Molecular diversity of the maize pathogen *Cercospora zeina* in South Africa

by

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1 Introduction

Cercospora zeina is the causal agent of a foliar disease of maize called grey leaf spot (GLS) in southern Africa (Meisel et al, 2009). Maize is the most important food crop in sub-Saharan Africa and the Americas. Consequently *C. zeina* is of great importance as a crop yield limiting pathogen and a threat to the future of food production (Latterell and Rossi, 1980; Meisel et al, 2009; Nowell et al, 1999; Nowell et al, 1998).

With the development of plant pathology as a discipline, it is important for plant pathologists to develop sufficient control strategies to prevent or limit the effects of a pathogen, such as *Cercospora zeina* (Agrios, 2005). This is performed by adequately understanding the infection process and biology of the pathogen. In 1944, Elvin Stakman described *Puccinia graminis* as a "shifty enemy" due to its ability to evolve from one season to another and continually overcome wheat defence mechanisms (Stakman, 1944). Subsequently understanding the population genetics of pathogens enables us to make deductions regarding population structure, origin, spread and evolution, providing valuable information for the development of effective control strategies (McDermott and McDonald, 1993; McDonald, 1997).

The first step in understanding the evolution of a pathogen population is to define the genetic structure of a population. The genetic structure of a population describes the genetic variation amongst individuals in a population (McDonald, 1997). Once the genetic structure of the pathogen population has been determined, it is possible to infer the origin, spread and mode of evolution. To determine the genetic variation within a population, molecular techniques that extract information from the DNA of an organism are used, such as genetic markers. Thus in

order to determine the population structure of *C. zeina*, genetically inferable markers and suitable samples need to be obtained.

1.1 *Cercospora zeina*, history and mystery

The collective pathosystem of any disease is of huge interest to agriculture, industry and the economy. Maize (*Zea mays*) is a crop of great importance globally as it serves as a staple diet for human and livestock consumption, and is a primary resource for bio-fuel production (Kunzemann, 2008). In South Africa, maize is important for local consumption but is also exported to countries such as Japan, Iran, Kenya and Venezuela (National Department of Agriculture, 2014).

Certain species in the genus *Cercospora sp*. are destructive fungal plant pathogens that can severely reduce the yield of a crop, which in turn threatens food security (Goodwin et al, 2001; Nowell et al, 1998; Nowell et al, 1999). *Cercospora zeina*, for example is the causal agent of GLS and can cause up to 65% yield loss (Kinyua et al, 2008; Kinyua et al, 2010; Meisel et al, 2009; Nowell et al, 1998; Nowell et al, 1999; Ward et al, 1998).

In order to meet the demand for maize in South Africa, plant pathologists and breeders aim at minimising yield loss due to GLS by understanding the plant-pathogen system. This is critical in order to make an informed decision pertaining to a viable and effective control strategy, particularly when faced with challenges exacerbated by climate change (Jones and Thornton, 2003).

1.1.1 Brief history into the discovery of grey leaf spot

In 1925 in Illinois, Tehon and Daniels collected maize material displaying GLS symptoms. They classified the causal agent of GLS in the United States as *Cercospora zeae-maydis* (Tehon and Daniels, 1925). This disease spread south to Brazil where it was characterised in 1934 (Brunelli

et al, 2008). The spread of this disease soon became of great economic importance in the 1970's as it caused significant yield loss of maize in the United States, Mexico, Costa Rica, Colombia, Venezuela, Trinidad, Peru, Brazil and Argentina (Latterell and Rossi, 1980). The movement from North America to South America was an important step in evaluating the potential spread and impact of this disease, and its global threat was realised in 1988 when GLS crossed the Atlantic to Natal, South Africa (Latterell and Rossi, 1980; Ward et al, 1997). By 2010, GLS had spread throughout the Americas, southern and eastern Africa, parts of eastern Europe, Asia and the Pacific Islands (Latterell and Rossi, 1980; Ward et al, 1998; Dunkle and Levy, 2000; Zhu et al, 2002; Okori et al, 2004; Meisel et al, 2009; Liu and Xu, 2013).

