of sorghum in areas where warm, humid climatic conditions are prevalent (Cardwell *et al.*, 1989; Thakur and Mathur, 2000). The disease is also a major constraint to sorghum production in West and Central Africa and is widely distributed in Sahel, Sudan and Guinea zones of West Africa (Pande *et al.*, 1991; Thomas *et al.*, 1992; Marley *et al.*, 2001). During severe epiphytotics, grain yield losses of 50% or greater occur on susceptible cultivars from the foliar phase of the disease (Thakur and Mathur, 2000; Ngugi *et al.*, 2000; Marley *et al.*, 2001). Grain yield losses of 41 to 67% have also been reported under experimental conditions in Mali (Thomas *et al.*, 1992). Grain yield losses due to panicle infection of susceptible cultivars range from 30-50% during severe epidemics (Thakur and Mathur, 2000). Grain yield losses are generally associated with a reduction in kernel number and size (Thomas *et al.*, 1992; Thakur and Mathur, 2000).

2.2.1 Symptoms and management practices of anthracnose

Anthracnose can infect all aerial parts of the plant (Thakur and Mathur, 2000; Hess *et al.*, 2002), but only leaf symptoms were recognized until 1943 when serious outbreaks of stalk rot were reported in Illinois (Koehler, 1943). Typical symptoms of foliar infection are small, circular, elliptical, or elongated spots (Thakur and Mathur, 2000). These lesions are generally 3-5 mm in length, but may be up to 20 mm long. The lesions develop gray to straw-colored centers with wide margins that are tan, orange, or red to blackish purple, depending on the disease response and the host plant color. Under conditions of high humidity or high levels of rainfall, the lesions increase in number and coalesce to cover most of the leaf area, and may show zonation. On the surface of the tan centers of the lesions, few to numerous, small, circular, concentric, erumpent black dots develop, which are fruiting bodies known as acervuli. The acervuli contribute to the spread of the disease (Thakur and Mathur, 2000).

Severe anthracnose infection can cause premature defoliation, reducing plant growth and affecting plant development (Thomas *et al.*, 1992; Thakur and Mathur, 2000). Sorghum anthracnose has been referred to as red leaf blight, stalk rot, panicle blight or seedling blight, depending on the site of infection. Foliar infection occurs more frequently and has been reported to be more severe than infection of other plant tissues (Marley and Ajayi, 2002). Infection in other tissue may occur in the absence or presence of foliar infection. Mid-rib infection may occur on cultivars that have little leaf damage, and this response may be independent of leaf infection (Thakur and Mathur, 2000). Symptoms of the mid-rib infection are elliptical to elongated lesions with acervuli. If mid-rib and foliar infections occur together, leaf damage, defoliation, and reduction of yield may be greater.

Infection often extends to leaf sheaths, where sunken elliptical lesions are formed (Thakur and Mathur, 2000).

2.2.2 Management of anthracnose

In areas favorable for high disease pressure, practices such as the elimination of alternative host weed species, use of resistant hybrids, early planting, ploughing under infected crop debris, intercropping and crop rotation can help control the disease by reducing the primary inoculum and thus preventing severe infection early in plant development (Ngugi *et al.*, 2001; Ngugi *et al.*, 2002). In contrast, minimum tillage practices increase the incidence of anthracnose in production fields, due to the survival of the pathogen on plant residue (Casela and Frederiksen, 1993). Effective control of anthracnose can be obtained by the use of fungicides; however, the pathogen might become highly resistant as reported for *C. graminicola* (Avila-Adame *et al.*, 2003). Furthermore, this practice is uneconomical for farmers in developing countries where sorghum is a major crop due to the low return to investment for cereals.

Genetic resistance is considered the ideal method of controlling diseases in cultivated plant species (Tenkouano *et al.*, 1993). The use of resistant cultivars is the most economical control method for sorghum anthracnose (Thakur and Mathur, 2000; Marley, 2004). Host plant resistance is an important component for the integrated disease management of leaf anthracnose, and can help reduce quantitative losses in sorghum grain yield (Hess *et al.*, 2002). The availability of stable sources of resistance with high yield and good grain quality is very important for maintenance of sustainable sorghum productivity, (Thakur and Mathur, 2000; Hess *et al.*, 2002).

Most sources of anthracnose resistance are considered to possess multiple genes (Thakur and Mathur, 2000). However, limited information is available on the genetics of host plant resistance and few sources have been used in sorghum improvement programme. Also, sources of horizontal resistance have been reported, which influence disease development. This type of resistance is expected to be inherited as a multigenic trait and significantly influenced by environmental conditions (Casela *et al.*, 1993). It is expressed as a reduced infection frequency, slow rate of disease development in the host and a slow rate of sporulation (Casela *et al.*, 1993). Although this type of resistance has importance, the use of horizontal resistance in hybrid production may have limited use as resistance would need to be incorporated into multiple parental lines. Thus, additional sources of resistance are essential for hybrid development.

2.3 Epidemiology of sorghum anthracnose

2.3.1 Infection cycle

Colletotrichum is a large genus of ascomycete fungi. It contains species that are amongst the most successful plant pathogenic fungi, causing significant economic damage to crops in tropical, sub-tropical, and temperate regions (Bailey and Jeger, 1992). The *Colletotrichum* pathogen survives on crop residues left on the soil surface especially panicles and seed. *Colletotrichum sublineolum* has a two-stage, hemibiotrophic infection process on sorghum, similar to that of *C. lindemuthianum* on bean (Wharton and Julian, 1996). The initial biotrophic phase is associated with intracellular infection vesicles and primary hyphae, which colonise many host cells before giving rise to necrotrophic secondary hyphae (O'Connell *et al.*, 1985; Wharton and Julian, 1996). In incompatible

interactions, host cells die soon after penetration by infection vesicles, and fungal development is restricted to a single epidermal cell (Wharton and Julian, 1996). Accumulation of red-pigmented cytoplasmic inclusions containing 3-deoxyanthocyanidin phytoalexins occurs in both compatible and incompatible interactions (Snyder *et al.*, 1991). This defense response, may however be expressed much earlier in incompatible interactions (Wharton and Julian, 1996; Lo *et al.*, 1999). During the disease spread up to infection period, conidia from sporulating lesions are mostly rain splashed onto the surfaces of the lower leaves of young plants. Since the disease is favored by warm, moist weather, disease severity increases during protracted periods of low light intensity and high humidity (Schall *et al.*, 1980).

The early stages of fungal development during the infection process are essentially the same for all *Colletotrichum* species and can be separated into stages including: (i) the deposition of conidia on plant surface, (ii) attachment of conidia to plant surfaces, (iii) germination of conidia, (iv) production of appressoria, (v) penetration of the plant epidermis, (vi) growth and colonisation of plant tissues, and (vii) production of acervuli and sporulation (Jeffries *et al.*, 1990; Prusky *et al.*, 2000). But the occurrence and relevance of each stage in the infection process may vary depending on the conditions of growth, the host tissue, the particular species, and or the fungal isolate (Bailey *et al.*, 1992).

Usually initial infection occurs at the base of the plant or on the roots from tiny black acervuli from the soil borne inoculum. Acervuli that appear on the upper leaves develop from the secondary spread of conidia which produces large numbers of spores in pinkish

masses during warm to hot, damp weather (Kollo, 1988). Spores from disintegrating debris are carried by wind or rain splash where they germinate and penetrate the tissues directly and start new infections.

2.3.2 Temporal and spatial attributes of anthracnose disease epiphytotics.

All plant disease pyramids typically comprise of the following key components. (i) pathogen, (ii) host resistance/susceptibility, (iii) role of environment, (iv) human activities, and (v) time (Okori, 2004). In the sorghum anthracnose pathosystem, pathogen variability, host resistance and environment appears to play the most crucial roles in epidemics that is every thing in the disease triangle. In general, the rate of development of any disease epidemics is driven by three factors that interact in time and space; (i) the initial amount of inoculum or disease, (ii) the rate of within season pathogen perpetuation and (iii) the proportion of healthy tissue remaining to be infected (Ward *et al.*, 1999). Inoculum in general originates from sporulation on the leaves or in the crop residues left in the field.

Anthrachnose spreads rapidly in the high rainfall and warmer agricultural areas (Shea, 1999). Other sources of inoculum include; infected seeds, stalk residues and /or adjacent growing crops or weeds (Casela and Frederiksen, 1993). Anthracnose is most severe on mature plants especially during the information of the panicle. The senescence of sorghum leaves plays an important role in the development of the disease. The telemorphic state (or sexual phase) of *Colletotrichum sublineolum* is not known and thus the anthracnose disease is presumed to be spread exclusively by conidia. Conidia can be

dispersal over longer distances as dry spore masses, but within smaller regions rain splash dispersal of moist conidia is largely responsible for the development of epidemics. The pathogen is seed borne and this plays a major part in establishing the pathogen within the crop (Cardwell *et al.*, 1989). Transmission of the pathogen on seed is presumably responsible for dissemination of over long distances.

2.3.3 *Colletotrichum* species diversity

The genus *Colletotrichum* is a member of the order Melanconiales of the class Coelomycetes and it is one of the most important genera of plant pathogens (Hawksworth, 1983). It has a worldwide distribution but is found mainly in subtropical and tropical regions (Bailey and Jegger, 1992). *Colletotrichum* have had a substantial impact on world agricultural production through their capacity to cause economic losses on a number of important cereal, legume, fruit and cash crops. The ability of many *Colletotrichum* species to cause latent or quiescent infections places them among the most important of post harvest pathogens.

The genus *Colletotrichum* was first described by Tode in 1790 under the name *Vermicularia*, but was later established as *Colletotrichum* by Corda in 1837. Despite the original clear description and excellent illustrations provided by Corda, the genus has been re-described under several different names, the two most common being *Vermicularia* and *Gloeosporium*. The names *Colletotrichum* and *Vermicularia* were used inter-changeably during the 19th and early 20th centuries for a range of species now accepted in the genus *Colletotrichum* (Sutton, 1992). Clements and Shear (1931) distinguished *Colletotrichum* from *Vermicularia* by the presence of marginal setae in

Colletotrichum, compared with setae dispersed throughout the conidiomata in *Vermicularia*. However, *Gloeosporium* species were not thought to produce setae, some could produce setae on certain substrates. This resulted in a large number of species being transferred from *Vermicularia* to *Colletotrichum*. There were also problems in distinguishing *Colletotrichum* from the morphologically similar *Gloeosporiums*, although *Gloeosporium* species were not thought to produce setae, some could produce setae on certain substrates. The names of some the *Gloeosporium* species have now been transferred to *Colletotrichum* as distinct acceptable species but the majority have been reduced to synonymy with the already well-known species.

The morphological features which identify the genus are its acervuli conidiomata, often with setae, producing elongated slimy conidia, and the presence of appressoria. Using this system around 900 'species' have been assigned to *Colletotrichum* (Sutton, 1992). Using host specificity, the number of species has reduced to about 37 (Sutton, 1992). The difficulties in species discrimination have resulted in some confusion in taxonomic grouping, for example von arx (1970) recognized eight host-specific species or 'types' in *C. gloeosporioides*, whilst Sutton (1980) recognized species such as *C. gloeosporioides*, *C. dematium*, and *C. capsici* as 'group species.' *Colletotrichum* species can be conveniently divided into two classes, one with straight conidia, and the other, with falcate conidia. Of the few species of *Colletotrichum* which undergo a sexual mode of reproduction (designated in the teleomorphic genus *Glomerella*), ascus states have frequently been associated with species producing straight conidia.

Although there are still problems with providing a workable taxonomy for this group, molecular biology is providing new insights into systematics, particularly in the delimitation of species and in defining inter- and intraspecific relationships (Bruns *et al.*, 1991). The most significant advances in taxonomy have been obtained from approaches based on the analysis of nucleic acids. These include comparisons of DNA sequences, analysis of restriction fragment-length polymorphisms (RFLPs), and the use of polymerase chain reaction (PCR)-based techniques to assess random amplified polymorphic DNAs (RAPDs) (Welsh and McClelland, 1990; Williams *et al.*, 1990). RFLP analysis has been used to determine the genetic relationship within *C. gloeosporioides* isolates which cause anthracnose on *Stylosanthes* species (Braithwaite *et al.*, 1990). RAPD analyses have been used to assess the molecular variation of 39 *C. gloeosporioides* isolates from different hosts, dividing this group into several sub-groups (Mills *et al.*, 1992). DNA sequence analyses have also been used to distinguish one group, consisting of *C. lindemuthianum*, *C. malvarum*, *C. obiculare*, and *C. Trifolii*, from all other *Colletotrichum* species (Sherriff *et al.*, 1995). They also verified the distinction between species with falcate conidia.

Genomic markers are proving to be of practical value for identification and diagnosis, providing a quick method for characterising variations and identifying genotypes within pathogen populations. By regularly sampling field material, researchers can follow the shifts in the genetic makeup of pathogen populations, which can then provide a dynamic picture of the interactions between host and pathogen types (Guthrie *et al.*, 1992).

2.4 Genetic diversity of *Colletotrichum sublineolum*.

Colletotrichum sublineolum pathotypes are highly variable and may change even while attempts are being made for classifying virulence (Casela *et al.*, 1995; 2001). It is therefore important that efforts to study pathogen changes attributed to genetic, physiological or environmental events are investigated. The development of efficient tools for promptly identifying new pathogen genotypes can help researchers follow changes in pathogen genotype structures and thus provide a dynamic picture of interactions between host and pathogen genotypes.

Colletotrichum sublineolum is generally regarded a “hyper variable pathogen” with respect to virulence (Frederiksen *et al.*, 1995). Many physiological races of *C. sublineolum* infecting sorghum have been identified in Brazilian fields and new races appear every year (Casela and Frederiksen, 1994; Casela *et al.*, 2001). In addition, phenotypic and physiological differentiations among *C. sublineolum* pathotypes are not always straight forward (Pande *et al.*, 1991; Frederiksen *et al.*, 1995). These effectively compound the breeding for new resistant varieties (Guthrie *et al.*, 1992). The knowledge concerning genetic diversity of *C. sublineolum* populations is crucial for disease management and for establishing the relationship among environmental variations and changes in pathotype aggressiveness (Wise *et al.*, 1995; Casela *et al.*, 2001). No studies have been conducted on *C. sublineolum* populations in Uganda. This is the focus of this study.

2.5 Population genetics of plant pathogens

The knowledge about the genetic structure of plant pathogen populations and how it relates to disease epidemics have been realised over the last 20 years (Wolfe *et al.*, 1987). This development in plant disease epidemiology has evolved with clear definition of the role of various disease pyramid factors and their concerted interactions on the amounts of incidence and severity of the disease epidemics. Indeed, the advent of molecular genetics and advances in computational biology has led to a large increase in pathogen population studies. Although the number of fungal studies has increased greatly, the most comprehensive work has focused on a small number of plant-pathogenic fungi. The majority of these fungi can be recognised easily by their fruiting bodies or disease symptoms on above ground plant parts.

