

Population studies of *Cercospora zeaemaydis* and related *Cercospora* fungi

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To Pamela, Paul, Pagiell and Sarah

Freedom to inquire into the nature of things is a rewarding privilege granted to a few by a permissive society (Horsfall & Cowling, 1980).

Abstract

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Grey leaf spot caused by *Cercospora zae-maydis* is considered a global threat to maize production. In Africa, the disease was first reported just over 10 years ago, but has rapidly spread to most maize growing countries of sub-Saharan Africa. Being a rapidly spreading new disease in the region, demands for a quick but effective control strategy. Since pathogen populations cause epiphytotic, it is logical that control strategies should target populations rather than individuals. Effectiveness of disease control strategies however, is dependent on the knowledge available about a pathosystem. The studies in this thesis attempted to elucidate the East African *C. zae-maydis* pathosystem using various population biology investigative approaches. In study I, the goal was to estimate genetic variability of East African *C. zae-maydis* and infer the role of evolutionary forces in the populations. Study II focused on elucidating critical elements of the grey leaf spot disease triangle by examining interactions between biotic and abiotic stresses of the pathosystem. Study III focused on estimation of genetic variability of *C. sorghi* in order to compare and infer evolutionary responsiveness of *C. zae-maydis*. Study IV investigated temporal dynamics in *C. zae-maydis* populations in response to disease management. In all the studies, neutral genetic markers were the main genetic tools utilized.

Study I showed that East African *C. zae-maydis* populations were predominantly of Type II haplotype, with a weak genetic structure between populations. East African *C. zae-maydis* was more variable than American Type I isolates. Concordance between neutral genetic markers, provided strong support for gene flow in the populations. Study II revealed that haplotype variability influenced epiphytotic. Presence of susceptible host and poor mineral nutrition also influenced epiphytotic patterns. In study III, no genetic structure of *C. sorghi* was detected and gene flow from wild hosts appeared to influence epidemics. A host species dependent evolution among *Cercospora* fungi was inferred by phenetics. Population parameters of *C. sorghi* were similar to *C. zae-maydis*, suggesting similar evolutionary responsiveness of the two fungi. In study IV, the data indicate population stability of *C. zae-maydis*. Taken together, these data suggest durability of resistant genotypes once deployed. Quarantine may reduce disease spread to new areas and being one epidemiological unit, suggests that regional efforts to abate epidemics are worthwhile.

Key word: AMOVA, AFLP, East Africa, gene flow, genetic variability, grey leaf spot, phenetics, population structure, rDNA, RFLP, sorghum, Uganda, *Zea mays*.

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Appendix

Papers I-IV

This thesis is based on the following papers, which will be referred to by their Roman numerals.

- I Okori, P., Rubaihayo, P.R., Fahleson, J., Adipala, E. and Dixelius, C. (2003) Assessment of genetic variation among East African *Cercospora zae-maydis* populations. *African Crop Science Journal* 11, 75-85.
- II Okori, P., Rubaihayo, P.R. Adipala, E. and Dixelius, C. (2003) Interactive effects of host, pathogen and mineral nutrition on grey leaf spot epidemics in Uganda. *European Journal of Plant Pathology* 110,119-128.
- III Okori, P., Rubaihayo, P.R., Adipala, E., Fahleson, J. and Dixelius, C. (2004) Genetic characterisation of *Cercospora sorghi* from cultivated and wild sorghum and its relationship to other *Cercospora* fungi. *Phytopathology* 94,743-750.
- IV Okori, P., Rubaihayo, P.R., Adipala, E., Fahleson, J. and Dixelius, C. (2004) Genetic structure and temporal dynamics of *Cercospora zae-maydis* populations in two agroecologies of Uganda. (Submitted)

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Commonly used Terminologies

Agroecology: Refers to a subset of natural ecologies that have been altered by human activity to promote agricultural activities.

Biogeography: Study of the geographical distributions of organisms, their habitats and the historical and biological factors, which produced them.

Disease efficiency: Refers to severity and progression of a disease as indicated by percent area affected after infection by a given pathogen.

Directional selection: Selection for an optimum phenotype resulting in a directional shift in gene frequencies of the character concerned and leads to a state of adaptation in a progressively changing environment.

Epidemiological fitness: Refers to ability to incite various amounts of disease in the same or different environments of one pathogen genotype relative to another.

Epiphytotics: A widespread and destructive outbreak of a disease of plants. It is used interchangeably with the term epidemics.

Episodic selection: Refers to the effect of any sudden environmental disturbance likely to lead to a significant alteration in a species population structure.

F-statistics: Statistical approaches developed by Sewal Wright and advanced others that measures the reduction in heterozygosity or increase in homozygosity expected with random mating at any level of a population hierarchy relative to another more inclusive level of the hierarchy.

Gene diversity: A measure of variation, also referred to as average heterozygosity. It refers to the probability that two randomly chosen genes from two randomly chosen individuals obtained from a population are different.

Haplotype: The collective genotype usually derived from a number of linked loci or alleles. It may also refer to the type species of a genus.

IDM: Integrated disease management. Holistic disease management approaches that employ a variety of management options that is cost effective and causes minimal environment damage.

Monophyletic group: A natural taxon composed of two or more species.

Multi-gene genealogy: Refers to the study of phylogenetic or ancestral lineages and relationships based on several genes or their fragments.

Parasitic fitness: Refers to the relative ability of a parasitic genotype or population to persist successfully over time in the same or different environments.

Pathosystem: A pathosystem is a subsystem of an ecosystem characterised by the phenomenon of parasitism. (Usually refers to hosts and their parasites).

Pathotype: A subset of a pathogen species, distinguished by common characters of pathogenicity, particularly in relation to the host range.

Population: Refers to a pool of conspecific individuals (pathogens) from which the next generation will be drawn, as delineated in space and time by allelic boundaries.

Population biology: A synthesis of plant disease epidemiology and population biology that integrates ecological, genetical and evolutionary principles within a population context.

Population structure: Refers to the extent to which large populations are subdivided into smaller subpopulations that may differ in allele frequency from the neighbouring subpopulations.

Sibling species: Pairs or groups of closely related and frequently sympatric species, which are morphologically indistinguishable but genetically different.

Sympatric species: Species or taxa occupying the same geographical area, habitat or geographically different but similar habitats.

Introduction

Maize (*Zea mays* L.) is the most important staple cereal in sub-Saharan Africa (SSA). At the current population growth rates and the wide scale adoption of intensive livestock production systems in developing countries, the annual demand for maize will increase at 2.4 % per annum, compared to other cereals, projected at 1.38% up to 2025 (Pinstrup-Andersen, Panya-Lorch & Resegrant, 1999). Currently, SSA has one of the highest per capita consumption of maize among developing countries, with maize largely consumed at household level (Pingali & Pandey, 2001). There is also increasing demand for maize in the region from intensive agricultural systems especially poultry and piggery. Overall, it is projected that aggregate demand for maize globally and SSA in particular, will overtake demand for rice or wheat by 2020 (Pinstrup-Andersen, Panya-Lorch & Resegrant, 1999). Accordingly, strengthening maize production is considered essential for food security in developing countries and SSA in particular (CIMMYT, 2002; Watkins & von Braun, 2003).

Maize production in SSA is curtailed by a number of biotic and abiotic stresses, which may cause losses of up to 80% (Pingali & Pandey, 2001; DeVries & Toenniessen, 2001). Abiotic stresses in SSA are mostly due to seasonal unreliability of rain-fed agriculture, poverty and limited access to remedial inputs. Conversely, biotic stresses are ever-present, and require effective management processes to support productivity and environmental protection. The main biotic constraints include pests, such as, stem borers (*Busseola fusca*, *Chilo partellus* *Sesamia calamistis* and *Heliothis armigera*). The major diseases include turicum leaf blight (*Exserohilum turcicum* Pass Leonard & Suggs), grey leaf spot (*Cercospora zea-maydis* Tehon & Daniels) and maize streak virus disease (Pingali & Pandey, 2001; DeVries & Toenniessen, 2001). In SSA, turicum leaf blight and grey leaf spot are the most important fungal diseases, and have been ranked as high research priority problems of maize production by CIMMYT and national maize research programmes in the region (Pingali & Pandey, 2001). The significance of these diseases vary with location and are most important when infection occurs at early crop growth stages or when the plant is most vulnerable, such as at grain filling, as in the case of grey leaf spot (White, 1999; Ward *et al.*, 1999).

For control, host resistance remains the most economically viable option for resource-constrained farmers in SSA. Unfortunately, host resistance is fraught with resistance erosion (Schumann, 1991), presumably due to an “arms race” between pathogens and their hosts (Stahl *et al.*, 1999; Stahl & Bishop, 2000), often manifested as “boom and burst” cycles. This arms race is an evolutionary response by pathogens to disease control measures and is maintained through mutation and recombination in pathogens and their host plants (Meyers *et al.*, 1998; Hulbert *et al.*, 2001; Vleeshouwers *et al.*, 2001). The durability of any crop protection system will therefore depend on the use of strategies that slow-down pathogen evolution. This work utilized population biology to gain insight into grey leaf spot epiphytotics and evolutionary processes of the pathogen, so as to improve disease management and promote maize grain productivity in East Africa.