With the advance in molecular and DNA technologies, it was discovered that there were three distinct causal agents of GLS on maize; *Cercospora zeae-maydis*, *Cercospora zeina* and *Cercospora sorghi var. maydis* (Groenewald et al, 2006; Carson and Goodman, 2006). Although all three causal agents have been documented in North America, only one (*Cercospora zeina*) has been recorded in Africa (Meisel et al, 2009). Furthermore, both *C. zeina* and *C. zeae-maydis* have been established as the causal agents of GLS in Brazil, Argentina and China (Brunelli et al, 2008; Liu and Xu, 2013).

1.1.2 Disease epidemiology

GLS on maize is predominantly caused by *Cercospora zeae-maydis* and *Cercospora zeina* (Crous et al, 2006). The disease is characterised by the visible sporulation phase of grey rectangular lesions, confined by the parallel vein network of the vascular system of the maize plant as seen in Figure 1A (Ward et al, 1999). These rectangular lesions may coalesce and cause whole leaf blighting as seen in Figure 1B (Ward et al, 1999). The damage caused to the foliar material substantially reduces the maize plants' ability to photosynthesise, and subsequently results in its inability to store sugars in the fruiting body (maize cob) resulting in yield loss (Kinyua et al, 2010; Nowell et al, 1998; Nowell et al, 1999; Ward et al, 1999).

Figure 1. Grey leaf spot (GLS) symptoms on maize leaves. A. Visual symptoms of GLS as confined by the parallel veins of the maize leaf, (Mpumalanga 2012, photo: Mischa Muller). B. GLS infection visualised by the grey rectangular lesions that have coalesced causing whole leaf blight, (Kwa-Zulu Natal 2013 photo: Miekie Haasbroek).

The infection progress for *Cercospora zeae-maydis* has been well characterised (Beckman and Payne, 1982). As *Cercospora zeina* is morphologically similar and causes the same disease, the infection process is thought to be similar to *Cercospora zeae-maydis*. Inoculum (conidia) initiates the infection process and germinates on the maize leaf under high humidity (Beckman and Payne, 1982). After germination, a germ tube is formed which initiates appressorial formation over the stomata through which the fungus penetrates into the leaf (Kim et al 2011; Beckman and Payne, 1982). Shortly after penetration, chlorotic dots appear on the maize leaves followed by the formation of rectangular discolouration that leads to a necrotic lesion, see Figure 2A. The dark melanised fungal conidiophores then emerge through the stomata and bear white, acicular, hyaline and septate conidia at their ends as seen in Figure 2B and C (Beckman and Payne, 1982; Beckman and Payne, 1983