From an epidemiology perspective, plant pathologists have been aware of the ability of pathogen populations to evolve since Stakman described the wheat rusts as “shifty enemies.” Agricultural ecosystems environment are in general subjected to changes induces by the use of resistant varieties, fungicide, fertilisers, irrigation, and crop rotation. These standard agricultural practices impose strong directional selection on pathogen populations leading to pathogen evolution. These control strategies must target populations instead of individuals if they are to be effective accordingly (Okori *et a.*, 2004). This provides rationale for plant pathologists to focus more effort on understanding the genetics of populations and how populations evolve in response to different control strategies. In general, many factors contribute to pathotype evolution. These factors include mutation, mating systems, gene flow or migration, population size, and selection. In plant pathology, the ultimate goal should be to determine which

evolution factors play major roles in pathogen evolution and how these evolutionary forces interact to determine the genetic composition and evolutionary potential of pathogen populations (Okori *et al.*, 2004). This study is an attempt to contribute to *C. sublineolum* population studies.

2.5.1 The genetic structure of populations

Genetic structure refers to the amount and distribution of genetic variation within and among populations (Okori *et al.*, 2004). The majority of fungal studies to date have focused on the genetic structure of populations (Peever and Milgroom, 1994). Defining the genetic structure of populations is the first logical step in studies of fungal population genetics because the genetic structure of a population reflects its evolutionary history and potential to evolve (Okori *et al.*, 2004). For evolution to occur by natural selection there must be variation in fitness among individuals. Fisher's fundamental theorem of natural selection, states that the mean fitness of a population is proportional to its variance (Fisher, 1930). This implies that the more variation there is in a population, the greater the adaptive capacity of that population or evolutionary potential of a population is proportional to the amount of genetic diversity in a population. However, Fisher's fundamental theorem applies only to genes that are known to have an effect on fitness. Selectively neutral DNA-based genetic markers such as restriction fragment length polymorphisms (RFLPs) or random amplified polymorphic DNAs (RAPDs) will not necessarily reflect the amount of genetic variation present in genes that condition fitness.

Genotypic diversity is one of several components estimated during analysis of the genetic structure of populations of microorganisms. Estimates of genotypic diversity are

functions of both the number of genotypes observed in a sample (genotype richness) and the evenness of distribution of genotypes within the sample. Two indices of genotypic diversity, Stoddart and Taylor (1988) and Shannon-Wiener (1949), have been used most frequently in the estimation of genotypic diversity of plant pathogen populations (McDonald *et al.*, 1996; Borchart *et al.*, 1998; Caffier *et al.*, 1999). These indices of diversity have been used in plant pathology to measure phenotypic diversity for example, for race (or pathotype) structure of rust fungi (Groth and Roelfs, 1989) and Oomycetes (Andrivon, 1994), as well as genotypic diversity for allozymes or other molecular markers (Goodwin *et al.*, 1993).

2.5.2 Tools and techniques used to study population genetics.

Genetic differentiation between populations arises due to the interaction between natural and sexual selection, migration and genetic drift. At neutral loci, the observed levels of population differentiation are usually assumed to represent equilibrium between mutation, migration and genetic drift (Wright, 1969). Variation in levels of neutral intraspecific differentiation will therefore largely reflect differences in dispersal and population size, although population structure will also be influenced by species-specific responses to biogeography history. Therefore the biological diversity observed within a population may be due to genetic variation, environmental variation or a combination of both. Evolutionary biologists frequently want to quantify the former as it is a prerequisite for an evolutionary response to selection.

Genetic variation of a population can be measured on the basis of either ecologically important traits or selectively neutral genetic markers. Molecular markers are identifiable DNA sequences found at specific locations of the genome. Molecular markers are being increasingly used to characterise fungal plant pathogen populations in terms of genetic diversity and phylogenetic relationships within and between species. Additionally, markers that are closely linked to avirulence genes are being increasingly sought to assist map based gene cloning. The main marker technologies used to study population and evolutionary biology presented below.

2.5.2.1 Restriction Fragment Length Polymorphism (RFLP)

These are bands that correspond to DNA fragments, usually within the range of 2–10 kb, which have resulted from the digestion of genomic DNA with restriction enzymes. DNA fragments are separated by agarose gel electrophoresis and are detected by subsequent Southern blot hybridisation to a labelled DNA probe. Labelling of the probe may be performed with radioactive isotopes or with alternative non-radioactive stains, such as digoxigenin or fluorescein. The locus specific RFLP probes consist of a homologous sequence of a specific chromosomal region. Probes are generated through the construction of genomic or complementary DNA (cDNA) libraries and therefore, may be composed of a specific sequence of unknown identity (genomic DNA) or part of the sequence of a functional gene (exons only). RFLP probes are maintained as clones in suitable bacterial vectors that conveniently allow the isolation of the DNA fragments they hold. Probes from related species may be used (heterologous probes). DNA sequence variation affecting the absence or presence of recognition sites of restriction enzymes, and insertions and deletions within two adjacent restriction sites, form the basis of length

polymorphisms. RFLPs are generally found to be moderately polymorphic. In addition to their high genomic abundance and random distribution, RFLPs have the advantages of showing codominant alleles and high reproducibility. The main drawbacks of RFLPs are the requirement of laborious and technically demanding methodological procedures and high expense. In general, if research is conducted with poorly studied groups of wild species or poorly studied crops (orphan crops) suitable probes may not yet be available, so considerable investments are needed for development. Moreover, large quantities (1–10 µg) of purified, high molecular weight DNA are required for each DNA digestion. Larger quantities are needed for species with larger genomes, and for the greater number of times needed to probe each blot. RFLPs are not amenable to automation and collaboration among research teams requires distribution of probes.

2.5.2.2 Minisatellites analysis

Minisatellites like RFLPs, also involves digestion of genomic DNA with restriction endonucleases, but they are conceptually a very different class of marker. Minisatellites consist of chromosomal regions containing tandem repeat units of a 10–50 base motif, flanked by conserved DNA restriction sites. A minisatellite profile consisting of many bands, usually within a 4–20 kb size range, is generated by using common multilocus probes that are able to hybridise to minisatellite sequences in different species. Locus specific probes can be developed by molecular cloning of DNA restriction fragments, subsequent screening with a multilocus minisatellite probe and isolation of specific fragments. Variation in the number of repeat units, due to unequal crossing over or gene conversion, is considered to be the main cause of length polymorphisms. Due to the high mutation rate of minisatellites, the level of polymorphism is substantial, generally

resulting in unique multilocus profiles for different individuals within a population. Minisatellite loci are also often referred to as Variable Number of Tandem Repeats (VNTR) loci. The main advantages of minisatellites are their high level of polymorphism and high reproducibility. Disadvantages of minisatellites are similar to RFLPs due to the high similarity in methodological procedures. If multilocus probes are used, highly informative profiles are generally observed due to the generation of many informative bands per reaction. In that case, band profiles can not be interpreted in terms of loci and alleles and similar sized fragments may be non-homologous. In addition, the random distribution of minisatellites across the genome has been questioned (Schlötterer, 2004).

2.5.2.3 Random Amplified Polymorphic DNA (RAPD)

These are DNA fragments amplified by the PCR using short synthetic primers (generally 10 bp) of random sequence. These oligonucleotides serve as both forward and reverse primers, and are usually able to amplify fragments from 1–10 genomic sites simultaneously. Amplified fragments, usually within the 0.5–5 kb size range, are separated by agarose gel electrophoresis, and polymorphisms are detected after ethidium bromide staining, as the presence or absence of bands of particular sizes. These polymorphisms are considered to be primarily due to variation in the primer annealing sites, but they can also be generated by length differences in the amplified sequence between primer annealing sites. The main advantage of RAPDs is that they are quick and easy to assay. Because PCR is involved, only low quantities of template DNA are required, usually 5–50 ng per reaction. Since random primers are commercially available, no sequence data for primer construction are needed. Moreover, RAPDs have a very high

genomic abundance and are randomly distributed throughout the genome. The main drawback of RAPDs is their low reproducibility (Schierwater and Ender, 1993). Hence highly standardised experimental procedures are needed because of their sensitivity to the reaction conditions. RAPD analyses generally require purified, high molecular weight DNA, and precautions are needed to avoid contamination of DNA samples because short random primers are used that are able to amplify DNA fragments in a variety of organisms. Altogether, the inherent problems of reproducibility make RAPDs unsuitable markers for transference or comparison of results among research teams working in a similar species and subject. As for most other multilocus techniques, RAPD markers are not locus-specific, band profiles cannot be interpreted in terms of loci and alleles (dominance of markers), and similar sized fragments may not be homologous.

2.5.2.4 Microsatellites

Like minisatellites, represent tandem repeats, but their repeat motifs are shorter (1–6 base pairs). If nucleotide sequences in the flanking regions of the microsatellite are known, specific primers (generally 20–25 bp) can be designed to amplify the microsatellite by PCR. Microsatellites and their flanking sequences can be identified by constructing a small-insert genomic library, screening the library with a synthetically labelled oligonucleotide repeat and sequencing the positive clones. Alternatively, microsatellites may be identified by screening sequence databases for microsatellite sequence motifs from which adjacent primers may then be designed. In addition, primers may be used that have already been designed for closely related species. Polymerase slippage during DNA replication, or slipped strand mispairing, is considered to be the main cause of variation

in the number of repeat units of a microsatellite, resulting in length polymorphisms that can be detected by gel electrophoresis. Other causes have also been reported (Matsuoka *et al.*, 2002). The strengths of microsatellites include the codominance of alleles, their high genomic abundance in eukaryotes and their random distribution throughout the genome, with preferential association in low-copy regions (Morgante *et al.*, 2002). Because the technique is PCR-based, only low quantities of template DNA (10–100 ng per reaction) are required. Due to the use of long PCR primers, the reproducibility of microsatellites is high and analyses do not require high quality DNA. Although microsatellite analysis is, in principle, a single-locus technique, multiple microsatellites may be multiplexed during PCR or gel electrophoresis if the size ranges of the alleles of different loci do not overlap (Ghislain *et al.*, 2004). This decreases significantly the analytical costs. Furthermore, the screening of microsatellite variation can be automated, if the use of automatic sequencers is an option.

One of the main drawbacks of microsatellites is that high development costs are involved if adequate primer sequences for the species of interest are unavailable, making them difficult to apply to unstudied groups. Although microsatellites are in principle codominant markers, mutations in the primer annealing sites may result in the occurrence of null alleles (no amplification of the intended PCR product), which may lead to errors in genotype scoring. The potential presence of null alleles increases with the use of microsatellite primers generated from germplasm unrelated to the species used to generate the microsatellite primers (poor “cross species amplification”). Null alleles may

result in a biased estimate of the allelic and genotypic frequencies and an underestimation of heterozygosity.

2.5.2.5 Inter Simple Sequence Repeats (ISSR)

These are DNA fragments of about 100–3000 bp located between, adjacent, oppositely oriented microsatellite regions. ISSR analyses have been used to increase the level of polymorphism detection (Coyne *et al.*, 2001, Borner *et al.*, 2004). ISSRs are amplified by PCR using microsatellite core sequences as primers with a few selective nucleotides as anchors into the non-repeat adjacent regions (16–18 bp). About 10–60 fragments from multiple loci are generated simultaneously, separated by gel electrophoresis and scored as the presence or absence of fragments of particular size. Techniques related to ISSR analysis are Single Primer Amplification Reaction (SPAR) that uses a single primer containing only the core motif of a microsatellite, and Directed Amplification of Minisatellite region. DNA (DAMD) that uses a single primer containing only the core motif of a minisatellite. The main advantage of ISSRs is that no sequence data for primer construction are needed. Because the analytical procedures include PCR, only low quantities of template DNA are required (5–50 ng per reaction). Furthermore, ISSRs are randomly distributed throughout the genome. Because ISSR is a multilocus technique, disadvantages include the possible non-homology of similar sized fragments. Moreover, ISSRs like RAPD, can have reproducibility problems.

2.5.2.6 Amplified Fragment Length Polymorphism (AFLP)

AFLPs are DNA fragments (80–500 bp) obtained from digestion with restriction enzymes, followed by ligation of oligonucleotide adapters to the digestion products and

selective amplification by the PCR. AFLPs therefore involve both RFLP and PCR. The PCR primers consist of a core sequence (part of the adapter), and a restriction enzyme specific sequence and 1–5 selective nucleotides (the higher the number of selective nucleotides, the lower the number of bands obtained per profile). The AFLP banding profiles are the result of variations in the restriction sites or in the intervening region. The AFLP technique simultaneously generates fragments from many genomic sites (usually 50–100 fragments per reaction) that are separated by polyacrylamide gel electrophoresis and that are generally scored as dominant markers. Selective Fragment Length Amplification and Selective Restriction Fragment Amplification are synonyms sometimes used to refer to AFLPs. A variation of the AFLP technique is known as Selectively Amplified Microsatellite Polymorphic Locus. This technology amplifies microsatellite loci by using a single AFLP primer in combination with a primer complementary to compound microsatellite sequences, which do not require prior cloning and characterisation. The strengths of AFLPs lie in their high genomic abundance, considerable reproducibility, the generation of many informative bands per reaction, their wide range of Applications, and the fact that no sequence data for primer construction are required. AFLPs may not be totally randomly distributed around the genome as clustering in certain genomic regions, such as centromeres, has been reported for some crops (Alonso-Blanco *et al.*, 1998; Young *et al.*, 1999; Saal and Wricke, 2002). AFLPs can be analysed on automatic sequencers, but software problems concerning the scoring of AFLPs are encountered on some systems. Disadvantages include the need for purified, high molecular weight DNA, the dominance of alleles, and the possible non-homology of co-migrating fragments belonging to different loci. In addition, due to the high number

and different intensity of bands per primer combination, there is the need to adopt certain strict but subjectively determined criteria for acceptance of bands in the analysis. Special attention should be paid to the fact that AFLP bands are not always independent. For example, in case of an insertion between two restriction sites the amplified DNA fragment results in increased band size. This will be interpreted as the loss of a small band and at the same time as the gain of a larger band. This is important for the analysis of genetic relatedness, because it would enhance the weight of non-independent bands compared to the other bands.

In this study, inter-simple sequence repeat were used because of their abundance, and the high degree of polymorphism between individuals within a population of closely related genotypes (Hokanson *et al.*, 1998; Lanham and Brennan, 1998). Those properties indicate their potential role as good in study of genome analysis.

2.6 Methods for studying genetic structure

2.6.1 Population differentiation

When a population is divided into isolated sub-populations, there is less heterozygosity than there would be if the population was undivided. Founder effects acting on different demes generally lead to sub-populations with allele frequencies that are different from the larger population (Hartl and Clark, 1997). Also, these demes are generally smaller in size than the original larger population. Thus since allele frequency in each generation represents a sample of the previous generation's allele frequency, there will be greater sampling error in these small groups than there would be in a larger undifferentiated population. Estimation of population differentiation based on the amounts of genetic

variation within and between populations is called population structure (Okori, 2004). Population structure refers to the amount and distribution of genetic variation that exist in a population of a given organism. It is based on the variation in heterozygosity (Hartl and Clark, 1997). Genetic drift pushes smaller demes toward different allele frequencies and allele fixation more quickly than would take place in a larger undifferentiated population. Some of the most widely used tools to study population structure are provided below.