Grey leaf spot disease of maize

Historical perspectives and importance

Grey Leaf Spot (GLS) caused by *Cercospora zea-maydis* Tehon & Daniels, is one of the most important foliar diseases of maize world-wide, which may cause yield losses of up to 60% (Ward *et al.*, 1999). The disease was first identified from specimens collected by Tehon and Daniels in 1925 in Illinois and confirmation of its identify performed by Chupp (1953). Disease symptoms are usually observed on the lower leaf surfaces. Lesions appear as small tan spots about 1-3 mm long that are rectangular to irregular in shape, often delimited by leaf veins and may appear grey in cast when heavily sporulating (Latterell & Rossi, 1983; Ward *et al.*, 1999). Early infections are indistinguishable from those caused by other fungal pathogens, except for a yellow halo visible when observed under transmitted light (Latterell & Rossi, 1983). Within 2 weeks such lesions gradually elongate appearing as streaks before developing their dark distinguished greyish brown rectangular shape (Fig. 1).

Since its first reporting in 1925, GLS was not considered a threat to maize production. Sporadic disease outbreaks were however reported in Virginia, (Roane, 1950), Kentucky and eastern Tennessee (Hyre, 1943) and South Carolina (Kingsland, 1963). Economic importance of the disease in the US, was recognized in the first half of the 1970's when it was described as the most destructive disease of maize after increased incidence and epiphytotics in North Carolina (Leonard, 1973). Since then, the disease has steadily spread in the US and other parts of the world. In Africa, first reports of GLS were made in 1990 in KwaZulu-Natal, South Africa (Nutter & Jenco, 1992) and since then, GLS has progressively spread northwards, and is now prevalent in most countries of Sub-Saharan Africa (Pratt, Lipps & Freppon, 1997; Ward *et al.*, 1999; Okori, Fahleson & Dixelius, 2001; Asea *et al.*, 2002). Today, GLS is considered a challenge to increased maize production in the region, and is ranked highly in national and international maize research agenda for the next decade (Pingali & Pandey, 2001; DeVries & Toenniessen, 2001).



Figure 1. Typical grey leaf spot lesions on susceptible maize plants.

The Cercospora complex and grey leaf spot

Members of the genus *Cercospora* are deuteromycetes and comprise one of the largest groups of plant pathogenic fungi (Pollack, 1987; Goodwin Dunkle & Zismann, 2001). Details of phylogenetic relationships between *Cercospora* fungi can be found elsewhere (Stewart *et al.*, 1999; Goodwin, Dunkle & Zismann, 2001). Many *Cercospora* are highly specialized phytopathogens, although, cross-infection by some species does occur, causing leaf spots on dicots and monocots (Chupp, 1953). In maize, GLS is caused by *C. zae-maydis*, although a related pathogenic species, *C. sorghi* var. *maydis* has been associated with the disease (Chupp, 1953; Carson, Goodman & Williamson, 2002). However, the impact of *C. sorghi* var. *maydis* on GLS epiphytotics is largely unknown (Carson, Goodman & Williamson, 2002). Recently, it has been shown that *C. zae-maydis* is composed of two genetically distinct but morphologically similar haplotypes or sibling species designated Type I and Type II (Wang, Levy & Dunkle, 1998). Preliminary investigations indicated that Type II was prevalent in Africa (Dunkle & Levy, 2000). Given that *C. sorghi* was obtained from Africa earlier (Chupp, 1953), and now that the GLS of maize is endemic in the continent, it was imperative to study the role(s) of these pathogens in the current epiphytotics.

Grey leaf spot epidemiology

Cercospora zae-maydis is a highly specialized necrotrophic pathogen of maize (Chupp, 1953; Stromberg & Donahue, 1986). The pathogen survives in infested crop debris during non-cropping periods, that incidentally, accounts for increased incidence and severity in no-till agricultural systems of the US (Payne & Waldron, 1983; de Nazareno, Lipps & Madden, 1992, 1993a). It should also be noted that GLS epiphytotics are highly dependent on prevailing environmental conditions (Beckman & Payne, 1983). In the tropics, especially in areas that experience two cropping seasons, continuous crop cultivation, late harvesting of maize and stacking of maize stalks in fields for later use as mulch, may support quick inoculum build-up and further spread of the disease (Asea *et al.*, 2002). Wind dispersal of spores and infested debris facilitate long distance transportation of the pathogen to newly planted maize crops. Significant losses occur if sufficient infested crop debris is disseminated by the wind and prevailing environmental conditions are highly favourable for disease development (Ward *et al.*, 1999). Interestingly, GLS epiphytotics under tropical conditions appear to occur at higher severity than in temperate regions. Recent studies reported relatively higher and shallower GLS dispersion gradients in the tropics (Asea *et al.*, 2002), than in temperate regions (de Nazareno, Lipps & Madden, 1993b), suggesting more rapid spread of the disease in the tropics. The high disease severity in Africa could also be attributed to a poorly understood but potentially important role of mineral nutrition in GLS epidemics (Smith, 1989; Ward, 1996). Taken together, it is evident that GLS, a relatively new disease in Africa, appears to occur at high severity, perhaps due to a number of mutually reinforcing elements of its disease pyramid such as susceptible varieties, supportive weather conditions, cropping patterns as influenced by human activity and other biophysical factors.

Elucidating the GLS disease pyramid in East Africa

The need for a pathosystems approach

A pathosystem is a subsystem of an ecosystem characterised by the phenomenon of parasitism (Robinson, 1987). Pathosystems reflect stabilised, co-evolved, interacting components of ecologies (Gliessman, 1995). Pathosystems can be classified as wild plant pathosystems, (lack human interference) or crop pathosystems (derivatives of natural pathosystems with human interference) (Robinson, 1987). Natural pathosystems are self-regulating and stable, whereas, crop pathosystems are typically unstable due to deliberate alterations by humans and are often represented as a disease tetrahedron or pyramid (Zadocks & Schein, 1979; Agrios, 1997). Crop pathosystems differ from natural pathosystems in three major aspects: (1) they are characterised by host species that are genetically diverged from wild relatives, (2) occurrence of high densities of genetically uniform host species within a small area and (3) the environment is usually altered in creation of the field in a deterministic manner for example by ploughing, fertilizer application, breeding and pesticide use *etc.* (Robinson, 1987). These alterations affect host-parasite relationships by creating unusual selection pressure that do not occur in the nature. This invariably disrupts natural ecosystem equilibrium resulting in disease or pest epiphytotics. This underscores the need to adopt an agroecological perspective in design of disease management strategies to reduce novel pathotype microevolution.

Disease management relies on ability to manipulate certain components of a pathosystem against the pathogen. The commonest evolutionary force manipulated against pathogens is selection. This implies that host plants and pathogens are the most-unstable parts of any pathosystem (Robinson, 1987; Schumann, 1991; Gliessman, 1995; McDonald & Linde, 2002). To develop durable economic and ecologically sustainable disease management programs, characterized by slow novel pathotype microevolution, a clear understanding of the evolutionary processes prevalent in the pathosystem is needed. Evolutionary investigative approaches provide means to study the micro-evolutionary processes of a pathosystem. For GLS, a new endemic disease in sub-Saharan Africa, the development of durable disease management strategies based on sound knowledge of the micro-evolutionary processes prevalent in the pathosystem is of utmost importance. Questions regarding micro-evolutionary processes are best addressed using population biology as was attempted in this thesis.

Role of population biology

Population biology describes a relatively holistic perspective of the ecological and evolutionary dynamics of plant and pathogen populations (Milgroom & Peever, 2003). In plant pathology, population biology is considered a synthesis of plant disease epidemiology and population genetics that focuses on all biological processes prevalent and influencing organism populations (Wöhrmann & Jain 1991; Milgroom & Peever, 2003; McDonald, 2004). Population biology is relevant to plant pathology because plant diseases are caused by populations of parasites. Epidemiology and population genetics are different but related subsets of

population biology (Milgroom & Peever, 2003; McDonald, 2004). Plant disease epidemiology is the study of the distribution and determinants of disease frequency in plant populations. Epidemiology focuses on spatial and temporal disease development patterns in pathogen populations and may not consider differences in behaviour or fitness of genetically distinct individuals in the population. Conversely, population genetics focuses on the processes that induce genetic change or evolution in populations over time and space. Population genetics deals mainly with genetic processes such as mutation, genetic drift, gene flow, mating systems and natural selection, which drive microevolution. In plant pathology, population genetics can provide explanations for evolutionary processes by addressing strategic questions related to pathosystems such as: How large the pathogen population is, what constitutes an epidemiological unit, what amount(s) of genetic variation exist in the population, what are the causes of genetic variation in the population, what control measures can be put in place to minimise rapid evolution of the pathogen and what is/are the anticipated response(s) to selection by the pathogen (Leung, Nelson & Leach, 1993; McDonald, 1997; Milgroom & Fry, 1997; McDonald & Linde, 2002). These questions are pertinent to GLS in Africa and the use of both epidemiology and population genetics could provide answers needed to control the disease.