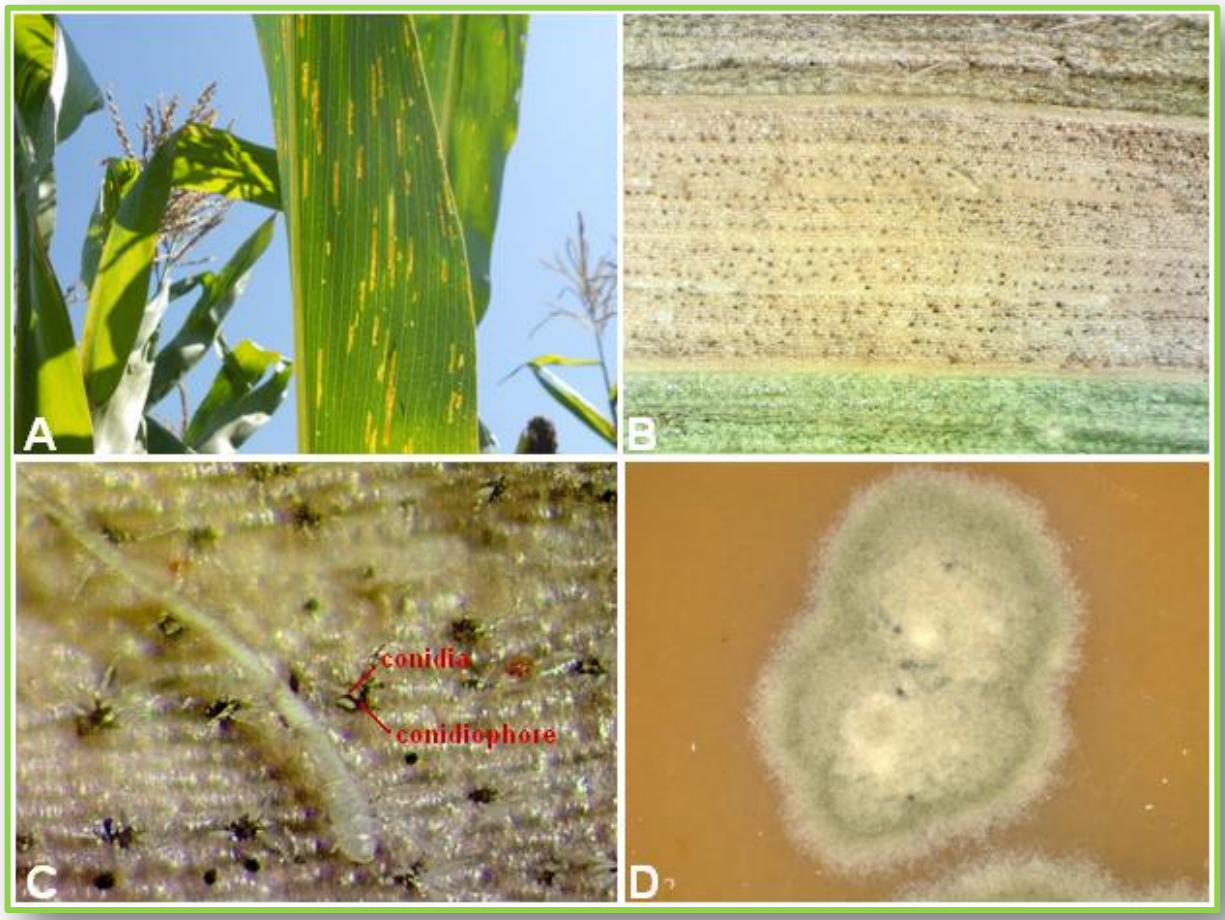


Figure 2. Grey leaf spot disease from leaf to culture. A. A maize leaf displaying GLS symptoms, Kwa-Zulu Natal (Cedara 2012, Photo: Mischa Muller). B. Magnification of a GLS lesion, the dark conidiophores are easily seen emerging from the stomatal opening (Photo: Mischa Muller). C. Melanised conidiophore and hyaline conidia of *Cercospora zeina* (100x magnification) emerging from the stomata (Photo: Mischa Muller). D. A colony of *C. zeina* grown on V8 media at 67X magnification (Photo: Mischa Muller).

1.1.3 Current control mechanisms

GLS symptoms develop in geographical regions that are characterised by warm and humid climates (Ward et al, 1999). The start of the infection season is initiated by conidial inoculum that has overwintered on infected maize debris from the previous season (Shi et al, 2007; Payne and Waldron, 1983; Ward et al, 1999). This knowledge would indicate that the control of such a

pathogen could be maintained through the practice of crop rotation, tillage and stubble management and fungicidal treatments (Munthali et al, 2003; Ward et al, 1998).

Crop rotation with legumes such as soybean and peanut is advantageous as soil nitrogen is depleted by the maize crop season and revitalised by the legume crop season. In addition, crop rotation breaks the propagation cycle of the fungus since the pathogen is host specific (Fischer et al., 2002). Tillage and stubble management is however not a sustainable option in sub-Saharan Africa where water is a scarce commodity and the effect of erosion, soil-water loss and declined soil fertility due to tillage is impractical (Fischer et al, 2002).