2.6.2 Wright's F statistic

The decline in heterozygosity due to sub-division within a population has usually been quantified using an index known as Wright's F statistic, also known as the fixation index (Hartl and Clark, 1997). The F statistic is a measure of the difference between the mean heterozygosity among the subdivisions in a population, and the potential frequency of heterozygotes if all members of the population mixed freely and non-assortatively (Hartl and Clark, 1997). The fixation index ranges from 0 (indicating no differentiation between the overall population and its subpopulations) to a theoretical maximum of 1, though in practice the observed fixation index is much less than 1, even in highly differentiated populations.

Fixation indexes can be determined for differentiated hierarchical levels of a population structure, to indicate, for example, the degree of differentiation within a population among groups of demes (F_{SG}), within groups among demes (F_{GT}), and within a population among demes (F_{ST}) (Hartl and Clark, 1997).

To determine the fixation index, the mean heterozygosity at each level must be determined. For a locus with two alternate alleles, allele frequency is symbolised as p and the alternative form of the allele is equal to $1 - p$.

2.6.2 Analysis of Molecular Variance (AMOVA)

Analysis of Molecular Variance (AMOVA) is a method of estimating population differentiation directly from molecular data and testing hypotheses about such differentiation. A variety of molecular data – molecular marker data (for example, RFLP or AFLP), direct sequence data, or phylogenetic trees based on such molecular data – may be analyzed using this method (Excoffier *et al.*, 1992).

AMOVA treats any kind of raw molecular data as a Boolean vector p_i , that is, a $1 \times n$ matrix of 1s and 0s, 1 indicating the presence of a marker and 0 its absence. A marker could be a nucleotide base, a base sequence, a restriction fragment, or a mutational event (Excoffier *et al.*, 1992). The sub-matrices are arranged in such a way that the diagonal of the larger matrix are pairs of individuals in the same population while those on the off-diagonal represent pairs of individuals from different populations. The sums of the diagonals in the matrix and sub-matrices yield sums of squares for the various hierarchical levels of the population. These sums of squares can then be analysed in a nested analysis of variance framework. A nested analysis of variance differs from a simple analysis of Variance (ANOVA) in that data is arranged hierarchically and mean squares are computed for groupings at all levels of the hierarchy. This allows for hypothesis tests of between-group and within-group differences at several hierarchical levels (Excoffier *et al.*, 1992; Excoffier, 2001). The variance components can be used to

calculate a series of statistics called phi-Statistics (Φ), which summarise the degree of differentiation between population divisions and are analogous to F -Statistics. Φ - Statistics are derived as shown in table 4 (Excoffier *et al.*, 1992; Excoffier, 2001).

Table 1. Amova frame work parameters of Φ -static at different levels of population hierarchy.

Level of population hierarchy	Φ -Statistic
Among demes within group	$\Phi_{SG} = \frac{\sigma_g^2}{\sigma_g^2 + \sigma_e^2}$
Among groups within population	$\Phi_{GT} = \frac{\sigma_g^2}{\sigma^2}$
Among demes within population	$\Phi_{ST} = \frac{\sigma_g^2 + \sigma_e^2}{\sigma^2}$

2.7 Sectional conclusions

Sorghum anthracnose caused by *Colletotrichum sublineolum* is one of the most devastating diseases affecting cultivated sorghum (Ali and Warren, 1987; Cardwell *et al.*, 1989; Pande *et al.*, 1991; Sherriff *et al.*, 1995; Thakur and Mathur, 2000). The disease is known to occur in epidemic proportions in many parts of the world, causing severe crop loss depending on the crop growth stage, degree of cultivar susceptibility and to a great extent, the conduciveness of the environment. The severity and significance of damages caused by this pathogen necessitates the development of strategies to control and manage the disease, so as to reduce crop loss and avert epidemics.

Host resistance has been used in the management of several diseases and thus breeding sorghum varieties with genetic resistance to sorghum anthracnose could be an effective and economical strategy for the control of the disease. However, *Colletotrichum sublineolum* is highly variable, rapidly breaks down resistance (Thakur, 1995). Thus breeding for durable resistance is one of the major challenges faced by sorghum breeders. One way to reduce rapid resistance breakdown is to slow down the rate of evolution of novel genotypes through the use of techniques that do not create very high selection pressure on the pathogen (McDonald and Linde, 2002). Since some resistance genes are effective only against particular pest or pathogen sub-populations, it is important to understand the structure of pest or pathogen populations in order to determine the best strategy for deployment of resistance and or incorporate the non-matching resistance genes to the existing pathogen. Consequently, information on pathogen population, diversity, and virulence are important for a successful sorghum-breeding program. Unfortunately, there is limited information regarding *Colletotrichum sublineolum*

populations in Uganda, yet the disease continues to impede production both locally and elsewhere. This study focuses on investigating genetic structure of *C. sublineolum* populations in Uganda to support plant breeding processes.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Introduction

3.2 Description of study area

Diseased sorghum leaves were collected from sorghum fields in sixteen districts of Uganda where sorghum and/or maize are commonly cultivated. These districts were selected to represent different agro-ecological zones, differentiated from each other by farming systems, edaphic factors; climatic factors, altitude and major vegetation cover (Wortmann and Eledu, 1999). Also it is important to note that farming systems tend to overlap between districts and agro-ecological zones.

The districts visited in the Northern moist Farmlands were Lira, Kaberamaido, Soroti, Dokolo, and Apac. (Wortmann and Eledu, 1999). This agro-ecological zone is characterised by mean annual temperatures of about 20⁰C and an annual precipitation of about 1200 mm received during one long rain season (Wortmann and Eledu, 1999; Ruecker *et al.*, 2003). The Northern moist Farmlands agro-ecological zone is on average 1073 meters above sea level. Two very similar farming systems exist in this agro-ecological zone; the annual cropping and cattle Northern system, practiced in Lira and the annual cropping and cattle Teso system, practiced in Soroti (Ruecker *et al.*, 2003). Both farming systems are characterised by unimodal rainfall seasonality, with annual precipitation potentials of 900 – 1200 mm of rainfall and temperatures ranging between 28 – 31⁰C (Ruecker *et al.*, 2003). Vegetation in both systems consists of moist *Combretum/Butyrospermum* and grass savannas; short grassland ideal for grazing. The major cereals include millet (*Eleusine coracana*), maize, and sorghum. Mixed agriculture

(crops and livestock) is practiced and the use of crop residues is very common in these systems. Soils are of poor to low productivity.

The district of Amuria is found in the North-Eastern Central Grass-Bush Farmland (Wortmann and Eledu, 1999). The North-Eastern Central Grass-Bush Farmland agro-ecological zone is characterised by the mean annual rainfall which varies from 1000mm – 1500mm and records a mean annual maximum temperature of 31.3°C and a mean minimum of 18°C. The agro-ecological zone lies approximately between 1,036meters – 1,127 meters above sea level. The soils are mainly of ferralitic type (sandy sediments and sandy loam). They are well drained and friable. Lowland contains widespread deposits of alluvium. The vegetation this district largely comprises of savannah grasslands dotted with shrubs and trees. It can generally be described as a wood land / shrub land – grassland vegetation dominated by *Acacia*, *Conbretum*, *Piliostigma*, *Butyrospermum paradoxum* and *Hyperenia* species. Geological surveys have revealed that most areas are underlain by rocks of basement complex of precambrian age, which include: granites, mignallites, gneiss, schists and quartzites.

The districts of Kumi and Pallisa are found in the Southern and Eastern Lake Kyoga Basin (Wortmann and Eledu, 1999). This agro-ecological zone receives an annual precipitation of 1200 mm, with mean temperatures of 28 – 31°C and is 1143 meters above sea level. The farming system prevalent in Kumi district is the annual cropping and cattle Teso system whereas, the farming system practiced in Pallisa is the Banana-millet-cotton system. Vegetation in the banana-millet-cotton system is moist *Combetrum*/ *Terminalia*/ *Butyrospermum* savannah with moderate biomass production. The major

cereals include millet, sorghum and maize. In the drier areas, livestock keeping is the main activity.

Iganga, Mbale, Masaka, and Tororo districts represented the Lake Victoria Crescent and Mbale Farmlands (Wortmann and Eledu, 1999). This agro-ecological zone is about 1106 meters above sea level with mean annual precipitation between 1200 – 1400 mm of rainfall received during two rain seasons (Ruecker *et al.*, 2003). The banana-millet-cotton system is the farming system prevalent in Tororo. Conversely, the farming system practiced in Iganga district is the intensive Banana-Coffee lake shore system. The Lake Victoria Crescent and Mbale Farmlands is characterised by soils of medium to high productivity. Vegetation is mainly forest-savanna mosaic with pastures suitable for intensive livestock production. Maize and bananas are the major crops in this agro-ecological zone; sorghum, millet, and sweet potatoes are secondary food security crops. Livestock are generally not integrated into the system but dairy cattle are gaining in prominence.

For the districts, which were visited in the Bushenyi – North Rukungiri Farmland, and Kabale-Rukungiri highlands agro-ecological zone consisted of Bushenyi, Kabale, and Rukungiri (Wortmann and Eledu, 1999). These agro-ecological zone receives annual rainfall exceeds 1875 mm, with mean temperatures of $< 20^{\circ}\text{C}$ and is 2123 meters above sea level. The relief is hilly, with steep slopes in some areas and the dominant soils are highly weathered ferrallitic and sandy loams with some sandy clay loams but nutrient supply is generally good and productivity is medium to high. In agro-ecological zone there is greater reliance on annual food crops (millet, sorghum and maize) since rainfall is

less stable than under the Banana-Coffee System. In the drier areas, livestock is a main activity. The farming system prevalent in these districts is Western Banana- Coffee-Cattle System. The major cereals include millet, sorghum and maize. In the drier areas, livestock is the main activity.

The districts of Nebbi and Arua are found in the West Nile farm lands (Wortmann and Eledu, 1999). This agro-ecological zone receives rainfall averages about 1250 mm annually and occurs in 140 to 170 days of the year, with mean temperatures of 28 – 31⁰C and is 1143 meters above sea level. The farming system prevalent in these districts is West Nile Cereal- Cassava- Tobacco system and the rainfall is adequate for most crops but the intensity of the dry season requires that drought tolerant annuals are cultivated (finger millet, sesame, cassava and sorghum). Tobacco and cotton are major cash crops. There is short grassland where communal grazing abounds. Intercropping is common with a wide variety of crops. The system is in the sub-humid zone and livestock activities are limited by the presence of tsetse fly (*Glossina sp*).

Hoima and Masindi district are found in the Western mid-altitude farmlands and Semiliki flats. This agro-ecological zone receives about 875-1000 mm of rainfall falling in 80 to 100 days, with mean temperatures of 28 – 31⁰C and is 1143 meters above sea level. Temperatures are high and vegetation varies from medium altitude moist forests, forest-savanna mosaic, savanna and swamps to post-cultivation communities. The forest-savanna mosaic is the most widespread and consists of a mixture of forest remnants, incoming savanna trees and a grass layer dominated by *Pennisetum purpureum*.

As per the above discription of the climatic conditions how that all the different agro-ecologies of Uganda comprises of different rain pattern and the vegetation cover as were as the height above the sea level hence influencing the environmental conditions with the different districts.



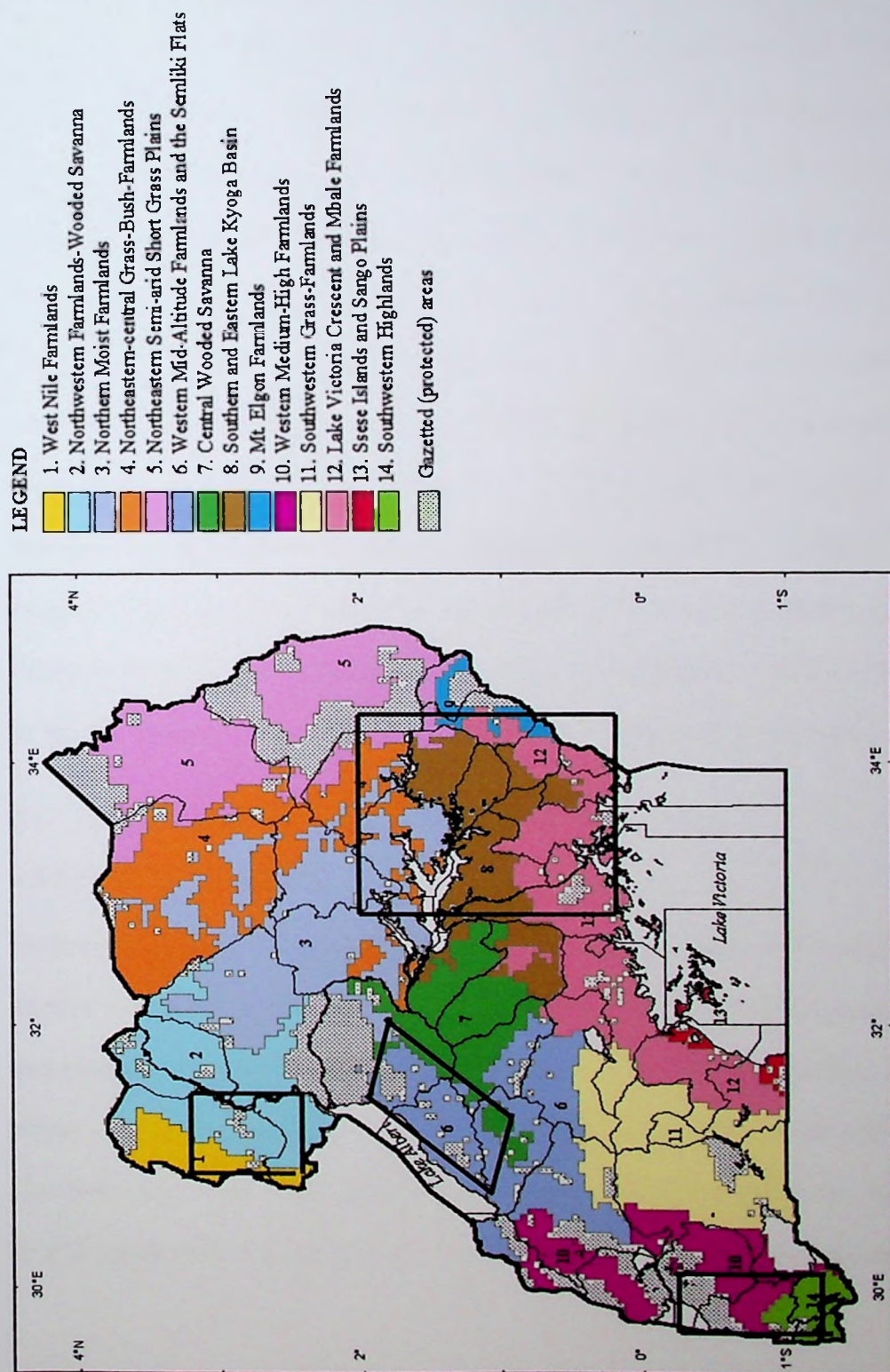


Figure 2. Map of Uganda showing an aggregation of the agro-ecological zones. Study areas are marked and enclosed in boxes.