Pathogen variability and epiphytotics

Diversity in pathogen populations

Sources of genetic variability

Successful breeding and deployment of resistant lines is dependent on understanding variability of pathogen populations. Populations are in fact, the basic units of evolution from which diversification needed to overcome host resistance is generated (Brown, 1995). In pathogen populations, microevolutionary processes drive resistance break down. Details of the factors that influence microevolution are well described elsewhere (Hartl & Clark, 1997). In this thesis only mention of their impacts on pathogen populations has been done. The factors include:

1. Mutation: Is a change in the DNA at a particular locus in an organism. Mutations are the ultimate source of new alleles in plant pathogen populations. Mutations are the primary source of new virulent pathotypes in pathogen populations especially under gene-for-gene relationships. In general, mutation rates are low and are conceivably of great importance in pathogens such as bacteria or viruses that occur in very large number in the same host (McDonald & Linde, 2002).

2. Genetic drift: Is a random process that can lead to unpredictable changes in pathogen populations over a short period of time. Genetic drift leads to fixation of alleles or genotypes in populations and therefore, tends to decrease overall levels of genetic variation (Hartl & Clark, 1997). Genetic drift is influenced by population size. Pathogen populations that undergo frequent reductions in size or bottlenecks will more rapidly experience the effects of genetic drift.

3. Gene flow: Is a process in which alleles (genes) or individuals (genotypes) are exchanged among geographically separated populations. Gene flow breaks down the boundaries that could otherwise isolate populations and promote fixation of different alleles in local populations. It is the average gene flow over generations that determine the extent to which dynamics of genes in different populations are independent (McDermont & McDonald, 1993). The impact of gene flow is greatest when asexual propagules or individuals (genotypes) are exchanged between populations. Such gene flow, involving individuals, introduces new selectively adapted alleles into new environments and is responsible for a number of epidemics that have plagued agriculture since the great potato famine of the 19th century (McDermont & McDonald, 1993; McDonald & Linde, 2002). In theory, the exchange of one individual or emigrant or more between populations per generation, is sufficient to prevent fixation of selectively adapted alleles in a population (Slatkin, 1987).

4. Mating system: Mating system affects the way that alleles are put together in each individual in a population. The mating system can be asexual, sexual or mixed and may range from strict inbreeding to obligate out-crossing. Mating system is relevant to sexual reproduction because of the central role of recombination in creation of new allelic combinations. Among fungi, occurrence of somatic recombination through processes such as heterokaryosis, accompanied by mitotic recombination and non-meiotic assortment of chromosomes by haplodization, during the parasexual cycle, may generate variation among fungi, which lack regular sexual recombination (Burdon, 1993; Burdon & Silk, 1997). Among the higher fungi, somatic recombination may be of particular importance when associated with episodic selection discussed in the next section (Braisier, 1995; Taylor, Jacobson & Fisher, 1999; Schardl & Craven, 2003). In general, pathogens that have both sexual and asexual reproduction benefit from the advantages inherent to both types of reproduction and pose a great threat to agricultural production.

5. Selection: This is a directional process that leads to an increase in frequency of selected alleles or genotypes in a population. Directional selection results in differential increases either in the frequencies of plant resistance alleles in natural ecosystems through coevolution, or increases in the frequencies of pathogen virulence alleles in agricultural ecosystems (Zhan, Kema & McDonald, 2004). Selection is more apparent in gene-for-gene pathosystems. In pathosystems that involve quantitative resistance, it appears that the effects of local selection are subtler, appearing as resistance erosion rather than total collapse, as is the case for qualitative resistance (McDonald & Linde, 2002). Furthermore, genotype-by-environment interactions compound detection of selection effects in pathosystems involving quantitative resistance. Wide distribution of selectively adapted alleles in pathogen populations may also further reduce detection of selection effects in pathosystems where quantitative resistance has been deployed. In agroecologies, episodic selection, simply defined as the effect of any sudden environmental disturbance that leads to a significant alteration in a species population structure is of particular importance. Episodic selection may be caused by disturbances such as exposure to new hosts, or vector, arrival of competitors, sudden climatological change or any of the named disturbances occurring due to geographical

translocation to new environments (Braiser, 1995, Braiser, Cooke & Duncan, 1999). There are some reports on the role of episodic selection in novel pathotype microevolution (Braiser, Cooke & Duncan, 1999; Schardl & Craven, 2003), its wider impact however remains to be investigated. Clonality especially among the ascomycetes may in fact be a strong indicator of direct effects of episodic selection especially where population bottlenecks occurred (Braiser, 1995; Taylor, Jacobson & Fisher, 1999).

Tools for studying genetic variability: neutral and selective markers

There are two basic types of genetic variation, ecologically important variation and selectively neutral variation. Ecologically important variation refers to traits that affect fitness and may therefore be under selection. Ecologically important traits e.g. fungicide sensitivity, have some advantages in population genetics. Firstly, they are under selection by disease control methods and thus, predictions on response(s) to selection by pathogens can be made directly, providing a test for durability of disease management practices (Milgroom & Fry, 1997). Secondly, ecologically important traits are usually amenable to integration with epidemiology and population genetics (Wolfe & McDermont, 1994; Brown, 1995; Milgroom & Fry, 1997). Population studies involving ecologically important traits may however be constrained by sample size limitations of the techniques, and the fact that the genes/traits under study may not represent genetic diversity of the entire population.

In contrast, selectively neutral variation refers to variation in traits that do not affect fitness and therefore are not under selection (Milgroom & Fry, 1997). Selectively neutral traits are affected by evolutionary forces such as; mutation, gene flow, genetic drift, mating system and selection, if linked to a selectable gene. Selectively neutral genetic markers similarly have a number of advantages. Their neutrality, permits usage in investigation of past and recent evolutionary processes in a population. They are easy to use, many samples can be handled per unit time, analyses can be combined with other techniques, when linked to gene loci of interest, they can be used in marker assisted breeding and are amenable to recent advances in genomics and bioinformatics. The most commonly used neutral traits are the DNA molecular markers discussed below.

Types of neutral molecular markers

Inference of phylogeny and other population genetic characteristics of a pathogen rely on presence of polymorphism that relates to evolutionary processes in a species. Neutral genetic markers satisfy these requirements and are thus widely used for these purposes. Today, there are a number of genome characterisation techniques based on selectively neutral DNA-based markers. Commonly used techniques include, random amplified polymorphic DNA (RAPD) (Williams *et al.*, 1990), restriction fragment length polymorphism (RFLP) (Bonierbale, Plaisted & Tanksley, 1988), amplified fragment length polymorphism (AFLP) (Vos *et al.*, 1995), inter simple sequence repeats (ISSR) (Pradeed-Reedy, Sarla & Siddiq, 2002), microsatellites, cleaved amplified polymorphic markers (CAPS) (Konieczny & Ausubel, 1993) and single nucleotide polymorphisms (SNPs)

(Brinkman & Leipe, 2001). Selectively neutral markers may also be regarded as dominant or co-dominant depending on ability to detect heterozygosity. These markers or techniques may also be used for DNA finger printing. Electrophoretic karyotyping using pulsed field gel electrophoresis (PFGE) is another useful genomic tool, especially for linkage analysis and genome characterisation (Mills & McCluskey, 1990; Zolan, 1995). Genetic variation can also be investigated through sequencing and analyses of specific genes for variation. For plant pathogens, the commonly sequenced regions are the internal transcribed spacer (ITS1 5.8S RNA gene ITS2) nuclear ribosomal DNA (rDNA), mitochondrial small sub-unit rDNA genes (mtSSU) (White *et al.*, 1990), β -tubulin gene (Skovgaard *et al.*, 2001), translation elongation factor 1 α (EF-1 α) coding region and introns (O'Donnell, Cigelnik & Nirenberg, 1998) among others. Sequencing of DNA/genes directly linked to a trait such as resistance or virulence could also be done and used in analyses.

Estimation approaches: systematics and population genetics

Genetic variation among pathogenic species is a product of evolutionary processes in population. As such, using evolutionary biology, one should be able to detect genetic variability. Evolutionary biology examines genetic variability using two interrelated sub-disciplines of population biology, that is, population genetics and systematics (phylogenetics) (Milgroom & Peever, 2003; McDonald, 2004). Systematics focuses on drawing phylogenetic inferences between species in the same or higher taxa based on morphological or genetic data. In plant pathology phylogenetics has been widely used to study speciation among and within species. Conversely, population genetics utilises information on genetic variation (gene and genotype frequencies) to elucidate the role(s) of evolutionary processes such as gene flow, genetic drift, selection, mutation and mating systems, usually within a species population at a micro-evolutionary scale. For most purposes, population genetics provides the most suitable tools to estimate genetic variability of pathogen populations. The success and accuracy of population genetics procedures however, depend on proper definition or description of what constitutes pathogen populations. In general, plant pathogens, especially fungi, may not satisfy the ecological definition that restricts population to members that can inter-breed. A more appropriate definition would be to consider a population as a pool of conspecific individuals from which the next generation will be drawn, as delineated in space and time by allelic boundaries (Milgroom & Fry, 1997). This definition provides for estimation of genetic variability on the basis of genetic structure, an attribute common among fungi. Genetic structure refers to the amount of genetic variation within and among populations. Defining genetic structure of populations is the first logical step to study fungal population, because genetic structure reflects the evolutionary history and potential to evolve (Leung, Nelson & Leach, 1993; McDonald, 1997; Milgroom & Fry, 1997). Population structure may be studied on the basis of multi-locus genetic diversity to make inference about reproductive biology or it may be performed on a spatial scale to understand genetic drift and gene flow (Milgroom, 1995). The information used to study genetic variability will however depend on the genetic markers used.