Fungicidal treatment of maize is important in combating the increased levels of fungal inoculum each season (Fischer et al, 2002; Ward et al, 1998). Fungicides such as Celest XL (complex of fungicides) are used to treat seeds to reduce inoculum germination on seed and to improve seed vigour against all potential pathogenic fungi (Aveling et al, 2007). Other fungicides used in South Africa as preventative sprays throughout the growing season are Abacus (BASF), Duett and Amistar (Syngenta). These fungicides contain triazole, which is an inhibitor of fungal germ tube development and strobilurin, which is an inhibitor of conidial germination (Bartlett et al, 2002; Sierotzki et al, 2007; Solorzano and Malvick, 2011; Ward et al, 1997). Although these fungicides are effective at preventing disease development, they are costly for small-holder farmers, leaving breeding for resistant varieties a more cost effective and economically viable strategy to control GLS in Africa (Derera et al, 2008; Ward et al, 1997; Ward et al, 1999).

Breeding for resistance to GLS in maize crops remains a principle focus of many research groups throughout the world (Bubeck et al, 1993; Poland et al, 2009; Shi et al, 2014). The movement from identifying one gene that confers resistance to multiple genes that confer resistance to GLS, has become an important progression in crop breeding (Poland et al, 2009; Shi et al, 2014). This progression has been instigated by the co-evolution of pathogens and hosts, where pathogens develop resistance to one gene more readily but rarely against many as

is the case with quantitative resistance (Munthali et al, 2003; McDonald, 2004). This has thus lead to mapping genomic regions that include genes responsible for pathogen recognition and resistance to prevent or minimise further infection (Berger et al, 2014). This form of mapping is termed quantitative trait loci (QTL) mapping and can assist breeders in developing cultivars that confer quantitative disease resistance against more than one pathogen (Zwonitzer et al, 2010). A recent QTL study presenting resistance to *C. zeina* has been performed on subtropical maize germplasm in South Africa (Berger et al, 2014).

In order to ensure effective management of a disease control strategy, three control measures need to be emphasised. The first aims to prevent an outbreak through the reduction of inoculum. The second aims to control disease development using inhibitory reagents such as fungicides. The third guarantees yield while maximising host defence through selective breeding. Implementation of these strategies will ensure the effective management of a pathogen and host interaction.

Cercospora zeina is a foliar pathogen of great economic importance that causes disease on maize in South Africa. To gain better understanding of this pathogen and its impact within South Africa, and in an attempt to manage its disease severity and spread, it is important to understand the pathogen population as a whole. This can be achieneved through the use of molecular markers to haplotype individuals in a population.

1.2 Molecular markers

When addressing questions regarding the structure or diversity within a population, molecular markers based on DNA sequence are used (Burgess, 2004; McDonald, 2004; Xu, 2005). The ideal molecular marker for this purpose is selectively neutral (thus genetic information obtained should not be biased), highly informative (the marker should be able to answer the question), reproducible (all isolates should provide the same information when tested with this marker in

any laboratory), relatively technically easy to use and preferably inexpensive (McDonald, 2004; Atallah and Subbarao, 2012).

1.2.1 Internal transcribed spacers (ITS)

Internal transcribed spacers (ITS) are non-functional DNA sequences found between structural ribosomal DNA (rDNA), i.e. the non-functional transcript sequence between the ribosomal ribonucleic acid (rRNA) on an RNA transcript (Baldwin, 1992). A polycistronic rRNA transcript read from 5' to 3' contains a 5' external transcribed sequence (5' ETS), 18S rRNA, ITS1, 5.8S rRNA, ITS2, 28S rRNA and finally the 3'ETS as illustrated in Figure 3 (O'Donnell, 1992). Genes that encode rRNA and ITS have a low mutation rate due their importance in primary cellular functions and thus are highly conserved within individuals of the same species (Bakkeren et al, 2000; Kumar and Shukla, 2005). This characteristic of ITS sequences makes them useful in elucidating relationships between species and related genera in all organisms (Baldwin, 1992; Chen et al, 2001).