3.3 Sampling strategy

Hierarchical sampling was used to collect anthracnose diseased sorghum leaf samples from nineteen districts within eight agro-ecologies. This was done to permit the capture of diversity at leaf, plant, fields within location and fields between locations. In the sampling structure, at least two districts were selected per agro-ecology and within each district; 15-20 farms were visited. Leaf samples were picked at 20 m intervals across each field, 80-100 diseased leaf sample were collected per district depending on field sizes and disease incidence. Global Positioning Systems (GPS) readings were taken at each sampling point using a GPS receiver model 315 (Magellan Navigation, Inc. Tulsa, Oklahoma, U.S.A) and the coordinates used to generate maps using the Geographic Information Systems (GIS) software Arc View 3.2a and spatial analyst 1.1 (Environmental Systems Research Institute, Inc. Seattle, WA, U.S.A). All diseased samples were taken from the most susceptible red seeded local variety, since it was the most common variety grown and exhibited the highest incidence and severity. In addition to that the farmer's lack of knowledge of the other varieties they cultivated.

3.4 Development of sorghum anthracnose disease maps

3.4.1 Disease assessment

Anthrachnose disease incidence data in each field was assessed by counting the total number of plants along a transect and expressing the proportion of diseased plants as percentage of the total. Severity of anthracnose in the field was rated on a scale of 1-5 where 1= low severity and 5 = high severity (Ward *et al.*, 1997; Ward *et al.*, 1999). This data was used to generate a disease maps using the GIS software Arc View 3.2a with spatial analyst by interpolating the surface from GPS points and the associated field

severity data using the inverse distance weighted interpolation method. Prior to interpolation, a power parameter of 5 was set to account for influence of surrounding points at each location (Environmental Systems Research Institute, Inc. Seattle, WA, and U.S.A).

3.5 Genetic variability of *Colletotrichum sublineolum* populations in Uganda

3.5.1 Fungal culture

Leaf samples were air dried for 7 days and stored at room temperature. Lesions which showed clear anthracnose symptoms were excised out from each leaf sample, surface sterilized using 0.5% sodium hypochlorite solution, rinsed twice in sterile distilled water, placed on moist filter paper in a Petri dish and incubated for 48 to 72 hours to stimulate sporulation. Conidia were dislodged from the lesions with the aid of stereo-microscope and then plated on Potato Dextrose Agar plates and cultured under natural light and darkness regimes for 48 to 72 hours at room temperature. Monoconidial cultures of the isolates were then established by sub-culturing germinated spore bearing distinct colony characteristics of *C. sublineolum* to fresh Potato Dextrose Agar plates (Difco Laboratories) mixed with 100 mg/ml of ampicilin. To obtain mycelium for DNA extraction, 200mls of potato dextrose broth (Difco Laboratories, Becton Dickinson, Microbiology Systems, Sparks, MD, USA) was inoculated with culture plugs of *C. sublineolum* and cultured at room temperature (25 ° C) for 8 days on a rotary shaker (Laboshake - laboratory shakers LS/RO 500 Gerhardt, Germany) mycelium was harvested by vacuum filtration after 8 days to prevent generation of high molecular weight carbohydrates that affects DNA isolation (Okori *et al.*, 2003). Mycelial fragment were stored at -80 ° C for DNA isolation.

3.5.2 Isolation and extraction of DNA

Genomic DNA extraction was performed by a modified extraction protocol (Okori *et al.*, 2003) using the hexadecyltrimethylammonium bromide (CTAB). Briefly, 500 mg of mycelial of each isolate were grain in a mortar using a pestle into which liquid nitrogen was powered, and transferred to 50 ml microcentrifuge tubes containing 5 ml of extraction buffer (0.1 M Tris-HCl pH 8.0; 0.2 M NaCl; 0.02 M EDTA; 1,0% SDS; 0.1% b-mercaptoethanol). Each tube was vigorously agitated to obtain a uniform suspension without lumps and then incubated for 15 min at room temperature. Afterwards, an equal volume of chloroform: isoamyl alcohol (24:1 v/v) was added to each sample, vigorously agitated, and incubated 10 min at room temperature. Cell debris was removed by centrifugation at 4 °C with 8000 rpm for 10 minutes. Aliquots of 5 ml of the supernatant layer were transferred to a 50 ml propylene tube and an equal volume of ice cold ethanol was added to each sample and gently inverted several times to precipitate nucleic acids. Nucleic acids were spooled out on a glass hook, briefly washed in 70% ethanol and dissolved with 0.5 ml of TE buffer. Genomic DNA was quantified for each sample by spectrometry and dilutions made to a final concentration of 20 ng.

Table 2. Origin and designations of the isolates used in the study

Isolate	Origin	Isolate	Origin	Isolate	Origin
Amu5	Amuria	Kabe25	Kaberaimaido	Ne18	Nebbi
Amu1	Amuria	Kabe17	Kaberaimaido	Ne5	Nebbi
Amu16	Amuria	Kabe3	Kaberaimaido	Ne7	Nebbi
Amu4	Amuria	Kabe21	Kaberaimaido	St17	Soroti
Amu15	Amuria	Kabe2	Kaberaimaido	St11	Soroti
Amu3	Amuria	Kabe14	Kaberaimaido	St18	Soroti
Amu2	Amuria	kabe20	Kaberaimaido	Tr10	Tororo
Ap2	Apac	Kabe4	Kaberaimaido	Tr35	Tororo
Ap3	Apac	Kabe22	Kaberaimaido	Tr13	Tororo
Ap5	Apac	Kabe7	Kaberaimaido	Tr35	Tororo
Ap8	Apac	kabe19	Kaberaimaido	Tr25	Tororo
Ap12	Apac	kabe29	Kaberaimaido	Tr17	Tororo
Ap18	Apac	Kabe1	Kaberaimaido	Tr12	Tororo
Ap4	Apac	Kabe8	Kaberaimaido	Tr27	Tororo
Ar1	Arua	Lr9	Lira	Tr13	Tororo
Ar28	Arua	Lr13	Lira	Tr16	Tororo
Ar15	Arua	Lr1	Lira	Tr3	Tororo
Ar12	Arua	Lr15	Lira	Tr20	Tororo
Ar16	Arua	Lr12	Lira	Tr2	Tororo
Bs1	Bushenyi	Lr3	Lira		
Bs3	Bushenyi	Lr11	Lira		
Bs4	Bushenyi	Lr8	Lira		
Bs9	Bushenyi	Lr10	Lira		
Bs19	Bushenyi	Lr18	Lira		
Bs6	Bushenyi	Lr3	Lira		
Bs14	Bushenyi	Ms18b	Masaka		
Bs2	Bushenyi	Ms19	Masaka		
Dok13	Dokolo	Ms14	Masaka		
Dok4	Dokolo	Ms17	Masaka		
Dok5	Dokolo	Ms15	Masaka		
Dok6	Dokolo	Ms11	Masaka		
Dok9	Dokolo	Ms2	Masaka		
Dok8	Dokolo	Ms22	Masaka		
Dok1	Dokolo	Ms8	Masaka		
Dok13	Dokolo	Ms17	Masaka		
Dok3	Dokolo	Ms5	Masaka		
Ho13a	Hoima	Ms18a	Masaka		
Ho29	Hoima	Ne9	Nebbi		
Ho13b	Hoima	Ne8	Nebbi		
Ho26	Hoima	Ne25	Nebbi		
Ho27	Hoima	Ne1	Nebbi		
Ho14	Hoima	Ne3	Nebbi		
Ho12	Hoima	Ne13	Nebbi		
Ho4	Hoima	Ne11	Nebbi		
Ho6	Hoima	Ne17	Nebbi		
Ho7	Hoima	Ne14	Nebbi		
Ho8	Hoima	Ne22	Nebbi		
Ho19	Hoima	Ne9b	Nebbi		
Kabe18	Kaberaimaido	Ne16	Nebbi		
Kabe21	Kaberaimaido	Ne23	Nebbi		
Kabe2	Kaberaimaido	Ne15	Nebbi		
Kabe7	Kaberaimaido	Ne19	Nebbi		

3.5.3 Inter Simple Sequence Repeats (ISSR) analysis

Polymerase Chain Reaction amplification reactions were performed in a total volume of 15 μ L. Each reaction mixture contained about 20 ng of template DNA, 2.5 mM $MgCl_2$, 0.5 μ M each primer, 0.2 mM each dNTP, and 1 U promega GoTaq DNA polymerase (Cape town, South Africa) in a reaction buffer containing 10 mM Tris-HCL pH 8.8, 50 mM KCl and 0.1% Triton X – 100.

Polymerase chain reaction amplification (PCR) was performed in a thermocycler (Geneamp systems, 9700, Applied Biosystems Foster City, CA 94404 USA) set to the following programmes. For primer BDB and DDC, 94⁰C for 2 min, 35 cycles of 94⁰C for 30 sec, 50⁰C for 30 sec, 72⁰C for 1 min, and a final extension of 72⁰C for 10 min. For primer DHB, 94⁰C for 2 min, 35 cycles of 94⁰C for 1 min, 48⁰C for 2 min, 72⁰C for 2 min, and a final extension of 72⁰C for 10 min. Also control tubes containing master mix without template DNA were included in each run and the reactions were repeated at least three times to check the reproducibility of the process. Amplification products were mixed with loading solution (dye) in the ratio of 1:5 and verified on 1% agarose gels stained with 10 mg/mL ethidium bromide in 1X Tris-borate EDTA (TBE) buffer. Electrophoresis was performed at 100 V for 2 hours and the resulting DNA fragments visualised by UV light (BIORAD Hercules, CA. U.S.A).

Table 3. Description of species specific primers used for PCR in this study.

Primer	Sequence (5' – 3')	T _m (⁰ C)
DHB	CGACGACGACGACGA	50
BDB	ACAACAACAACAACA	40
DDC	CCACCACCACCACCA	50

T_m, = Melting temperature calculated from 4(G+C) + 2(A+T). All primers were supplied by Invitrogen Life Technologies.

3.5.4 Data analyses

Statistical analyses were performed using Genstat 5 version 3.2, 1995 (Lawes Agricultural Trust: Rothamsted Experimental Station, UK). Field incidence and severity data were subjected to one-way analysis of variance (ANOVA) (Steel *et al.*, 1997). Field severity was also subjected to nested analysis of variance using MINITAB release 15 version 15.0.0.1, 2007 (Minitab Inc, Pennsylvania, USA).

3.5.5 Phylogenetic and population structure analysis

For ISSR analysis, each fragment was considered as a single locus with two alleles, present and absent. Data generated from each primer combination were pooled to generate one multilocus data set comprising 37 fragments that subsequently was used in genetic analyses. Data generated from ISSR analysis was subjected to phylogenetic analysis by employing the Neighbour-joining method in TREECON version 1.3b (Van de Peer and De Wachter, 1994). Robustness of the dendrogram was tested by bootstrap analysis of 1000 replicates.

For population genetic analyses, the 123 fungal isolates were structured into eight populations divided by their agro-ecological zone of origin. Genetic variation in fungal collections was also measured by Nei's gene diversity, which measures the probability of obtaining two different alleles at a locus when two haplotypes are sampled from a population (Nei, 1973), and Nei's measure of genetic distance (Nei, 1987). Statistical comparisons of gene diversity estimates were performed using t-test (Nei 1987). A null hypothesis indicating presence of population structure between populations was analyzed using Wright's population differentiation index F_{st} . The fixation index as described by Wright equals to the reduction in heterozygosity expected with random mating at any one

level of population hierarchy relative to another more inclusive level of hierarchy (Wright, 1969). In this study, F_{st} was estimated using the analysis of molecular variance (AMOVA) framework (Excoffier, Smouse and Quattro, 1992) and implemented in Arlequin (Schneider *et al.*, 2000). AMOVA estimates fixation indices in form of ΦF_{st} , which attempts to correct for the effects of sampling a limited number of organisms from a limited number of populations. Theoretically, the fixation index has a maximum of 1 and a minimum of 0. Further tests for population differentiation were performed using exact tests at the 5% significance level as implemented in Arlequin (Raymond and Rousset, 1995). Significance of ΦF_{st} as estimated in AMOVA was tested using non-parametric permutation procedures in Arlequin (Schneider *et al.*, 2000). Gene diversity i.e. the probability that two randomly chosen haplotypes are different in a sample was also calculated. ($\hat{H}$) and its sampling variance $V(\hat{H})$ were computed as:

$$\hat{H} = \frac{n}{n-1} \left(1 - \sum_{i=1}^k p_i^2 \right)$$

$$V(\hat{H}) = \frac{2}{n(n-1)} \left\{ 2(n-2) \left[\sum_{i=1}^k p_i^3 - \left(\sum_{i=1}^k p_i^2 \right)^2 \right] + \sum_{i=1}^k p_i^2 - \left(\sum_{i=1}^k p_i^2 \right)^2 \right\}$$

Where n is the number of gene copies in the sample, k is the number of haplotypes and P_i is the sample frequency of the i -th haplotype (Nei, 1987). The $\hat{H}$ values were compared by t-test (Nei, 1987).

CHAPTER FOUR

RESULTS

4.1 Incidence and severity of anthracnose in Ugandan agro-ecologies

Analysis of variance (ANOVA) results showed that there was variation in incidence and severity of sorghum anthracnose disease. Both incidence and severity varied significantly ($P \leq 0.05$) between agro-ecologies, reflecting the effect of different cropping patterns on sorghum anthracnose epiphytotics (Table 4). Districts within agro-ecologies showed a significant variation ($P \leq 0.05$) in both incidence and severity of sorghum anthracnose. This might be attributed to the different crop management practices carried out by the farmers within the different agro-ecologies. Similar observations were made for fields within the districts with significant differences at $P \leq 0.05$ occurring for both incidence and severity (Table 4).

Table 4: Mean squares of sorghum anthracnose incidence and severity at the different hierarchical levels of the study.

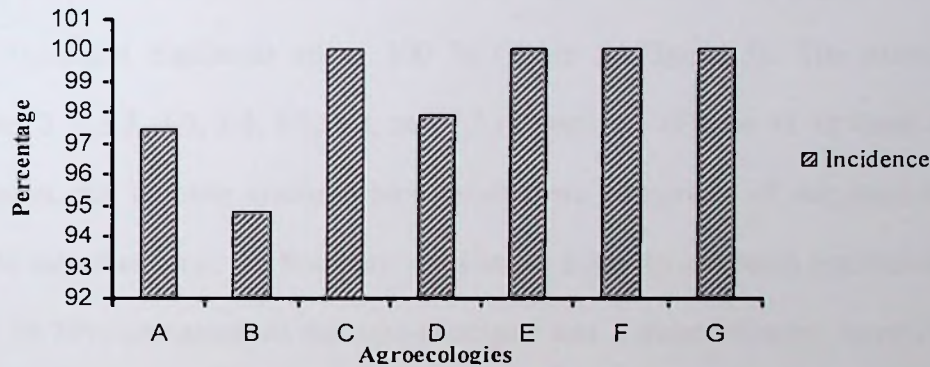
Source of variation	Mean square		
	d.f	incidence	severity
Agro ecological zone	6	248.24*	7.1874*
Districts	19	173.31*	6.9178*
Fields	43	181.18*	4.1330*

* indicate mean squares significant at the $P \leq 0.05$ level

Table 5. Sorghum anthracnose mean incidence and severity in the different agro-ecologies of Uganda.