Experimental design and analytical tools

Sampling strategies

Statistical inference on any data set can only be meaningful if tenets of the analysis are upheld. In population genetics, the starting point is to sample well enough to capture genetic variation in a given population. A key question that must be addressed before a sample is taken is: “To what population will inferences be made.” Another critical question is, “What type of genetic marker will be used to study the variation?” Neutral genetic markers are the most commonly used tools today. Neutral genetic markers however, vary in proportion of the genome coverage and therefore, affect sample sizes that can be used in any study (Leung, Nelson & Leach, 1993). Consequently, it is vital that sample sizes should be of the magnitude that permits reliable estimation (Weir, 1996; Excoffier, 2004). In the absence of any knowledge about the population structure of a pathogen, the best strategy to employ is the hierarchical sampling strategy (Milgroom, 1995; McDonald, 1997). Hierarchical sampling defines the spatial scale that covers the dispersal distance of the pathogen. This is usually a good starting point for most pathogens.

Population structure: Analytical considerations

Population structure refers to the extent to which large populations are subdivided into smaller subpopulations that may differ in allele frequency from the neighbouring subpopulations. Population structure is a useful indicator of the amount of genetic variation between and within populations. Defining population structure is in fact, the first logical step in prediction of evolutionary responses of pathogen populations. However, since population structure is obtained by partitioning of genetic variation, inference of population structure is highly dependent upon the tools used to estimate genetic diversity. Different types of markers can yield different relationships among isolates from the same population (Leung, Nelson & Leach, 1993; McDonald & McDermont, 1994). In general, we gain more insight with techniques that have higher discriminating power. One of the consequences of population structure on an organism's population is the reduction of heterozygous individuals relative to the average proportion of heterozygous genotypes expected under random mating. Sewal Wright (1951), developed statistics for estimating reduction in heterozygosity or the inbreeding effect of population substructure at each population hierarchy, otherwise called Wright's F statistics or fixation index. Wright's F statistics measures the reduction in heterozygosity or increase in homozygosity expected with random mating at any level of a population hierarchy relative to another more inclusive level of the hierarchy (Wright, 1978). The genetic symbol for the fixation index is F embellished with subscripts, denoting levels of hierarchy being compared. The F statistics include: F_{IS} (estimates the reduction in heterozygosity in individuals relative to the sub-population from which they originate), F_{ST} (estimates the reduction in heterozygosity of a subpopulation relative to the total population from which they originate) and F_{IT} (estimates the reduction in heterozygosity of individuals relative to the total population from which they originate).

To measure the effects of population subdivision or population structure, F_{ST} also referred to as population fixation index, is most appropriate (Hartl & Clark, 1997). F_{ST} can be computed for both haploid and diploid data in a fixed or random model framework, depending on the objectives of the study. In general, Wright's F statistics has undergone several modifications aimed at improving the statistic. The modifications are based on diverse viewpoints of F_{ST} derived from Sewall Wright's original idea that F statistics represents correlation of genes within classes (intra-class) with respect to genes between classes (inter-class). Cockerham (1969, 1973) and Weir & Cockerham (1984), showed that one could decompose the total variance of gene frequencies into variance components associated with different subdivision levels within the framework of analysis of variation (ANOVA). Excoffier, Smouse & Quattro (1992), modified the earlier ANOVA framework (Cockerham, 1969, 1973; Weir & Cockerham, 1984; Long, 1986; Cockerham & Weir, 1993) to generate analysis of molecular variance (AMOVA) based on covariance components instead of computing "variance components" of the earlier methods. This approach takes into consideration the impact of mutation in generation of variation, is easily adapted to a number of molecular data types and does not require normality of distribution, a prerequisite for ANOVA (Excoffier, Smouse & Quattro, 1992; Excoffier, 2004; Rousset, 2004). 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. Statistical inference about F_{ST} as estimated using the AMOVA approach could be done through numerical re-sampling methods such as bootstrapping. AMOVA is commonly implemented in Arlequin (Schneider, Roessli & Excoffier, 2000). A second commonly used modification to Wright's F_{ST} , designated G_{ST} , is based on gene diversity (defined as the probability that two randomly chosen genes from a population are different (Nei, 1973, 1977)). This approach is particularly useful with exhaustively collected samples or when population sizes are highly unequal (Excoffier, 2004). Conversely, the intraclass correlation approaches such as AMOVA are particularly useful when the population sizes are small, supposed so, or is totally unknown (Excoffier, 2004), as is common with many plant pathogen. Further tests for population differentiation can be performed using exact test (Raymond & Rousset, 1995). The test is analogous to Fisher's exact test on a 2x2 contingency table, but extends to include all populations by genotypes (populations x genotypes) being tested. For haplotypic data, as is the case for most fungi, the tables are built from haplotype frequencies. The procedure tests for the probability of observing a table less likely or equally likely under panmixia.

Other measures of genetic variation in populations includes Nei's gene diversity or average heterozygosity at discrete loci (Nei, 1987). Gene diversity measures the probability of obtaining two different alleles at a locus when two haplotypes are sampled from a population (Nei, 1973, 1987). Gene diversity is especially suitable for inbred populations where there are very few heterozygotes, but there may be several different homozygotes (Weir, 1996). A gene diversity of 1 means the diversity is so high that any two alleles sampled from a locus are different, in converse to genetic uniformity were it would be 0 (Nei, 1973). Genotype diversity is another informative parameter of the genetic variability within population. In species reproducing both sexually and asexually, genotype

diversity can be used to estimate the relative extent of clonal contribution within a population, or between populations (Stoddart & Taylor, 1988). Thus both genotype diversity and gene diversity are indeed appropriate for measuring variability, especially among asexually inbred populations (Weir, 1996; McDonald, 1997). Additionally, on the basis of population structure, indirect or direct estimates of gene flow (Nm) between populations can be calculated (Slatkin & Barton, 1989; McDermond & McDonald, 1993). A caveat however, in Nm estimation and the interpretation thereto, is that non-zero Nm estimates do not always imply presence of migration, since the estimation procedure may generate an $Nm > 0$ even when no individuals are exchanged (Rousset, 2004). Gene flow can occur either by spore movement as in the case of long distance transport of asexual or sexual spores or by movement of individuals between different populations. Movement of individuals is generally more dangerous in terms of influencing population structure and epidemics than spore movement. This is because the exchange of individuals between two populations is equal to movement of selectively adapted alleles into new environments. This type of gene flow is also referred to as genotype flow and has been the major cause of some of the most severe plant disease epidemics (McDonald & Linde, 2002).

Phylogenetic analyses

Depending on the research question, data may also be subjected to molecular phylogeny. Molecular phylogeny refers to the study of evolutionary relationships among organisms (Felsenstein, 2004). Inferring phylogeny at best is an estimation procedure of the evolutionary history based on incomplete information contained in a given data set (Swafford *et al.*, 1996). Nevertheless, molecular phylogeny can be used to resolve questions related to population structure, analysis of mating systems, gene evolution, genetic relatedness, species boundaries and hybridisation (Hills, Moritz & Marble, 1996). Phylogeny can be estimated using two main approaches, that is, character-based and distance-based methods. Distance-based methods use the amount of dissimilarity between data sets to derive trees. In general, distance-based methods would reconstruct the right evolutionary tree if all genetic divergence events were accurately recorded in the sequence (Swofford *et al.*, 1996). Distance-based methods compute pair-wise distances or quantitative comparisons between two species or sequences. Thereafter, data from characters are discarded and only genetic distances used in subsequent analysis to derive trees (Felsenstein, 2004). This process improves speed and can recover the correct tree but is limited by the fact that the process utilizes information on mutation rate variability less efficiently than say likelihood methods (Felsenstein, 2004). Additionally, the fact that divergence encounters an upper limit when sequences become mutationally saturated reduces the likelihood of inferring realistic evolutionary phylogeny using distance-based methods (Brinkman & Leipe, 2001). Examples of distance-based methods include, neighbour joining (NJ), the unweighted pair-group method with arithmetic average (UPGMA) and methods that optimise additivity of distance trees such as minimum evolution (ME). Character-based methods derive trees that optimise the distribution of the actual data patterns for each sequence and tend to be computationally demanding. Tree distances are not fixed as they are determined by each tree topology. Examples of

character-based methods include maximum parsimony and maximum likelihood approaches. Swofford *et al.* (1996), Brinkman & Leipe (2001) and more recently Felsenstein (2004), provide excellent reviews of available methods. Sequences may also be analysed for base-pair changes and in that case, older populations with existing population structure will have accumulated more mutations and thus more base-pair differences.