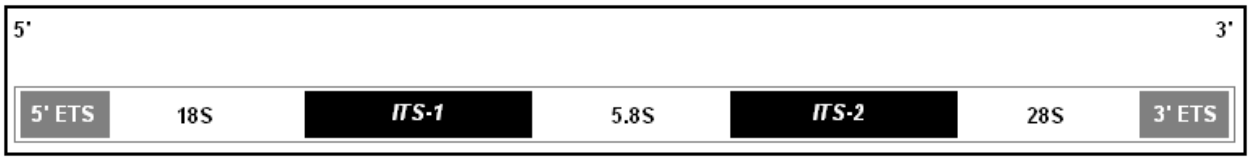


Figure 3. Polycistronic rRNA transcript read from 5' to 3' contains a 5' external transcribed sequence (5' ETS), 18S rRNA, ITS1, 5.8S rRNA, ITS2, 28S rRNA and finally the 3'ETS.

Several taxon-specific primers for fungi have been described that allow selective amplification of ITS sequences (Gardes and Bruns, 1993; Meisel et al, 2009). Such primers have been developed and used for *Cercospora* species and have enabled scientists to distinguish two species that cause GLS on maize, namely *Cercospora zeae-maydis* and *Cercospora zeina* (Dunkle and Levy, 2000; Crous et al, 2006).

1.2.1.1 Molecular Markers used for population studies

There are numerous molecular markers available for determining the diversity of populations of specific organisms. A summarised record of technologies used to determine the population diversity of fungal organisms, was executed from searching the following journal archives; Fungal Biology (previously known as Mycological Research), Molecular Ecology, Molecular Ecology Research (previously known as Molecular Ecology Notes) and Fungal Genetics and Biology (previously known as Experimental Mycology), over the periods January 1996 to May 2013. This attempt aimed to summarise the history of technologies used to elucidate populations of fungal organisms, over the past few decades, and a bar graph was constructed to represent this data (Figure 2).