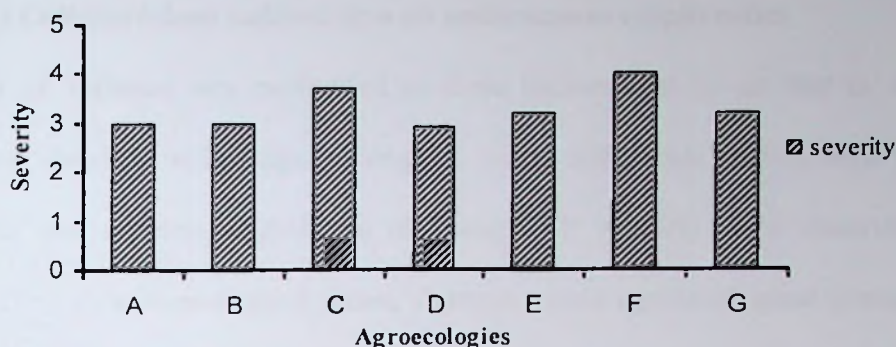
Study site	Incidence (%)	Severity
Agro-ecological zone		
Lake Victoria crescent and Mbale farmland	97.5	3.0
Southern and Eastern Lake Kyoga basin	94.8	3.0
Northern moist Farmland	100.0	3.7
West Nile farm land	97.9	2.9
Western Mid-Altitude Farmlands and the Semiliki Flats	100.0	3.2
Bushenyi - Northern Rukungiri Farmland (South Western Highland)	100.0	4.0
Kabale- Rukungiri highlands	100.0	3.2
Mean	98.5	3.2
SED	2.3	0.3
CV %	6.7	29.3

%CV= Coefficient of variation, SED= Standard Error of Difference of mean



A= Lake Victoria crescent and Mbale farmland, B= Southern and Eastern Lake Kyoga basin, C= Northern moist Farmland, D= West Nile farm land, E= Western Mid-Altitude Farmlands and the Semiliki Flats, F= Bushenyi – Northern Rukungiri Farmland, G= Kabale- Rukungiri highlands

Figure 3 Sorghum anthracnose mean incidence in different agro-ecologies of Uganda.



A= Lake Victoria crescent and Mbale farmland, B= Southern and Eastern Lake Kyoga basin, C= Northern moist Farmland, D= West Nile farm land, E= Western Mid-Altitude Farmlands and the Semiliki Flats, F= Bushenyi – Northern Rukungiri Farmland, G= Kabale- Rukungiri highlands

Figure 4. Sorghum anthracnose mean severity in different agro-ecologies of Uganda.

The highest sorghum incidences were registered in Northern moist farmland, Western mid-altitude Farmlands and the Semiliki flats, Bushenyi- Northern Rukungiri Farmland and Kabale-Rukungiri highlands all at 100 % (Table 5, Figure 3). The associated severities were 3.7, 3.2, 4.0, 3.4, 3.1, 3.4, and 3.2 respectively (Figure 4). In these agro-ecological zones, the farming system which exists there comprised of sorghum-maize intercrops. On the other hand, the Southern and Eastern Lake Kyoga basin registered low incidence of 94.76% compared all the agro-ecologies and a mean severity score of 2.9 (Figure 3 , 4). In this agro-ecology, the cropping pattern comprised blocks of sorghum and maize fields grown side by side and rarely intercropped.

Analysis of disease levels at district level revealed that Tororo, Bushenyi, Rukungiri, Kabale, Masaka, Dokolo, Amuria, Kaberamaido, Lira, Hoima, Apac, Soroti and Masindi had the highest incidence of 100% and mean severity of 3, while Mbale registered the lowest incidence of 89% and severity of 2.2 (Table 6, Figure 5, 6).

4.2 Effect of *Colletotrichum sublineolum* on anthracnose epiphytotics

Nested analysis of variance was performed at three hierarchical levels that is, agro-ecological zones, districts within agro-ecological zones and fields within both agro-ecological zones and districts. Significant differences ($P \leq 0.05$) were observed in anthracnose severity in agro-ecological zones, districts within agro-ecological zones and fields within districts (Table 8). The analysis confirmed what was observed on incidence and severity of disease in all the sorghum growing districts. Infact, all the three levels of hierarchy tested – agro-ecology, district and field had significantly different levels of epiphytotics (Tables 6, 7).

Table 6. Sorghum anthracnose mean incidence and severity at the different district where the study was conducted.

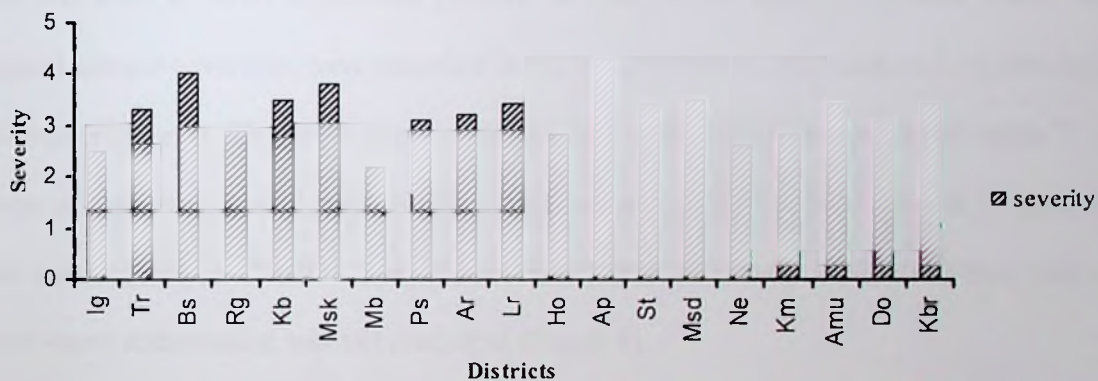
Study site	incidence (%)	severity
<u>District</u>		
Iganga	95.5	2.5
Tororo	100.0	3.3
Bushenyi	100.0	4.0
Rukungiri	100.0	2.9
Kabale	100.0	3.5
Masaka	100.0	3.8
Mbale	89.0	2.2
Pallisa	95.2	3.1
Arua	99.0	3.2
Lira	100.0	3.4
Hoima	100.0	2.8
Apac	100.0	4.3
Soroti	100.0	3.4
Masindi	100.0	3.5
Nebbi	96.7	2.6
Kumi	94.4	2.8
Amuria	100.0	3.4
Dokolo	100.0	3.1
Kaberamaido	100.0	3.4
Mean	98.5	3.1
SED	2.5	0.3
CV %	6.5	27.2

%CV= Coefficient of variation, SED= Standard Error of Difference of mean



Ig=Iganga, Tr= Tororo, Bs=Busshenyi, Rg=Rukungiri, Kb=Kabale, Msk=Masaka, Pa=Pallisa, Mb= Mbale Ar= Arua, Lr=Lira, Ho= Hoima, Ap=Apac, St= Soroti, Msd=Masindi, Ne=Nebbi, Km=Kumi, Amu= Amuria, Do= Dokoro, Kbr=Kaberamaido.

Figure 5. Sorghum anthracnose mean incidence in different district levels of Uganda



Ig=Iganga, Tr= Tororo, Bs=Busshenyi, Rg=Rukungiri, Kb=Kabale, Msk=Masaka, Pa=Pallisa, Mb= Mbale Ar= Arua, Lr=Lira, Ho= Hoima, Ap=Apac, St= Soroti, Msd=Masindi, Ne=Nebbi, Km=Kumi, Amu= Amuria, Do= Dokoro, Kbr=Kaberamaido.

Figure 6. Sorghum anthracnose mean severity in different agro-ecologies of Uganda.

Table 7. Nested analysis of variance for severity, base on 3 levels of hierarchy used in study.

Source	df	Sequential Sum of Squares	Adjusted Mean Squares	F-Value
Agro-ecology	6	57.85	6.45	9.93*
District (Agro-ecology)	10	56.56	5.73	8.81*
Fields(Agro-ecology District)	24	50.44	2.10	3.23*
Error	419	272.30	0.65	
Total	146	437.14		

District (Agro-ecology) = District nested within agro-ecological zone.

Field (Agroecology District) = District nested within both agro-ecological zone and District.

** = significant at ($P \leq 0.05$).

4.3 Spatial distribution of sorghum anthracnose in Uganda using disease map.

The disease severity/incidence data and GPS recordings were used to construct a disease map. The disease map illustrates severity and incidence levels over the agro-ecologies and was used to study epidemics patterns at time of the study. The data shows that highest disease severities were recorded in the wet humid regions such as Lira and Apac districts (Figure 7). The cooler agro-ecologies had relatively lower severity (Figure 7). In terms of incidence virtually all districts recorded very high levels of disease (Figure 8). The vast majority of districts studied recorded 100% incidences in fields. There was no field where anthracnose was not recorded (Figure 8).

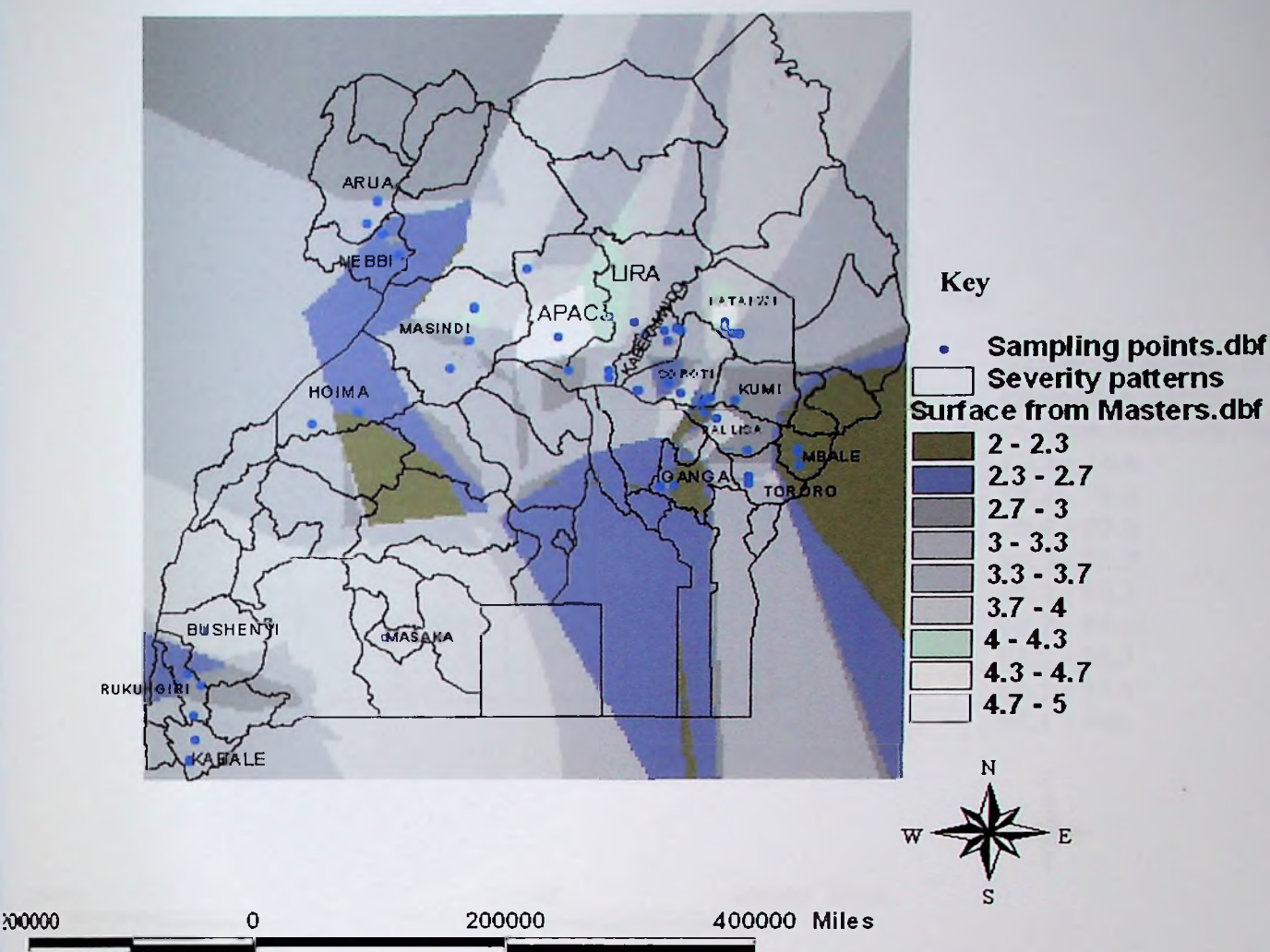


Figure 7. Disease map showing sorghum anthracnose severity patterns in various agro-ecologies of Uganda.

Note: The variations in colour/shades over the map are used to typify disease severity patterns in the study areas. Districts that are labeled are the ones where the study was conducted.