Managing variable phytopathogens

Integrated disease management (IDM) refers to the use of available disease control methods, with a goal of improving yield, a cleaner environment and slow resistance erosion (Brent, 1995; Hamblin, 1995; Agrios, 1997). Increasing emphasis is today being placed on IDM as the most suitable disease strategy control for sustainable agriculture. IDM is conceivably, economically viable for resource-poor farmers, who are the majority in sub-Saharan Africa (Bentley, Castano-Zapata & Andrews, 1995; Nelson *et al.*, 2001). Agroecologies however, are never in total equilibrium, consequently, disease control measures alter the balance of evolutionary forces in a pathosystem, promoting pathotype evolution (Fig. 2). In Figure 2, crop epiphytotics result when epidemic pathogen populations develop from the effects of clonal speciation as driven by microevolution (Cycle 1). Once initiated, control measures, through directional selection, distort the population structure, triggering further clonal speciation (Cycle 2) (Brasier, 1995; Taylor, Jacobson & Fisher, 1999). During clonal speciation, directional selection, as influenced by evolutionary forces, leads to emergence of new selectively adapted pathotypes that induce new epidemics and the process starts all over again.

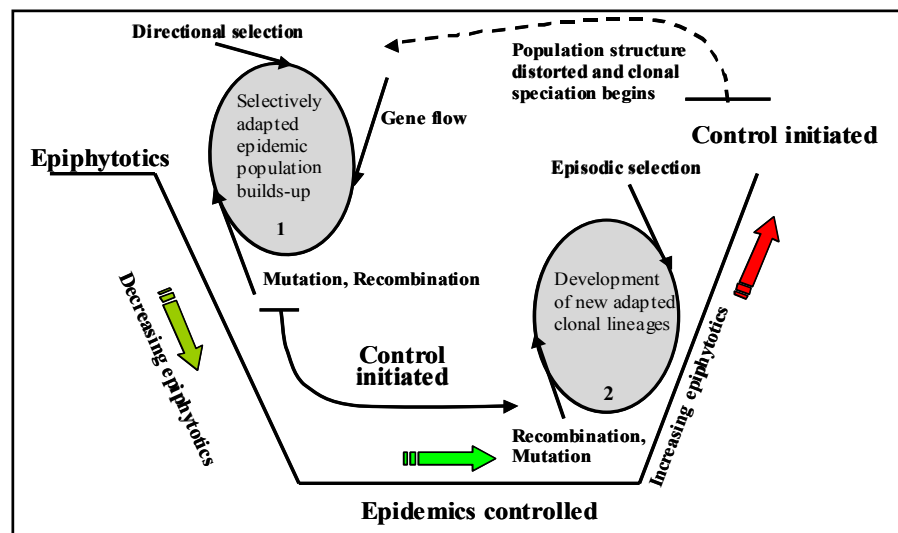


Figure 2. Schematic representation of evolutionary processes prevalent in agroecologies. Colour changes from light green to dark green and then finally to red refer to magnitudes of epiphytotics from low to high levels in the pathosystem.

This process is more pronounced in gene-for-gene pathosystems, but appears as resistance erosion in the case of quantitative resistance. The long-term success of IDM in agriculture when controlling variable pathogen will consequently, depend on usage of crop management practices in the right balance. To achieve this, the proper design of an IDM that leans on epidemiology and population genetics is necessary. These two disciplines of population biology will elucidate the rate determining components of disease epiphytotics and evolutionary potential of the pathogen, providing for appropriate design of control strategies. Population biology has been successfully used in North America to control stem rust of wheat (*Puccinia graminis* f.sp *tritici*) and other pathosystems (Agrios, 1997; Milgoom & Peever, 2003). Disease management in the 21st century will therefore have to depend on population biology, as we increasingly adopt monoculture systems.

For sub-Saharan Africa, the challenge today, is how to integrate data being generated from population genetics and epidemiological studies of various pathogens for effective IDM design. For most diseases in sub-Saharan Africa, there is paucity of information on pathogen populations, when compared to that available elsewhere. The challenge therefore is to begin conducting some of these studies in the region. In this way, we will shift disease management to well designed control strategies based on a wealth of information generated locally. Also importantly, though IDM is suggested as being holistic in design and application, very little is known of pathosystem interactions that may favour evolution of novel pathotypes under such management. There are very few studies on selection under different farming systems and consequently, lack of much needed information to formulate realistic hypotheses about pathotype evolution and more importantly, resistance breakdown (Ennos & McConnell, 1995). This is an area of research that plant pathologists need to move into if we are to strengthen the fight against plant pathogens, once described as “treacherous and shifty enemies” by E.C. Stakman. Overall, the integration of population studies in the design of crop disease management systems is absolutely necessary if we are to improve the durability of disease control strategies. This is essential in both asexual and sexually propagated fungi for the long-term success of any IDM.

Aims of the Study

Grey leaf spot of maize is a new disease in East Africa and considered a threat to production. Being a new disease in the region, a number of questions arise; (1) are East African *C. zae-maydis* populations introduced from elsewhere, (2) what is their population structure, (3) what could be the most active evolutionary force in the populations, (4) what is the potential for novel pathotype evolution and (5) what management methods can be used to slow down disease spread and novel pathotype evolution? The overall goal of this study was to identify and characterize individual and combined effects of components of the East African *C. zae-maydis* pathosystem as a first step towards development of an IDM for grey leaf spot.

Specific study aims

1. To investigate the population genetic variability of East African *C. zea-maydis* using neutral genetic markers.
2. To elucidate interactive effects of the GLS disease triangle components under tropical conditions.
3. To gain insights into the evolutionary potential of *C. zea-maydis* through comparative population studies of *C. sorghi*.
4. To investigate temporal variation in genetic variability of Ugandan *C. zea-maydis* populations in response to disease control.

Results and Discussions

This thesis reports studies of the East African *C. zea-maydis* pathosystem. The research is based on three population biology tools *i.e.* population genetics, phylogenetics and plant disease epidemiology. Investigations were based on fungal isolates collected from East and Southern Africa (Fig. 3). Implications of the results for broad applications in GLS control in Uganda and the region are discussed.

Genetic variability of East African *C. zea-maydis* populations as revealed by neutral genetic markers (I)

This study was conducted using two phases. In the first phases, population genetic variability of East African *C. zea-maydis* was examined using the neutral genetic markers, amplified fragment length polymorphism (AFLP) and restriction fragment length polymorphism (RFLP). In the second phase, phylogenetic analysis was performed to formulate a hypothesis on *C. zea-maydis* biogeography. Multi-gene genealogies have been used previously to study biogeography (O'Donnell & Cigelnik, 1997; O'Donnell, Cigelnik & Nirenberg, 1998). To study biogeography, sequence data from three genomic regions, *i.e.* the nuclear internal transcribed spacer region (ITS) ribosomal DNA (rDNA) (White *et al.*, 1990), mitochondrial small sub-unit (mtSSU) rDNA (White *et al.*, 1990) and mitochondrial Cytochrome b (CYT b or *cob*) (Yokoyama *et al.*, 2000) were used. The nuclear and mitochondrial rDNA genes and intergenic regions were chosen because their evolutionary properties permit efficient phylogenetic inference (Jorgensen & Cluster, 1988; White *et al.*, 1990; Berbee & Taylor, 2001).

For population genetic analysis, samples from Uganda, Kenya, Rwanda and Zimbabwe were used. Genetic variability of *C. zea-maydis* was investigated by testing for genetic structure and other population genetic attributes based on AFLP and RFLP data. Two levels of hierarchy in genetic structure were tested *i.e.* at continent level (America and African or at haplotype level (Type I and Type II).

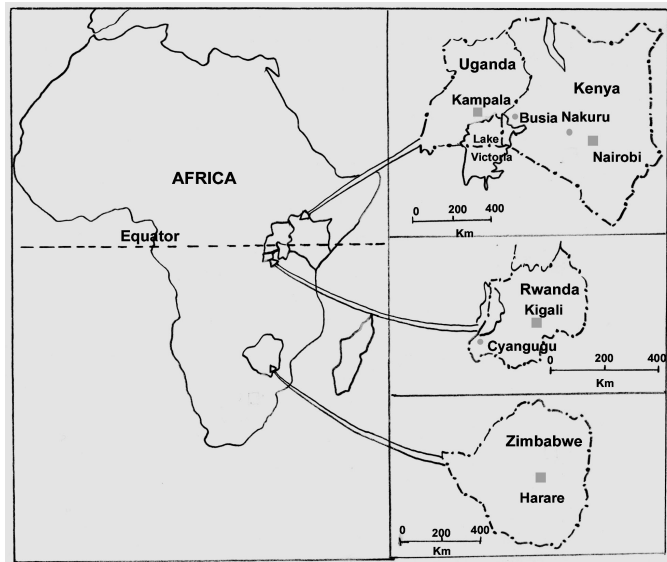


Figure 3. Map showing East and Southern African countries (Uganda, Kenya, Rwanda and Zimbabwe) from where *Cercospora zae-maydis* or *Cercospora sorghi* isolates used in the studies reported in this thesis were obtained.