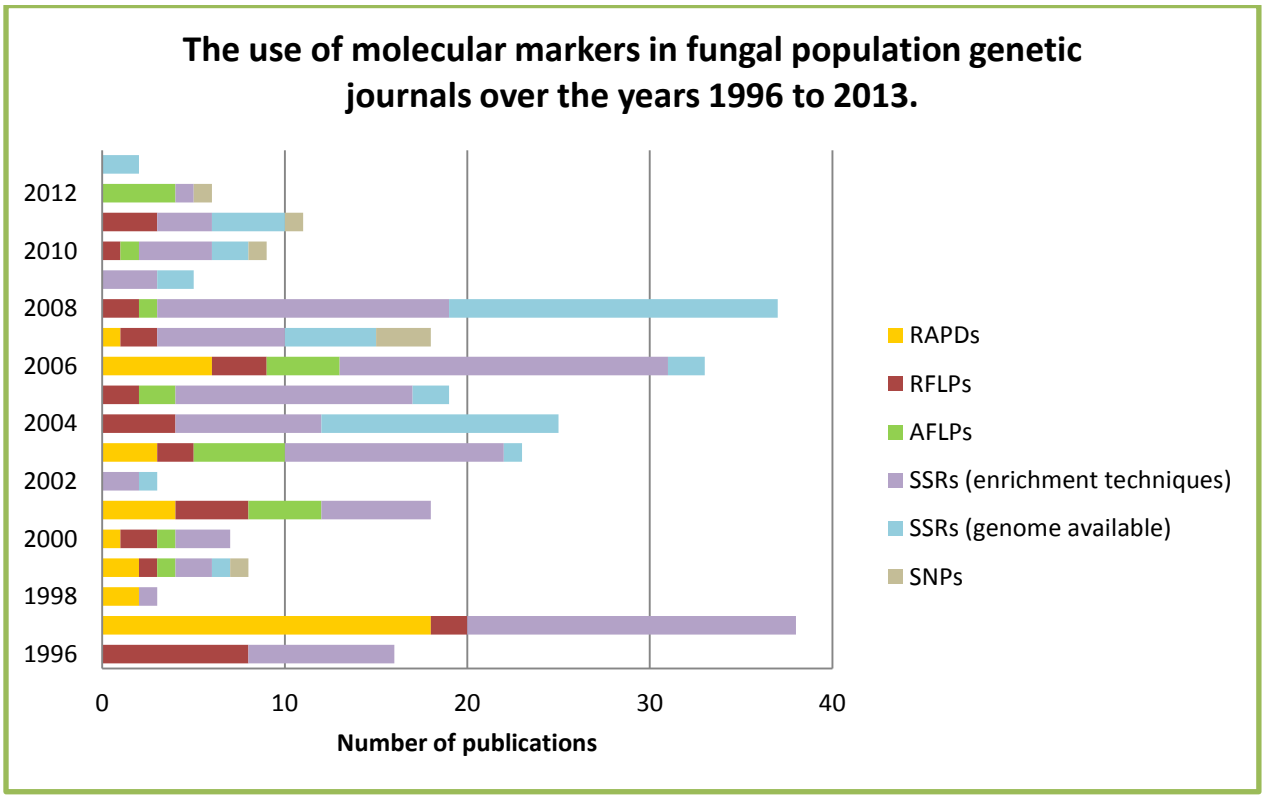


Figure 4: A bar chart summarising the use of different molecular markers in fungal population studies journals over the period January 1996 to May 2013.

From the graph indicated in Figure 4, it is apparent that there has been a movement from the use of RFLPs and RAPDs to the use of SSR markers over time. The pattern can be explained by the improvements of technologies and expansion of biological knowledge. This gradual movement from RFLPs to SSR markers will be discussed further in this review.

1.2.1.1.1 Random Amplified Polymorphic DNA (RAPD)

Random Amplified Polymorphic DNA (RAPDs) is a PCR technique that uses synthetic arbitrary short primers (8-13 oligomers) to amplify segments of genomic DNA from complementary areas within the genome to produce a distinctive gel pattern profile for an individual (Burnett, 2003; Gryta et al, 2000; Meng et al, 1999; Selosse et al, 1999). These primers are relatively specific as they bind to complementary segments of the genome; however the target sequence amplified is unknown. These profiles have been used to differentiate between species in the same genus such as *Glomus caledonium, G. fistulosum, G. mosseae* and *G. versiforme* (Longato and Bonfante, 1997). RAPDs have also been used to distinguish between isolates within the same species. Inglis and colleagues used RAPDs to determine the genetic similarity of isolates of *Cercospora caricis* from Brazil and the Unites States (Inglis et al, 2001).

RAPDs were popular in the 1990's as execution of this technique is straightforward, requires little preparation time, results are obtained relatively quickly, and data interpretation is relatively easy and straightforward but gel images are sometimes difficult to score properly (McDonald, 1997). Although RAPDs are useful for analysis of clonal and haploid organisms, there are a few negative aspects to using RAPDs. RAPDs are not popular amongst diploid studies as they are predominantly dominant, some co-dominant markers still provide less information than other markers. This poses disadvantages. Firstly, this technique should preferably not be used when studying higher eukaryotes (polyploidy level). Secondly, as these markers are mostly dominant, results are underestimated as recessive alleles are not easily detected.

Additionally, due to the sensitivity of this technique (i.e. binding of primers to complementary genomic sequence), all variables need to be kept constant as small elements like low DNA quality will give rise to results being irreproducible between laboratories (McDonald, 1997). These factors have lead to the reduced use of RAPDs from 2007 onwards (Figure 4).