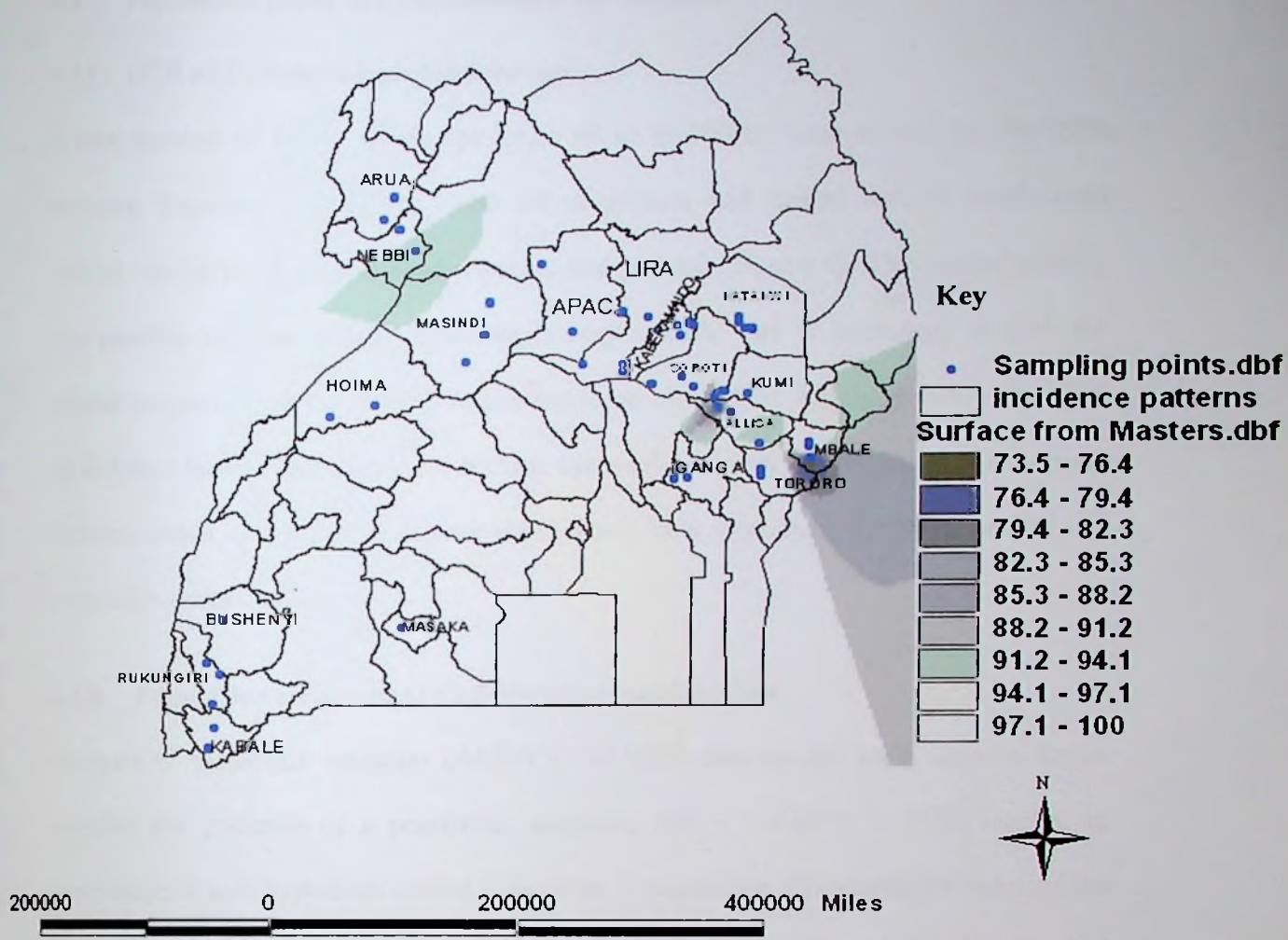


Figure 8. Disease map showing sorghum anthracnose incidence patterns

Note: The variations in colour/shades over the map are used to typify disease severity patterns in the study areas. Districts that are labeled are the ones where the study was conducted.

4.4 Population study of *Colletotrichum sublineolum*

4.4.1 PCR of *Colletotrichum sublineolum*

A total number of bands within the range of 50 to 300 bp were scored for the ISSR analyses. Repetitions using the same set of primers and fungal isolates consistently yielded similar polymorphic band numbers and patterns (Figure 9). This reproducibility was possible because primer annealing is very specific due to homology to both the adapter sequence and the restriction site sequence. Amplified fragments lower than 200 bp and faint bands were neglected, because the resolution was insufficient to discriminate between bands of various molecular sizes. These data were used for phylogenetic and population genetic analyses.

4.4.2 Population structure of *Colletotrichum sublineolum*

Analysis of molecular variation (AMOVA) of ISSR data on the basis agro-ecologies revealed the presence of a population structure, ($\Phi F_{ST} = 0.08$ $P = 0.05$) leading to acceptance of null hypothesis stating a presence of population differentiation between the agro-ecologies in Uganda (Table 8). The results of the AMOVA analysis also revealed the allelic variation (92.5%) was shared between populations.

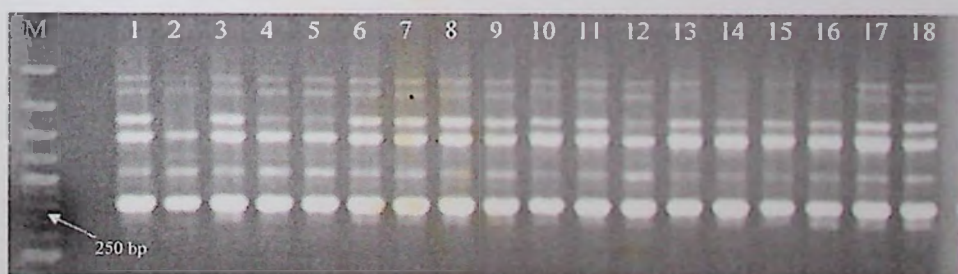


Figure 9. Polymorphic band patterns of different isolates for different agro-ecologies of Uganda i.e. M = 1 kb ladder (Fermentas Life Sciences).

Table 8. Analysis of molecular variation *Colletotrichum sublineolum* from seven Ugandan agro-ecological zones.

Source of Variation	d.f.	Sum of Squares	Variance components	Percentage of variation
Among groups	6	65.03	0.23	4.06
Among populations within groups	5	33.69	0.19	3.44
Within populations	104	539.62	5.19	92.49
Total	115	638.35	5.61	

$\Phi F_{ST} = 0.08$ ($P = 0.001$) based on 1000 permutations

Average gene diversity over all loci ranged from 0.192 to 0.335 showing high diversity within population rather than between populations (Table 9). The number of polymorphic loci were similar for the population studied (a locus is considered polymorphic if the most common allele has a frequency of less than 95% in all of the populations analysed) (Table 9).

Table 9. Gene diversity and number of polymorphic loci among Ugandan *Colletotrichum sublineolum* population based on Inter Simple Sequence Repeat polymorphism.

District of origin	Number of polymorphic loci ^b	Average gene diversity ($\bar{H}$) ^a
Arua	20	0.254
Masaka	29	0.264
Hoima	33	0.312
Nebbi	32	0.335
Tororo	30	0.274
Bushenyi	21	0.224
Apac	27	0.192
Soroti	18	0.324
Lira	26	0.235
Dokolo	23	0.228
Amuria	23	0.260
Kaberamaido	32	0.283

^a average gene diversity computed according to Nei (Nei, 1987) and Tajima (Tajima, 1993)

^b The number of polymorphic loci in comparison to the total number of 37 ISSR loci studied

4.4.3 Phylogenetic analysis

No significant clusters were found as demonstrated by bootstrap values less than 95% (Figure 10). Nevertheless the data shows some trend in the clustering of isolates. In general isolates, from a similar region for example Northern and North-Eastern Uganda which comprised the Northern moist farm lands tended to cluster together (Figure 10). For example isolates marked Lira, Dokolo, Kaberamaido all clustered together. A similar trend was observed for the isolates from other agro-ecologies separated by wide geographical distances clustered together for example the isolate Tr2 from Tororo clustered largely with isolates from Arua located more than 600 Km. This pattern suggests presence of local populations as supported by the AMOVA analysis in section 4:3:1.

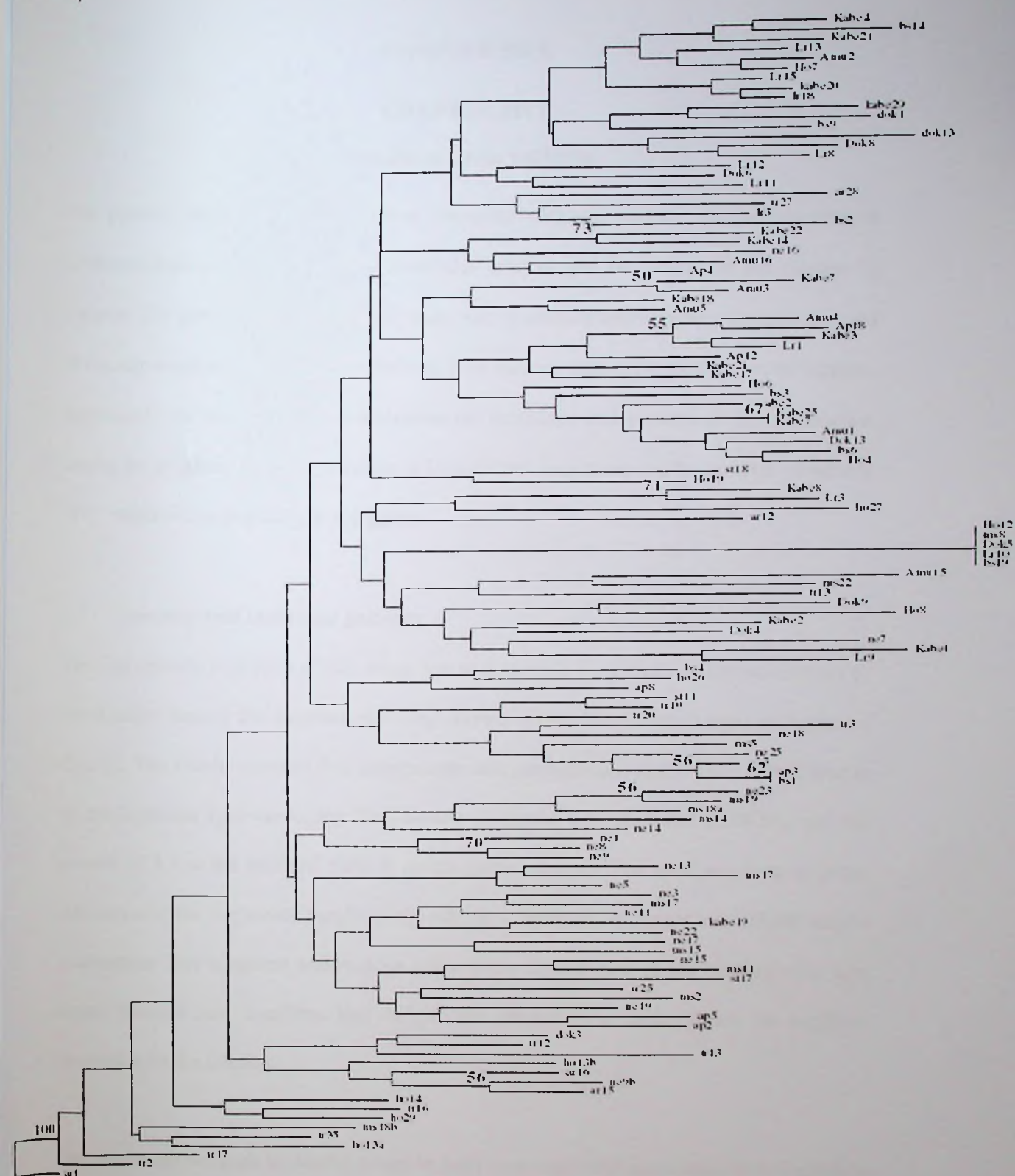


Figure 10. Neighbour-Joining dendrogram of 124 *C. sublineolium* isolates derived from Inter-Simple sequence Repeats (ISSR). Branch length is proportional to the genetic distance which is indicated by the bar. Only bootstrap values above 50 are indicated.

CHAPTER FIVE

CHAPTER FIVE

GENERAL DISCUSSION

The present study for the first time provides data on genetic characterisation of *Colletotrichum sublineolum* using molecular markers and the status of the disease in Uganda. The general objective of this study was to characterise sorghum anthracnose and its causative agent *C. sublineolum* derived from various agro-ecological zones of Uganda. Specifically the study sought to determine the incidence and severity of *C. sublineolum* among the sorghum growing districts of Uganda and also to assess the genetic variability of *C. sublineolum* population in Uganda.

5.1 Severity and incidence patterns

The first specific objective of this study was to determine the incidence and severity of *C. sublineolum* among the sorghum growing districts within the different agro-ecologies of Uganda. The results showed that anthracnose was predominant and widely distributed in all the Ugandan agro-ecologies. The disease incidence was observed at 98.5% and the severity of 3.3 in the farmers' field in all the agro-ecologies. The local sorghum landrace cultivars and the improved sorghum all exhibited anthracnose symptoms. These results demonstrate that sorghum anthracnose has a wider distribution in the country than was earlier thought and confirms that indeed the disease is a major threat to sorghum production in the country.

One reason for the high incidence could be high inoculum level associated with Uganda's farming systems (Okori, 2004). Farmers generally, do not practice closed seasons (do not plant the crop in one season of the year) and grow the crop year to year. This leads to

quick inoculum build-up. From the literature, *C. sublineolum* can survive from season to season as mycelia, sclerotia or chlamydospores on infected crop debris or in the soil (Casela and Frederiksen, 1993). Moreover alternative hosts and volunteer crops may also provide sources of primary inoculum. Seed transmission has been reported for *C. sublineolum* (Cardwell *et al.*, 1989).

Anthrachnose is a polycyclic disease, whose epidemics are favoured by high rainfall and humidity, moderate temperatures, and the presence of large amounts of inoculum (Frederiksen, 1986). However, severe epidemics can occur, even in sub-optimal conditions, where highly pathogenic strains infect susceptible host cultivars (Pande *et al.*, 1991). Previous studies on epidemiology of anthracnose have indicated that the disease is mostly severe with mature plants (Ashok-Mishara *et al.*, 1992). These facts on *C. sublineolum* suggest that the high incidence detected throughout this study could be compounded by effects of inoculum build-up and susceptible cultivars. This would be expected of a pathosystem involving an aggressive pathogen and a susceptible host (Agrios, 1997; Okori 2004).

Anthrachnose was generally observed to occur in all areas studied. Earlier reviews on sorghum anthracnose suggest that the disease occurs in all sorghum growing areas of the world with completely different climatic conditions (Ali and Warren, 1992). In the Uganda anthracnose-sorghum pathosystem, the interactive role of the environment, plant mineral nutrition and variety effects on epidemics under different conditions have been reported (Frederiksen *et al.*, 1995). The pathogen is reported to cause severe damage during the rainy season in a wide climatic range from the hot (35 °C) and dry savannas Niger and Mali to the cool (12 °C) and wet (2200 mm average rainfall) highlands of

Rwanda (Frederiksen *et al.*, 1995). Casela and Frederiksen (1993) observed that *Colletotrichum* produce small sclerotia embedded in stalks of mature infected sorghum plants. These sclerotia appear to be an important means by which the organism survives in many sorghum growing regions. Such a phenomenon may account for high disease incidence and severity observed during this study.

In this study, agro-ecologies experiencing warm and humid conditions had high disease incidence and severity perhaps due to favourable weather conditions in general. However, there was a generally high incidence and severity perhaps due to susceptible varieties cultivated by the farming communities. On average, disease severity was 3, suggesting susceptibility of sorghum varieties to *C. sublineolum*. The vast majority of farmers/farms visited during the study, cultivated sorghum for subsistence purposes. There was limited evidence of adoption of elite resistant varieties in the majority of farms visited. Even districts close to the sorghum research programme of NASARI registered high incidences. For example Apac, Soroti, Amuria, all had high severity close to 4 indicative of limited adoption. The low severities in Western Nile farmlands and Western mid altitude compared to the rest of the country could be attributed unfavorable climatic condition of low humidity due to the high temperature ranging from 28 – 31 ° C (Wortmann and Eledu, 1999).

Nested ANOVA revealed significant differences in anthracnose severity at the agro-ecological zone level, district within the agro-ecology and fields within districts. These findings allude to the relative importance of variations within field condition that might impact on anthracnose epiphytotics relative to the broader differences between agro-ecological zones (Wang and Szmidt, 2000). Other studies have suggested local adaptation as a possible explanation to the predicted severities obtained at different locations the

main factor appears to be attributable mainly to host availability. Local adaptation has been confirmed among isolates and/or races of the bean pathogen *Colletotrichum lindemuthianum* (Gandon *et al.*, 1996; Kaltz and Shykoff, 1998; Capelle and Neema, 2005).