A weak population structure was detected at continent level hierarchy using either AFLP or RFLP (Table 1). In both cases, most of the variation was attributed to within population rather than between population differences. It is interesting to note that for RFLP data, negative variance components were obtained. As discussed by Excoffier (2004) and Rousset (2004), AMOVA estimates F_{ST} in form of covariance that can be negative, as opposed to true variance components, which are positive, and are derived from decomposition of sum of variances. Negative variance components in the case of asexually reproducing organisms, such as *C. zae-maydis* suggest high levels of gene flow between populations and in the case of sexually reproducing organisms, it indicates tendency to outbreed (Excoffier, 2004; Rousset, 2004). In this study, gene flow estimates were very high providing support for this theory. The highest levels of gene flow estimates were between Ugandan and Rwandan populations. The shortest distance between two study sites in both countries was over 700 km. This may implicate humans in GLS dissemination. Concordance of the two neutral genetic markers (RFLP & AFLP) provides support for gene flow as discussed in paper I. In some instances, populations that may not have experienced gene flow can indeed have non-zero Nm values (Rousset, 2004). Accordingly, these results may be indicative of extensive GLS epiphytotic, attributed to epidemiological *C. zae-maydis* populations, exposed to large populations of susceptible genotypes in the region. Human efforts in consonance with other factors may then have played a crucial role in disease spread, hence concordance of the data generated by AFLP and RFLP.

Table 1. Summary of analysis of molecular variance of 80 *Cercospora zeaemaydis* isolates based on 276 AFLP and 670 polymorphic RFLP DNA fragments

| Source of variation | Variance component | ^a Φ _{ST} | Percentage variation |
|--|--------------------|------------------------------|----------------------|
| AFLP data | | | |
| Grouped as African and United States isolates | | | |
| Among groups | 0.67 | 0.07* | 2.66 |
| Among population within groups | 1.18 | | 4.67 |
| Within populations | 23.40 | | 92.68 |
| Classified as Group I and II | | | |
| Among groups | 4.98 | 0.19* | 17.16 |
| Among populations within groups | 0.62 | | 2.14 |
| Within populations | 23.39 | | 80.69 |
| RFLP data | | | |
| Grouped as African and United States isolates | | | |
| Among groups | -5.73 | 0.01* | - |
| Among population within groups | 6.51 | | 5.73 |
| Within populations | 112.98 | | 99.32 |

^a Φ_{ST} computed according to Excoffier, Smouse & Quattro (1992).

*Significant at (P≤0.05) as tested by 1051 permutations in Arlequin (Schneider, Roessli & Excoffier, 2000).

Phylogenetic analysis were performed using PAUP*4.0b10 (Swafford, 1998). The study involved three genomic regions and it was thus of interest to test if they inferred similar evolutionary history. Combinability of the data was tested using partition homogeneity test (PHT). PHT tests whether trees from two or more data sets are significantly different (Felsenstein, 2004). For PHT, all parsimony uninformative data were excluded from analysis. Parsimony analyses consisted of heuristic searches with 1000 random addition sequences and branch swapping option that employs the tree bisection-reconnection (TBR) algorithm. Maximum trees was set to 5000 and 1000 random replications were performed using bootstrap analysis. PHT revealed significant differences in tree topologies for ITS, mtSSU, CYT b (*cob*) (P = 0.01) leading to non-combination of the data sets. Pair-wise PHT analyses of the data sets revealed that only SSU and CYT b (*cob*) could be combined (P = 0.16). ITS data revealed monophyly of isolates. The *Cercospora sorghi* isolates (CS14, CS24 and CS14) used in the study, were distinct from *C. zeaemaydis* (Fig. 4). The phylogenetic tree generated from the combined data set (mtSSU and CYT b (*cob*), had different topology but with monophyly suggested and clear species distinction. The American *C. zeaemaydis* isolates IL2, In24 and NY2 (characterized as Type I (IL2, In24) and Type II (NY2) (Wang, Levy & Dunkle, 1998) were more related to each other, although, NY2 was indeed related to East African *C. zeaemaydis*.

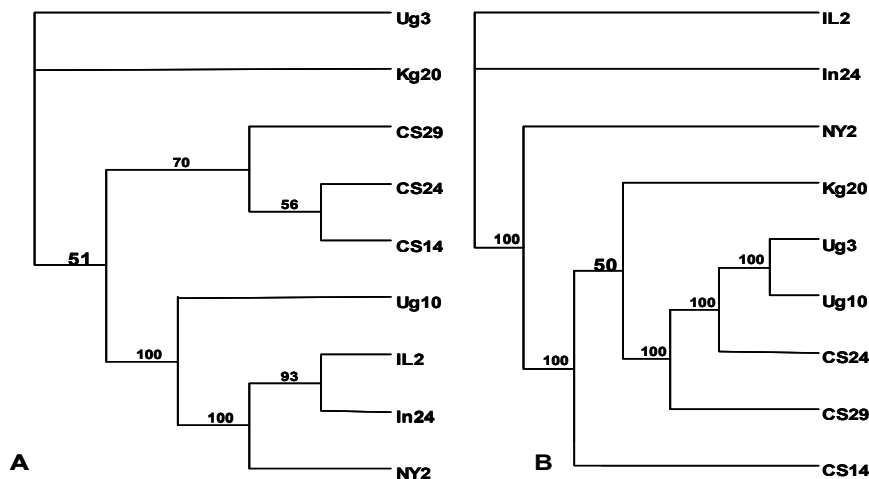


Figure 4. Phylogenetic analysis of *Cercospora zae-maydis* (Type: I IL2, In24 and Type II: NY2, Ug3, Ug 10, Kg20) and *Cercospora sorghi* (CS14, CS24, CS29), based on ITS, mtSSU and CYT b (*cob*) sequences. (A) The most parsimonious tree based on ITS (550bp sequence), Consistency index (CI) = 0.80, Retention index (RI) = 0.82. (B) Phylogenetic tree based on combined mtSSU and CYT b sequences (501, 433 bp respectively), CI = 0.80, RI = 0.80. Bootstrap support for major nodes of bifurcations have been emphasised by larger font type-face.

Among *Phytophthora*, similar observations based on nuclear and mitochondrial genes, has been attributed to rapid evolution in the two genomes (Kroon *et al.*, 2004). Due to limited distribution in the USA, Africa has been proposed as the source of Type II *C. zae-maydis* (Dunkle & Levy 2000). One explanation for this theory is that Africa is the centre of origin for sorghums and millets, thus a likely source of *C. sorghi*, a close relative of Type II *C. zae-maydis*. The multi-gene genealogical data suggests a common origin of *C. zae-maydis*. But, do not indicate that *C. sorghi* is the progenitor of Type II *C. zae-maydis* as shown by species distinction. Since the two *C. zae-maydis* sibling species are sympatric in the US, it is logical to suppose a common origin. The question of Africa being the source of Type II is compounded by reports that by 1886, *C. sorghi* had been isolated from various grass species in the US (Chupp, 1953) and by 1925, *C. zae-maydis* had been reported (Tehon & Daniels, 1925). Of the three genomic regions studied, the ITS is most useful for interspecies studies (Berbee & Taylor, 2001). The ITS data supports a species distinction among these fungi (this study) and by others (Goodwin, Dunkle & Zismann, 2002). The mitochondrial genes similarly support species distinction, although the data suggests extensive heterogeneity perhaps due to recombination (Berbee & Taylor, 2001; Zhan, Kema & McDonald, 2004) or horizontal gene transfer (Walton, 2000). These preliminary data therefore suggest a common origin of *C. zae-maydis*. Biogeography of this pathogen could be further studied using: more genes, sequence analysis and tracking mutations, and investigation of gene flow and phylogeny using networks or maximum

likelihood based methods. On the whole, this study has described for the first time African populations of *C. zea-maydis* and also attempted to provide insights into possible *C. zea-maydis* biogeography. Since epiphytotics are a result of interactions of disease pyramid components (Zadocks & Schein, 1979; Agrios, 1997), elucidation of these interactions is worthwhile and is the focus of the next study.

Interactive effects of grey leaf spot disease triangle components under tropical conditions of Uganda (II)

Grey leaf spot epidemics appears to be more severe in Africa than the US (Ward *et al.*, 1999; Asea *et al.*, 2002). Moreover the disease appears to have spread faster in Africa than in the US where it took over 50 years before reaching epidemic proportions (Latterell & Rossi, 1983; Ward *et al.*, 1999). The biological meaning of these differences in epiphytotics between the US and Africa is not known. The GLS disease pyramid has been studied extensively in the US (Ward *et al.*, 1999), but only limited studies have been conducted in sub-Saharan Africa. Elucidation of the GLS disease pyramid could reveal the most critical elements accounting for the current epidemics (Fig. 5). This information can in turn, be used to improve or design durable GLS management programmes. At the initiation of this study it was necessary to investigate the role(s) of mineral nutrition, pathogen variability and host interactions in the current GLS epidemics. These three comprise the three planes of the GLS disease triangle as impacted upon by man to form the pyramid (Fig. 5). Study I in this thesis had shown that African populations of *C. zea-maydis* were variable and that gene flow may account for long distance spread of the disease. The role(s) however, of host resistance and mineral nutrition in the current epiphytotics in Uganda and the region were largely unknown.