5.2 Genetic variability of *Colletotrichum sublineolum*

The aim of the study was to assess the genetic variability of *Colletotrichum sublineolum* population in Uganda. Determination of genetic or population structures provides insights into the degree of genetic diversity of a pathogen or any other organism and lays a foundation for its population genetic characterisation. DNA markers have provided a powerful tool for this type of study. Over the last decade, DNA markers have been used to quantify genetic variation (Balardin *et al.*, 1997; Sicard *et al.*, 1997), decipher spatial distribution (Milgroom and Lipari, 1995), and distinguish among forces that generate and maintain genetic variation for several agriculturally important fungi (Zhang *et al.*, 1987). In this study, an estimate of Wright's population fixation ΦF_{ST} value was 0.08. In other fungal pathosystems, population differentiation indices within this range have been interpreted as indicative of moderate genetic differentiation between populations (Keller *et al.*, 1997; Gagné *et al.*, 2001). The presence of a moderate population structure among the populations, suggests that the pathogen population in this study have been genetically isolated from each other. The populations deriving from the same original population have got to be reproductively and genetically isolated from each other to permit selection and fixation of unique alleles that will account for divergence and presence of genetic structures (Hartl and Clark, 1997). Uganda is located close to the secondary center of diversity of sorghum and millet. It is therefore not surprising that this study finds a

genetically diverse population of *Colletotrichum sublineolum* within the various agro-ecologies.

It is expected that in centers of diversity pathogens and their hosts have co-evolved and as such contain diverse population. Interestingly, the data also shows that all population had very high gene diversity on an average of 0.2. Gene diversity is a good tool for estimating genetic variability among organisms that reproduce asexually (Weir, 1996). Such data as found in this study suggest that presence of highly variable clonal populations of *C. sublineolum*. Other studies have shown that *C. sublineolum* largely reproduce asexually and population structures may be found even within the same field (Rosewich *et al.*, 1998). On the basis of population structure, gene flow, and presence or absence of sexual phases, the potential evolutionary risk associated with fungal plant pathogens can be predicted (McDermont *et al.*, 1993). The presence of a population structure among Ugandan *C. sublineolum* from a management point of view suggests that the evolutionary responsiveness of this pathogen to selective disease control strategies may be moderate.

The population dynamics of a species is driven by interactions among mutation, migration, genetic drift, mating system, and selection, but it is rare to have sufficient empirical data to estimate values for all of these forces and to allow comparison of the relative magnitudes of these evolutionary forces. The data from phenetic analysis alludes to local populations of the pathogen as indicated by clusters of isolates from the same agro-ecologies. These data suggest limited gene flow or atleast a strong effect of selection and perhaps genetic drift in the Uganda *C. sublineolum* population leading to population differentiation.

Taken together, these results imply that for long-term disease management, host disease resistance should form a core component of disease management due to moderate evolutionary responsiveness of *C. sublineolum*. Spatial characterisation of anthracnose epidemics could elucidate disease carry over. This information could help improve disease control in the tropics and elsewhere. Moreover, the presence of population structure suggests that there is a local population of *C. sublineolum* in Uganda. Thus disease management strategies should target deployment of resistant lines as first line of defense, and coupled with cultural practices this should provide long term disease management.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

In the present study, genetic variability of *C.sublineolum* population in Uganda was studied to gain insights of its evolutionary history and potential to evolve. This thesis is based on the premise that population structure, gene flow, and study of the presence or absence of sexual phases, can be used to assess the potential evolutionary risk associated with fungal plant pathogens (McDermont and Donald, 1993). It is hoped that the results presented here in and the conclusions and recommendations drawn shall be useful in the design and implementation of disease management strategies for sorghum anthracnose in the different agro-ecological zones of Uganda.

The generally high severity and incidence of anthracnose suggest that sorghum anthracnose is a major threat to production in virtually all agro-ecologies of Uganda. More over, the high severities suggest cultivation of very susceptible varieties. No evidence was found for massive adoption of elite varieties perhaps accounting for the high disease incidence recorded. This combination of susceptible genotypes and good weather (warm-humid) supports high disease severity and incidence. It is therefore, obvious that deployment of disease resistant varieties will go along way in management of sorghum anthracnose.

6.1 Future research perspectives

What is clear from this study, is the fact that *C. sublineolum* is a highly variable pathogen devastating sorghum production. In this study neutral genetic markers (ISSR) were used to investigate population differentiation. Neutral genetic markers have their major weakness of being selectively neutral and thus cannot detect effects of selection in

populations. "Neutral" in this case means that the genetic variation observed is not thought to make a difference to the fitness of the organisms, and therefore it is not affected by natural selection. For example, in *C. sublineolum*, some populations from one district say Lira, might have neutral genotypes that are quite similar to each other, but substantially distinct from the neutral genotypes of the other district like populations from Masaka district. This district distinction is mainly at the DNA level, the idea is that these two groups have been evolving independently of each other for a long time, and therefore they might have unique adaptations, even if it's not apparent what these adaptations are. Hence further research with the use of selectable markers such as race differentials will provide better insights on the impact of sorghum variety selection in Uganda. There is thus a need to conduct further studies using race differentials. More over there is need to further characterise the sorghum anthracnose epidemics. These two issues will improve on management of sorghum anthracnose in Uganda and indeed Eastern Africa.

REFERENCE:

- Ali, M.E.K. and Warren, H.L. 1987. Physiological races of *Colletotrichum graminicola* on sorghum. *Plant Disease* 71: 402-404.
- Ali, M.E.K. and Warren, H.L. 1992. Anthracnose of sorghum. in sorghum and millet diseases: a second world review. de milliano, W. A.J., frederiksen, R.A. and bengston, G.D. (eds.) patancheru, A.P. 502324, india. International Crop Research Institute for the Semi-arid Tropics. Pages 203-208.
- Adaskaveg, J. E. and Forster, H. 2000. Occurrence and management of anthracnose epidemics caused by *Colletotrichum* species on tree fruit crops in California Pages 317-336. In: Prusky, D., Freeman, S. and Dickman, M.B. (ads.), *Colletotrichum: host Specificity, pathology, and Host-Pathogen Interaction*,. The American Phytopathological society. St. Paul MN.
- Agrios, G. N. 1997. *Plant Pathology*. 4th edition. Academic Press, New York.
- Alonso-Blanco, C., Peeters, A.J., Koornneef, M., Lister, C., Dean, C., van den Bosch, N., Pot, J. and Kuiper, MT. 1998. Development of an AFLP based linkage map of Ler, Col and Cvi *Arabidopsis thaliana* ecotypes and construction of a Ler/Cvi recombinant inbred line population. *Plant Journal* 14:259-271.
- Andrивon, D. 1994. Race structure and dynamics in populations of *Phytophthora infestans*. *Canadian Journal on Botany* 72:1681-1687.
- Ashok-Mishra, Siradhana, B. S. and Mishra, A. 1992. Effect of host age on sorghum anthracnose. Gujarat Agricultural University Research Journal 18: 135-6.
- Avila-Adame, C., Olaya, G. and Köller, W. 2003. Characterisation of *Colletotrichum graminicola* isolates resistant to strobilurin-related Quinone outside inhibitor fungicides. *Plant Disease* 87:1426-1432.
- Bailey, J. A. and Jegger, M. J. 1992. *Colletotrichum: biology, pathology and control*. CAB International, Wallingford, United Kingdom.
- Balardin, R. S., Jarosz, A. M. and Kelly, J. D, 1997. Virulence and molecular diversity in *Colletotrichum lindemuthianum* from South, Central, and North America. *Phytopathology* 87:1184-1191.
- Bray, E. A., Bailey-Serres, J. and Weretilnyk, E. 2000. Responses to abiotic stresses. In W Gruissem, B Buchannan, R Jones, eds, *Biochemistry and Molecular Biology of Plants*. American Society of Plant Physiologists, Rockville, MD, pages 1158-1249.
- Braithwaite, K. S., Irwin, J. A. G. and Manners, J. M. 1990. Ribosomal DNA as a molecular taxonomic marker for the group species *Colletotrichum gloeosporioides*. *Australian Systematic Botany* 3:733-738.

- Bruggers, R.L. and Elliott, C. C. H. 1989. *Quelea quelea – Africa's bird pest*. Oxford: Oxford University Press.
- Bornet, B., Muller, C., Paulus, F. and Branchard M. 2004. High informative nature of Inter Simple Sequence Repeat (ISSR) sequences amplified with tri- and tetra-nucleotide primers from cauliflower (*Brassica oleracea* var. *botrytis* L.) DNA. *Genome* 45: 890-6.
- Caffier, V., Brändle, U. E. and Wolfe, M. S. 1999. Genotypic diversity in barley powdery mildew populations in Northern France. *Plant Pathology* 48:582-587.
- Cardwell, K. F., Hepperly, P. R. and Frederiksen, R. A. 1989. Pathotypes of *Colletotrichum graminicola* and seed transmission of sorghum anthracnose. *Plant Disease* 73: 255-257.
- Cardwell, K. F. 1989. Variation in virulence of *Colletotrichum graminicola* and competition between monoconidial isolates. PhD dissertation. Texas A&M University. 170 pp.
- Casela, C. R., Ferreira, A. S., Zeller, K. A. and Levy, M. 1995. Pathotype variation in the sorghum anthracnose fungus: a phylogenetic perspective for resistance breeding Pages 257-276. In: Leslie, J. F. and Frederiksen, R. A. (eds.). *Disease analysis through genetics and molecular biology: interdisciplinary bridges to improved sorghum and millet crops*. Ames: Iowa state university press.
- Casela, C. R., Ferreira, A. S. and Santo, F. G. 2001. Differences in competitive ability among races of *Colletotrichum graminicola* in mixtures. *Fitopatologia Brasileira, Brasília* DF, 26: 217-219,
- Casela, C. R. and Frederiksen, R. A. 1994. Variabilidade patogênica em isolamentos monospóricos de *Colletotrichum graminicola*, agente causal da anthracnose do sorgo, originadas de uma única lesão e de culturas monospóricas. *Fitopatologia Brasileira, Brasília*, DF, 19: 149-153.
- Casela, C. R., Frederiksen, R. A. and Ferreira, A. S. 1993. Evidence for dilatory resistance to anthracnose in sorghum. *Plant Disease* 77: 908-911.
- Capelle, J. and Neema, C. 2005. Local adaptation and population structure at a micro-geographical scale of a fungal parasite on its host plant. *Journal of Evolutionary Biology* 8: 1445-1454.
- Clements, F. E. and Shear, C. L. 1983. The genera of fungi. New York. Wiley, Page 379.
- Coyne, K. J., Hutchins, D. A., Hare, C. E. and Cary, S. C. 2001. Assessing temporal and spatial variability in *Pfiesteria piscicida* distributions using molecular probing techniques. *Aquatic Microbial Ecology* 24: 275-5.
- Esele, J. P. E. 1995. Foliar and head diseases of sorghum. *Africa Crop Science Journal*. 3:185-189.

- Excoffier, L., Smouse, P.E. and Quattro, J.M. 1992. Analysis of molecular Variance inferred from metric distances among DNA haplotypes: Application to human mitochondrial DNA restriction data. *Genetics* 131: 479-491.
- Excoffier, L. 2001. Analysis of population subdivision, Pages 271-307. In: Balding, D.J., Bishop, M. and Cannings, C., eds. *Handbook of Statistical genetics*. John Wiley & Sons United Kingdom.
- FAO, 1991. Food and Agricultural Organization of United Nations.
- FAO Statistics, 2003. Food and Agricultural Organization of United Nations.
- FAO 2004. Food and Agricultural Organization of United Nations.
- FAO Statistics, 2005. Food and Agricultural Organization of United Nations.
- Fisher, R.A. 1930. *The Genetical Theory of National Selection*. Claredon Press, Oxford, UK.
- Frederiksen, S. 1986. Revision of *Taeniatherum* (Poaceae). *Nordic Journal of Botany* 6:389-397.
- Frederiksen RA. 1991. *Compendium of Sorghum Diseases*. St. Paul, MN. *American Phytopathological Society*. Page 82.
- Frederiksen, R. A., Thomas, M. D., Bandyopadhyay, R. and Mughogho, L. K. 1995. Variable pathogens of sorghum. Pages 11-23. In: Leslie J. F. and Frederiksen, R. A. (eds). *Disease Analysis through Genetics and Molecular Biology: Interdisciplinary Bridges to Improved Sorghum and Millet Crops*. Iowa State University Press, Ames.
- Freeman, S. 2000, Genetic diversity and host specificity of *Colletotrichum* species on various fruits. Pages 131-144. In: Prusky, D. Freeman, S. and Dickman, M.B. (eds.), *Colletotrichum: host Specificity, pathology, and Host-Pathogen Interaction*. The American Phytopathological society St. Paul MN.
- Gagné, P., Yang, D. Q., Hamelin, R. C. and Bernier, L. 2001. Genetic variability of Canadian populations of the Sapstain fungus *Ophiostoma piceae*. *Phytopathology* 91:369-376.
- Gandon, S., Capowiez, Y., Dubois, Y., Michalakakis, Y. and Olivieri, I. 1996. Local adaptation and gene-for-gene coevolution in a metapopulation model. *Proceedings of the Royal Society of London B* 263: 1003-1009.
- Ghislain, M., Spooner, D. M., Rodríguez, F. Villamon, F. Núñez, C. Vásquez, C. and Bonierbale, M. 2004. Selection of highly informative and user-friendly microsatellites (SSRs) for genotyping of cultivated potato. *Theoretical and Applied Genetics* 108:881-890.

- Groth, J. V. and Roelfe, A. P. 1989. The analysis of genetic variation in populations of rust fungi. Pages 318-339. In: Leonard, K. J. and Fry, W. E. (eds) *Plant Disease Epidemiology*. Volume 2: *Genetics, Resistance, and Management*. McGraw-Hill Publishing Company, New York.
- Goodwin, S. B., Saghai Maroof, M. A., Allard, R. W. and Webster, R. K. 1993. Isozyme variation within and among populations of *Rhynchosporium secalis* in Europe, Australia and the United States. *Mycological Research* 97:49-58.
- Guthrie, P.A.I., C.W. Magill, R.A. Frederiksen and G.N. Odvody, 1992. Random amplified polymorphic DNA markers: a system for identifying and differentiating isolates of *Colletotrichum graminicola*. *Phytopathology* 82: 832-835.
- Hartl, D.L. and Clark, A.G. 1997. *Principles of Population Genetics*. 3rd Edition, Sinauer Associates. Inc. Sunderland, Massachusetts, USA.
- Harris, H. B., Johnson, B. J., Dobson, J. W. and Luttrell, E. S. 1964. Evaluation of anthracnose on grain sorghum. *Crop science* 4:460-462.
- Hawksworth, D. L., Sutton, B. C. and Ainsworth, G. C. 1983. *Ainsworth and Bisby's Dictionary of the fungi*. Commonwealth Mycology Institute Surrey England.
- Hess, D. E., Bandyopadhyay, R. and Sissoko, I. 2002. Pattern analysis of sorghum genotype x environment interaction for leaf, panicle, and grain anthracnose in Mali. *Plant Disease* 86:1374-1382.
- Holliday, P. 1980. *Fungus Diseases of Tropical Crops*. Cambridge University Press, Cambridge United Kingdom.
- Hokanson, S. C., Szewc-McFadden, A. K., Lamboy, W. F. and McFerson, J.R. 1998. Microsatellite (SSR) markers reveal genetic identities, genetic diversity and relationships in a *Malus x domestica* borkh. Core subset collection. *Theoretical Applied Genetics*. 97: 671-683.
- International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) 2001. *Applied Genetics* 97 671-683.
- Jeffries, P., Dodd, J. C., Jeger, M. J. and Plumbey, R. A. 1990. The biology and control of *Colletotrichum* species on tropical fruit crops. *Plant Pathology* 39: 343-366.
- Kaltz, O. and Shykoff, J. A. 1998. Local adaptation in host-parasite systems. *Heredity* 81: 361-370.
- Keller, S. M., Wolfe, M. S., McDermont, J. M. and McDonald, B. A. 1997. High genetic similarity among populations of *Phaeosphaeria nodorum* across wheat cultivars and regions in Switzerland. *Phytopathology* 87:1134-1139.
- Koehler, B. 1943. Disease threatening broom corn production in Illinois. *Plant Disease*

Reporter 27:70-73.

- Kollo, I. A. 1988. Influence of leaf age on anthracnose development in *Zea mays*. MS thesis. Purdue University, West Lafayette, IN.
- Lanham, P.G. and Brennan, R.M, 1998. Characterisation of genetic resources of redcurrant (*Ribes rubrum* Ribesia) using anchored microsatellite markers. *Theoretical Applied Genetics* 96 917-21.
- Marley, P.S., Thakur, R. P. and Ajayi, O. 2001. Variation among foliar isolates of *Colletotrichum sublineolum* of sorghum in Nigeria. *Field Crops Research* 69: 133-142.
- Marley, P.S. and Ajayi, O. 2002. Assessment of anthracnose resistance (*Colletotrichum graminicola*) in sorghum (*Sorghum bicolor*) germplasm under field conditions in Nigeria. *Journal of Agricultural Science* 138: 201-208.
- 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.
- McDermont, J. M. and McDonald, B. A. 1993. Gene flow in plant pathosystem. *Annual Review of Phytopathology* 31: 353-373.
- McDonald, B. A. and Linde, C. 2002. Pathogen population genetics, evolutionary potential and durable resistance. *Annual Review of Phytopathology* 40: 349-379.
- McDonald, B. A., Mundt, C. C. and Chen, R. S. 1996. The role of selection on the genetic Structure of pathogen populations: Evidence from field experiments with *Mycosphaerella graminicola* on wheat. *Euphytica* 92: 73-80.
- Milgroom, M. G. and Lipari, S. E. 1995. Spatial analysis of nuclear and mitochondrial RFLP genotypes in populations of the chestnut blight fungus, *Cryphonectria parasitica*. *Molecular Ecology* 4:633-642.
- Mills, P. R., Sreenivasaprasad, S. and Brown, A. E. 1992. Detection and differentiation of *Colletotrichum gloeosporoides* isolates using PCR. *FEMS microbiology Letter* 98: 137-144.
- Moffat, A. S. 2002. Finding new ways to protect drought-stricken plants. *Science* 296: 1226–1229.
- Morgante, M., Hanafey, H. and Powell, W. 2002. Microsatellites are preferentially associated with nonrepetitive DNA in plant genome. *Nature Genetics* 30: 194–200.
- Mundy, P. J. and Jarvis, M. J. F. 1989. *Africa's feathered locust*. Harare: Boabab Books Harare, Zimbabwe.

- Ngugi, H. K., Julian, A. M., King, S. B. and Peacocke, B.J. 2000. Epidemiology of sorghum anthracnose (*Colletotrichum sublineolum*) and leaf blight (*Exserohilum turcicum*) in Kenya. *Plant Pathology* 49: 129-140.
- Ngugi, H. K., King, S. B., Abayo, G. O. and Reddy, Y. V. R. 2002. Prevalence, incidence, and severity of sorghum diseases in Western Kenya. *Plant Disease* 86: 65-70.
- Nei, M. 1973. Analysis of gene diversity in subdivided populations. *Proceedings of the National Academy of Sciences of the United States of America* 70: 3321-3.
- Nei, M. 1987. Molecular Evolutionary Genetics. Columbia University Press, New York.
- Ngugi, H. K., King, S. B., Holt, J. and Julian, A. M. 2001. Simultaneous temporal progress of sorghum anthracnose and leaf blight in crop mixtures with disparate patterns. *Phytopathology* 91:720-729.
- O'Connell, R. J., Bailey, J. A. and Richmond, D. V. 1985. Cytology and physiology of infection of Phaseolus vulgaris by *Colletotrichum* infection processes. Page 57-76. In: Prusky, D., Freeman, S. and Dickman, M. B. (eds.), *Colletotrichum: Host Specificity, and Host-Pathogen Interaction*. The American Phytopathological Society. St. Paul MN.
- Okori, P., Fahleson, J., Rubaihayo, P. R., Adipala, E. and Dixelius, C. 2003. Assessment of genetic variation among East African *Cercospora zea-maydis* populations using AFLP and RFLP. *African Crop Science Journal* 11: 75-86.
- Okori, P. 2004. Population studies of *Cercospora zea-maydis* and related *Cercospora* fungi. Uppsala: Swedish University of Agricultural Sciences for PhD Thesis.
- Pande, S., Mughogho, L.K. Bandyopadhyay, R. and Karunakar, R.I. 1991. Variation in pathogenicity and cultural characteristics of sorghum isolates of *Colletotrichum graminicola* in India. *Plant Disease* 75: 778-783.
- Pingali, P. L. 2001. CIMMYT 1999-2000 World Maize Facts and Trends. Meeting World Maize Needs: Technological Opportunities and Priorities for the Public Sector. Mexico, D. F.: CIMMYT.
- Peever, T. L. and Milgroom, M. G. 1994. Genetic Structure of *Pyrenophora teres* populations determined with random amplified polymorphic DNA markers. *Canadian Journal of Botany* 72: 915-913.
- Perfect, S. E., Hughes, H. B., O'Connell, R. J. and Green, J. R. 1999. *Colletotrichum*: a model genus for studies on pathology and fungal- plant interactions. *Fungal Genetic Biology* 27: 186-198.
- Poehlman, M. J. and Sleper, D. A. 1995. Germplasm resources and conservation. Pages 243-255. In: *Breeding Field Crops*. 4th ed. Iowa State University Press. Ames, Iowa.

- Prusky, D., Kobilier, I., Ardi, R., Beno-Moalem, D., Yakoby, N. and Keen, N. T. 2000. Resistance mechanisms of subtropical fruits and *Colletotrichum gloeosporioides*. Pages 232-244 In: Prusky, D., Freeman, S. and Dickman, M.B. (eds.), *Colletotrichum: Host Specificity, Pathology, and Host-Pathogen Interaction.. The American Phytopathological Society*. St. Paul MN.
- Ramaiah, K.V., Parker, C., Vasudeva Rao, M. J. and Musselman, L. J. 1983. *Striga* Identification and Control Handbook. Information Bulletin No. 15. ICRISAT. Page 52.
- Raymond, M. and Rousset, F. 1995. GENEPOP: population genetics software for exact tests and ecumenicism. *Journal of Heredity* 86: 248-249.
- Ruecker, G. R., Park, S. J., Ssali, H. and Pender, J. 2003. Strategic Targeting of Development Policies to a Complex Region: A GIS-Based Stratification Applied to Uganda. ZEF - Discussion Papers On Development Policy No. 69 (p. 41). Bonn, Germany: Center for Development Research. <http://www.zef.de>
- Rosewich, U. L., Pettway, R.E., McDonald, B. A., Duncan R. R. and Frederiksen, R. A. 1998. Genetic Structure and temporal dynamics of a *Colletotrichum graminicola* population in a sorghum disease nursery. *Phytopathology* 88: 1087-1093.
- Saal, B. and Wricke, G. 2002. Clustering of amplified fragment length polymorphism markers in a linkage map of rye. *Plant Breeding* 121: 117-123.
- Schneider, S., Roessli, D. and Excoffier, L. 2000. Arlequin version 2.000: *A software for population genetics data analysis*. Genetics and Biometry Laboratory, University of Geneva, Switzerland.
- Schall, R. A., Nicholson, R. L., and Warren, H. L. 1980. Influence of light on maize anthracnose in the greenhouse. *Phytopathology* 70: 1023-1026
- Shannon, C. E. and Weaver, W. 1949. *The Mathematical Theory of Communication*. University of Illinois Press, Urbana, IL.
- Shea, G. 1999. Managing lupin anthracnose. *Journal of Agriculture of Western Australia* 40: 24-30.
- Sherriff, C., Whelan, M. J., Arnold, G. M. and Bailey, J. A. 1995. rDNA sequence analysis confirms the distinction between *Colletotrichum graminicola* and *C. sublineolum*. *Mycology Research* 99:475-478.
- Sicard, D., Buchet, S., Michalakakis, Y. and Neema, C. 1997. Genetic variation of *Colletotrichum Lindemuthianum* in wild population of common bean. *Plant Pathology* 46: 355-365.
- Songa, J. M., Bergvinson, D. and Mugo, S. 2001. Impacts of Bt-gene based resistance in maize on non-target organisms in Kenya. Characterisation of target and non-target organisms of Bt-gene-based resistance in two major maize growing regions in

- Kenya. Insect Resistant Maize for Africa (IRMA). Annual report 2000. IRMA Project Document N0. 4. Pages 16-21.
- Sutton, B. C. 1992. The genus *Glomerella* and its anamorph *Colletotrichum*. Page 1-26 in: *Colletotrichum*. Biology, Pathology and Control. J. A. Bailey and M. J. Jeger, eds. CAB International, Wallingford, UK.
- Sutton, B. C. 1980. *The Coelomycetes. Fungi Imperfecti with pycnidia, acervuli and stromata*. Surrey, England: Commonwealth Mycological Institute. Page 696.
- Souza-Paccola, E. A., FavAro Fa'varo, L. C. L., Bomfeti, C. A., Mesquita, S. F. P. and Paccola-Meirelles, L. D. 2003. Cultural Characterisation and Conidial Dimorphism in *Colletotrichum sublineolum*. *Journal of Phytopathology* 151: 383-388.
- Steel, R. G. D., Torrie, J. H. and Dickey, D. A. 1997. *Principles and procedures of statistics: a biometrical approach*, 3rd edition, McGraw Hill New York Pages 1-675.
- Stoddart, J. A., and Taylor, J. F. 1988. Genotypic diversity: Estimation and prediction in samples. *Genetics* 118: 705-711.
- Tenkouano, A., Miller, F. R., Hart, G. E., Frederiksen, R. A. and Nicholson, R. L. 1993. Phytoalexin assay in juvenile sorghum: an aid to breeding for anthracnose resistance. *Crop Science* 33: 243-248.
- Thakur, R. P. 1995. Status of international sorghum anthracnose and pearl millet downy mildew virulence nurseries. Pages 75-92. In: Leslie, J.F. and Frederiksen, R.A. (eds) *Disease Analysis through Genetics and Biotechnology, inter disciplinary bridges to improved sorghum and millet crops*. Iowa State University Press.
- Thakur, R. P. and Mathur, K. 2000. Anthracnose. In: *Compendium of Sorghum Diseases*. (Eds. R.A. Frederiksen and G.N. Odvody). *The American Phytopathological Society*, St. Paul, MN, USA, Pages 10-12.
- Thakur, R. P. 2004. The shifty anthracnose pathogen continues to challenge sorghum. www.icrisat.org/text/research/grep/homepage/grephomepage/archives/anthracnose.htm.
- Thomas, M. D. 1992. Sorghum diseases in Western Africa. Pages 25-29 In: *Sorghum and Millets Diseases: A Second World Review*. W. J. A. de Milliano, R. A. Frederiksen, and G. D. BergSton, eds. ICRISAT, Patancheru, India.
- Timmer, L.W. and Brown, G. E. 2000. Biology and control of anthracnose diseases of citrus. Pages 300-316. In: Prusky, D., Freeman, S. and Dickman, M.B. (eds.), *Colletotrichum: host Specificity, pathology, and Host-Pathogen Interaction*. The American Phytopathological society. St. Paul MN.

- Uddin, W., Serlemitsos, K. and Viji, G. 2002. A temperature and leaf wetness duration based model for prediction of gray leaf spot perennial ryegrass turf. *Phytopathology* 93: 336-343.
- USDA, 2006. Grain world market and trade December 2006.
- Van de Peer, Y. 2004. Computational approaches to unveiling ancient genome duplications. *Nature Review of Genetics* 5: 752-63.
- Von Arx, J.A. 1970. A revision of the fungi classified as *Gloeosporium*. *Bibliotheca Mycologica* 24: 1.
- Ward, J. M. J., Liang, M. D. and Rijkenberg, F. H. J. 1997. Frequency and timing of fungicide applications for the control of gray leaf spot in maize. *Plant Disease* 8: 41-48.
- Ward, M. J. J., Stromberg, E. L., Nowel, C. D. and Forrest, N. W. Jr. 1999. Gray leaf spot: A Disease of Global Importance in Maize Production. *Plant Disease* 83: 884-895.
- Wharton, P. S. and Julian, A. M. 1996. A cytological Study of compatible and incompatible interactions between *Sorghum bicolor* and *Colletotrichum sublineolum*. *New Phytopathology* 134: 25-34.
- Welsh, J. and McClelland, M. 1990. Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Research* 18: 7213-7218.
- Weir, B. S. 1996. *Genetic data analysis II*. Sinauer associates Inc. Sunderland MA, USA page 445.
- Weir, B. S. and Cockerham, C. C. 1984. Estimating *F*-statistics for the analysis of population structure. *Evolution* 38: 1358-1370.
- Wise, M. G., Shimkets, L. J. and McArthur, J. V. 1995. Genetic Structure of a lotic population of *Burkholderia* (*Pseudomonas*) *cepacia*. *Applied Environmental Microbiology*, 61: 1791-1798.
- Wright, S. 1969. *Evolution and the Genetics of Populations, variability within and among natural populations*, Volume 4. University of Chicago Press: Chicago USA.
- Wolfe, M. S. and Caten, C. E., 1987. *Populations of Plant Pathogens: Their Dynamics and Genetics*. Blackwell Scientific Publications, Oxford.
- Wortmann, C. S. and Eledu, C. A. 1999. *Uganda's Agro-ecological zones: A guide for policy makers and planners*. Centro Internacional de Agricultural Tropical. Kampala, Uganda.
- Young, N. D. 1999. A cautiously optimistic vision for marker-assisted breeding. *Molecular Breeding* 5:505-510.

Zhang, Q., Webster, R. K., and Allard, R. W. 1987. Geographical distribution and associations between resistance to four races of *Rhynchosporium secalis*. *Phytopathology* 77:352-357.