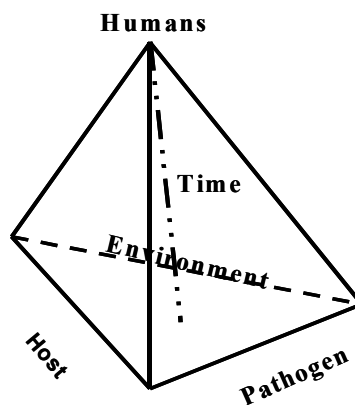


Figure 5. Schematic diagram of the disease pyramid, showing interrelationships of factors involved in epiphytotics. For GLS, host factors = maize variety resistance status; Pathogen factors = *C. zea-maydis* pathotype, (Type I or Type II and within-pathotype variability); Environment = soil fertility and general farming environment. The pyramid considers how all these factors are influenced in time by human activities (Zadocks & Schein, 1979).

Elsewhere, reports on the role of mineral nutrition are inconsistent. In the US Smith (1989), reported increased GLS severity with nitrogen application, whereas Carrera and Grybauskas (1992) found no association between nitrogen application and GLS severity. In South Africa, Ward (1996) reported increased GLS severity on nitrogen application and no effect by potassium. In general, African soils are heavily leached and have severe nitrogen and phosphorus deficiency (Pingali & Pandey, 2001). It was thus conceivable to hypothesize that poor mineral nutrition accounted in part, for the current GLS epiphytotics in Africa, compared to US where fertilizer application is common. It was also of interest to study the role(s) of the second disease triangle component, pathogen related factors, in the current epiphytotics. For this aspect, the objective was to elucidate the role of pathogen variability on epidemics. This was done by examining parasitic fitness of the fungus. Parasitic fitness refers to the relative ability of a parasitic genotype or population to persist successfully over time (Nelson, 1979). Parasitic fitness is related to epidemiological fitness, which refers to the epidemiological competence of a pathogen genotype relative to another. Pathogen genotypes with higher parasitic fitness in general, have high epidemiological fitness and thus induce high amounts of disease within a short time (Nelson, 1979). Parasitic fitness may be measured variously, but in this study, disease efficiency (amount of disease that develops from a given genotype of a pathogen) was used. In the US, a possible role of *C. zea-maydis* variability in parasitic fitness has been reported (Bair & Ayers, 1985; Carson, Goodman & Williamson, 2002). The host factor was also crucial in all these trials. The goal of this study was therefore to elucidate the interactive effects of biotic (host and pathogen) and biophysical (mineral nutrition) factors in the current GLS epiphytotics under Ugandan conditions. Experiments were conducted at two GLS hot spots of Uganda (Masaka District Farm Institute, Kameyamigo and Makerere University Agricultural Research Institute, Kabanyolo). Controlled experiments were also conducted in the greenhouse and growth chambers. The results showed that isolates varied in parasitic fitness. No infection pattern however, could be attributed to specific isolates. Under both field and controlled conditions sporulation efficiency, an index of parasitic fitness, was influenced by host resistance. Disease efficiency was similarly influenced by host resistance and mineral nutrition as indicated by slower disease progress in fertilized plots compared to untreated plots (Fig. 6). Details of the results are presented in paper II of this study. Overall, these results show that in Uganda, the current GLS epidemics may be attributed to presence of large populations of susceptible maize being cultivated on infertile soils and the interaction of these factors in turn, favour disease development. Large populations of susceptible hosts may enhance rapid disease spread by supporting survival of variant *C. zea-maydis* clones. Given that *C. zea-maydis* is a poor base competitor in soil surfaces (Ward *et al.*, 1999), and being a deuteromycete, it is conceivable to surmise that deployment of resistance and other management options will be effective against the fungus in the long run. There is paucity of knowledge on the long-term impact of disease management on GLS especially in the tropics and those aspects are the focus of the next two studies of this thesis.

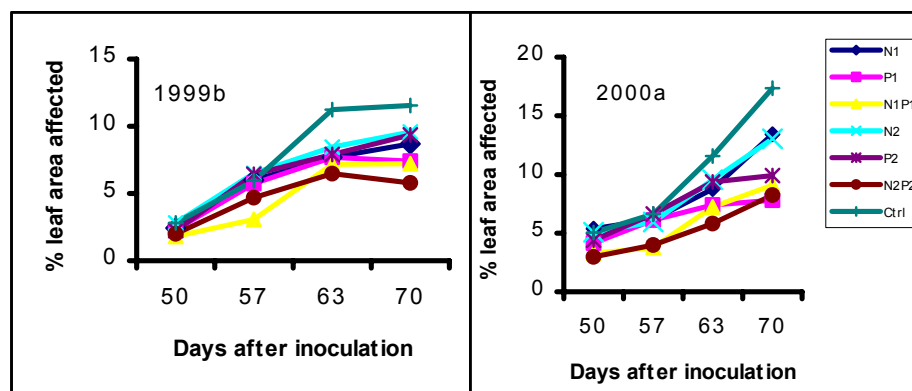


Figure 6. Disease progress curves GLS on maize subjected to various nutrient augmentation treatments, at Makerere University Agricultural Research Institute Kabanyolo (MUARIK) during 1999b crop growing season and 2000a crop growing seasons. Data is based on treatment means of various nutrient combinations applied. (For details see Paper II).

Population studies of Ugandan *C. sorghi* populations (III)

Sorghum (*Sorghum bicolor* (L.) Moench), an important staple cereal in many parts of Africa, was presumably domesticated in Eastern Africa (Hankook, 1992). It is therefore conceivable that both crop and pests/pathogens have coevolved for a long time in the region. Sorghum and maize are cross-attacked by several pests and diseases such turicum leaf blight (TLB) (*Exserohilum turcicum* Pass. Leonard & Suggs), grey leaf spot (GLS) (*Cercospora sorghi* var *maydis*, Ell. & Ev), downy mildew (*Peronoscleropora sorghi* (Western & Uppal) Shaw), stem borers (*Busseola fusca* Fuller (Lepidoptera: Noctuidea) and *Chilo partellus* (Swinhoe) Lepidoptera: Crambidae) (DeVries & Toeniessen, 2001; Gitau *et al.*, 2002). In theory, sorghum could provide an excellent experimental system to study evolutionary processes of crop-pathogen interactions that may also apply to maize. In the first two studies of this thesis, the focus was to understand the interactive roles of GLS disease triangle components in the current epidemics. In this section, the study goal was to infer evolutionary responsiveness of *C. sorghi*, for comparison with *C. zea-maydis*, in order to elucidate pathotype-host evolutionary responses of *C. zea-maydis*.

Cercospora sorghi the cause of GLS in sorghum is widely distributed in sub-Saharan Africa (Odvody, 1986; Doggett & Prasada Rao, 1995). Epidemics of GLS in sorghum are sporadic but in some cases widespread (Emechebe, 1975; Mbwaga, 1993; Thomas, 1991). Epidemics of GLS in maize is remarkably similar to that of sorghum, being also sporadic and widespread (Ward *et al.*, 1999). The factors accounting for the pattern of GLS epidemics in sorghum are not known. However, it has been suggested that genetic variability of *C. sorghi* may influence epiphytoics, but there is paucity of information to that effect (Duncan & De

Millano, 1995). In GLS of maize, severity and incidence has been partially attributed to pathogen variability (Bair & Ayers, 1986; Carson, Goodman & Williamson, 2002). By combining population genetics and epidemiology it should be possible to investigate the role of genetic variability in epiphytotics. This however, requires a two-stage process whereby, the genetic variability of the pathogen is first investigated, followed by utilization of variant pathotypes to study epidemic patterns. This study is a report on the first stage of investigations in which the role of pathotype variability in GLS of sorghum was studied. Infested leaf samples were collected from all possible sources of the pathogen such as cultivated sorghum and its wild progenitors *Sorghum bicolor* var. *verticilliflorum* (Steud.), *S. bicolor* var. *arundinacium*, and *S. halapense* (Chupp, 1953, Doggett & Prasada Rao, 1995). The isolates from wild relatives were included to investigate their possible role in GLS epiphytotics on sorghum. It was hypothesized that genetic analysis of *C. sorghi* collected from wild and cultivated sorghum may provide a thesis for diversity and overall genetic structure of the pathogen that may also apply to *C. zae-maydis*. Due to the monophyletic nature of *Cercospora* fungi (Goodwin, Dunkle & Zismann, 2001), other *Cercospora* species were included for comparative purposes. The ITS and mtSSU rDNA and AFLP were used for the study. AFLP data showed absence of genetic structure, which was attributed to gene flow in these populations. Polymerase Chain Reaction-Restriction Fragment length Polymorphism (PCR-RFLP) of the ITS and mtSSU revealed conspecificity of all *C. sorghi*, suggestive of a potential role of wild grasses in GLS epiphytotics of sorghum. The data also provided evidence for host-species driven speciation among *Cercospora* fungi as previously proposed (Goodwin, Dunkle & Zismann, 2001). This study therefore provides evidence for limited genetic variability of *C. sorghi*. The findings are similar to those in study I of this thesis and would therefore suggest that both pathogens, in theory, have slow evolutionary responsiveness. From a disease management point of view this suggests that resistance is expected to be durable once deployed against these fungi (McDonald & Linde, 2002). Further details and implications of this study are presented in paper III of this thesis.

Temporal variation in genetic variability of Ugandan *C. zae-maydis* populations (IV)

Study I of this thesis, showed that Type II *C. zae-maydis*, the predominant haplotype in Africa is genetically variable. Study II showed that genetic variability of the pathogen may partially account for epiphytotics. Study III, demonstrated that *C. sorghi* and *C. zae-maydis* had similar population structure and caused cognate epidemics. This implied that though new in the region, the overall population genetic attributes of *C. zae-maydis* were expected to be similar to *C. sorghi*. The results also showed that among *C. zae-maydis* and *C. sorghi*, genetic variation was attributed to unique clones/individuals in populations rather than between population differences. It was therefore of interest to investigate temporal changes in genetic variability of *C. zae-maydis*, the key pathogen being investigated in this thesis.

The rationale for this study stems from the fact that phytopathogens are viewed as natural ecology sub-systems or pathosystems (Robinson, 1987), and reflect stabilised, co-evolved, interacting components of ecologies (Gliessman, 1995). Agroecologies on the other hand, are purposely-altered natural ecosystems, established for agricultural production (Gliessman, 1995). Such alterations invariably disrupt ecosystem equilibrium and often manifest as disease or pest epidemics. By studying temporal dynamics in population structure, one is able to deduce evolutionary responsiveness and hence, predict durability of resistance being used (Zhan, Mundt & McDonald, 2001). Grey leaf spot is a new disease in sub-Saharan Africa and efforts to curtail epidemics are being implemented. It was therefore logical to anticipate evolutionary response by *C. zae-maydis* to disease control measures being implemented.

Populations of *C. zae-maydis* were collected from 2001 to 2003 and analysed for genetic variability using fluorescent AFLP. The data were analysed using various population genetic tools. Details of the methods are presented in papers I, III and IV. The results indicated little or no genetic differentiation ($\Phi_{ST} \leq 0.05$) for populations from the same location over the study period. Between locations, the data suggests small effects of local selection in the populations ($\Phi_{ST} 0.09$, $P = 0.01$). Gene diversity and genetic distance estimates similarly indicated limited population differentiation. Pair-wise comparisons using Φ_{ST} , gene diversity and genetic distance, showed a reduction in genetic diversity in younger populations, suggestive of minor effects of selection and genetic drift. In general, the data suggest that micro-evolutionary processes at the study sites, in a subtle manner, were promoting genetic homogeneity of populations. These analyses imply that Ugandan *C. zae-maydis* populations at the two study sites were stable over the study period. The pathogen *C. zae-maydis*, like other necrotrophic fungi, undergoes cyclic annual demographic changes that impact on the evolutionary forces operative in pathogen populations. Our data shows that the net result of such changes, with regard to overall change in population genetic attributes, as inferred from neutral genetic markers, is slow with subtle changes perhaps prevalent. The fluctuations in Ugandan *C. zae-maydis* population genetic attributes may also be indicative of the possible role(s) of other evolutionary processes that influence variation in fungal pathogens. The overall impact of such evolutionary processes however, appears not to markedly influence population variability, at least, during this study period. Nevertheless, to make definite inference about responses to selection, long-term experiments of 5 to 10 year periods may be worthwhile.

Conclusions

This thesis presents four studies that utilized tools of population biology to elucidate the *C. zae-maydis* pathosystem in Uganda and Eastern Africa in general. These studies, some of which are pioneering in the region, provide useful data for improved management of GLS. Overall, the following findings of this thesis will be central for durable management of GLS in Uganda and Eastern Africa.

1. The region can be generally considered as one epidemiological unit. This implies that disease control regimen and resistance sources may be effective at various locations in the region. As such, concerted disease management and breeding efforts at regional level makes sense and should be promoted.
2. The role of pathogen variability in epidemic patterns is subtle, being more marked in some locations than others. This may be indicative of strong environmental influences on infection process and inoculum accumulation. It may also indicate environmental influence on GLS resistance, which is reportedly quantitative and prone to such interactions. Multi-location trials may improve selection for stable genotypes across agroecologies of the region.
3. For breeders, the data suggest that disease resistance is expected to last long due to low evolutionary potential of *C. zea-maydis*. However, other evidence from these studies, suggest prevalence of extensive gene flow in *C. zea-maydis* populations. Extensive gene flow may increase the effective population size of the pathogen and thus potential to evolve new pathotypes. Regional efforts to abate epidemics through quarantine and maize genotype rotation by breeders and farmers may therefore reduce the evolutionary-risk posed by the pathogen. It is however important to note that due to large genotype-by-environment interactions associated with GLS resistance, screening of introduced materials under various environments is worthwhile.
4. From a disease management point of view, the data suggest that *C. zea-maydis* epidemiological efficiency in the tropics is influenced extensively by host factors such as susceptibility and poor mineral nutrition. As such, deployment of resistant lines in combination with appropriate mineral nutrition and other cultural practices are suggested strategies that could reduce inoculum pressure, especially for resource-constrained farmers of East Africa.

Future Perspectives

Today, GLS is prevalent in most maize growing regions of Africa and has been classified as a key threat to increased grain production in sub-Saharan Africa. It is my considered opinion the data presented in this thesis provide at least reasonable information upon which suitable control strategies may be designed. But like all other studies, there is still a lot that we do not know and include the following.

Fluctuations in GLS epidemic patterns sometimes do not correlate well with predictions. Sometimes, epidemics do not occur even when the weather is conducive. There are no proper explanations for such occurrences although inoculum density is reportedly a key factor but the role of this in pathogenesis is unknown.

Akin to the question of genetic variability and pathogenesis is the role of the perylnequinone toxin cercosporin, implicated in pathogenesis among *Cercospora* fungi (Daub & Ehrenshaft, 2001). *C. zea-maydis* Type II generally do not produce cercosporin, while Type I produce. Interestingly, pathogenesis is similar

for both haplotypes of *C. zea-maydis*. Using thin layer chromatography we have observed that Type II produce trace amounts of cercosporin. When infected lesions were cultured on potato dextrose agar, we observed that Type II produce a pink reddish pigmentation, which stops with subsequent sub-culture. Cercosporin is reddish to pink in colour. This would suggest that Type II *C. zea-maydis* perhaps use complex host recognition systems to regulate cercosporin production. Other workers have reported reduced cercosporin production in rich nutrient media for Type I *C. zea-maydis* (Shim & Dunkle, 2003) and in *C. kikuchii* (Ehrenschaft & Upchurch, 1993). Recent studies show that cercosporin biosynthesis in *C. zea-maydis* is regulated by mitogen activated protein (MAP) kinase dependent pathways (Shim & Dunkle, 2003). MAP kinases are involved in various plant metabolic pathways, where they link stimuli that are activated by external sensors to cellular processes (Jonak *et al.*, 2002). This recent report is the first step to understand pathogenesis of *C. zea-maydis*, but clearly a lot more remains unknown.

Among cereals, resistance remains the most economical control strategy for improving production. Central to cereal improvement is the development of marker assisted breeding systems (MAS). The success of MAS nevertheless, depends on availability and reliability of genetic markers (Peleman & van der Voort, 2003). One source of genetic markers for MAS is quantitative trait loci (QTL), mapped for various traits including GLS (Bubeck *et al.*, 1993; Saghai Maroof *et al.*, 1996; Clements, Dudley & White, 2000). QTLs however may be unreliable and depend on how well the mapping work was done (Kearsey & Farquhar, 1998). Comparative genomics provides an alternative for gene discovery for MAS, by using well-characterised plant genomes. Markers developed on the basis of known genes are reliable and could easily be adopted for MAS (Peleman & van der Voort, 2003). Now that the Maize Genomics Consortium has begun to sequence the maize genome, comparative genomics could play a crucial role in understanding various physiological phenomena and on the basis of synteny, lead to identification of useful markers for use in MAS.

Lastly, effective long-term control will depend on a clear understanding of the *C. zea-maydis* biogeography. The tools used in this study only provide insights and are heavily constrained by numbers and the inference that can be made. If it is true that the disease was introduced into Africa from the US and spread northwards in sub-Saharan Africa, extensive studies could perhaps reveal this in the region. Undertaking such a study remains desirable because it will provide answers to the fourth element of the GLS disease pyramid, role of humans in disease spread. Up till now, all we have made are indirect deductions, but such a study will unravel more.

These four areas in my opinion are the current research challenges. Any attempts to address these issues is thus of utmost importance. I would like to end by quoting the great Physicist Maria Sklodowska-Curie who once said ***“in any piece of work, one never notices what has been done, one can only see what remains to be done.”***

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