1.2.1.1.2 Restriction Fragment Length Polymorphisms (RFLP)

Restriction Fragment Length Polymorphisms (RFLPs) were the first molecular marker to be used in detecting polymorphisms between individuals as illustrated in Figure 4 (Botstein et al, 1980). RFLPs are based on a DNA-DNA hybridisation technique, which exploits restriction enzymes to digest the genome at specific recognition sites giving rise to DNA fragments of various sizes. These fragments are separated by agarose gel electrophoresis followed by a Southern blot. Variations between isolates are detected by using a probe that binds to the target complementary sequences (Saiki et al, 1985; Inglis et al, 2001).

RFLPs have been popular from their conception in 1996 to 2011 (Figure 4), as these markers are co-dominant and are more reproducible than RAPDs (McDonald, 1997). RFLPs have been used to determine the diversity of isolates within a species such as *Mycosphaerella graminicola* (Torriani et al, 2008). In addition, RAPDs and RFLPs were used in conjunction to determine the lineages of isolates from different geographical regions such as in *Cercospora caricis* and *Cercospora beticola* (Inglis et al, 2001; Groenewald et al, 2007). This technique requires technical competencies that RAPDs do not, which makes it more challenging. RFLPs are reproducible and interpretation is relatively uncomplicated (McDonald, 1997).

1.2.1.1.3 Amplified Fragment Length Polymorphism (AFLP)

Amplified Fragment Length Polymorphism (AFLP) is a PCR-based DNA fingerprinting technique designed to rapidly screen the genetic diversity of isolates within a population (Mueller and

Wolfenbarger, 1999; Vos et al, 1995). There are numerous advantages to using this technique. It requires no prior sequence knowledge and is therefore useful to detect variation within a population where no genome sequence is available (Koebner, 2010; Mueller and Wolfenbarger, 1999; Vos et al, 1995). The technique is comprised of three steps; the restriction of carefully purified DNA followed by the ligation of specific adapters to the restricted ends, selective amplification of restricted fragments using primers designed to anneal to the adapter sequences, and finally banding pattern detection (such as silver staining of polyacrylamide gels) (Vos et al, 1995). This technique is used to detect dominant polymorphisms from a distinct locus, and is scored by presence or absence of bands within the banding pattern (Koebner, 2010).

AFLPs have been used to differentiate between species within the same genus such as *Puccinia graminis, P. striiformis,* and *P. triticina;* as well as *Coniophora* sp. (Keiper et al, 2003; Skrede et al, 2012). In addition, AFLPs have also been used to study the genetic variation within fungal populations of *Cladosporum fulvum, Agaricus bisporus, Amanita francheti* and *Aspergillus flavus* (Foulongone-Oriol et al, 2010; Majer et al, 1996; Ramirez-Camejo et al, 2012; Redecker et al 2001). Most importantly AFLP profiles have been used to distinguish populations of *Cercospora zeae-maydis* and *Cercospora zeina* from various geographical regions comparing Ugandan, Zimbabwean and North American isolates (Dunkle and Levy, 2000; Brunelli et al, 2008).

As with RAPDs, DNA quality and quantity is important for successful digestion and amplification, thus making AFLP technology highly sensitive and reproducibility challenging (Koebner, 2010; McDonald, 1997; Vos et al, 1995). Gel analysis can be problematic due to the closely arranged banding patterns, which when not carefully analysed may lead to uncertainty of pattern profiles. Firstly, banding pattern profiles are independently scored, thus there is room for overestimation of variability due to the nature of genome recombination. Secondly, there is room for underestimation of diversity as fragments are treated in a dominant manner, such that co-dominance is analysed monomorphically, and further cannot be confirmed as being

derived from the same locus (Koebner, 2010; Majoer et al, 1996; Mueller and Wolfenbarger, 1999; Vos et al, 1995). However AFLPs have been useful in differentiating between different species and thus have been extensively used (Figure 4).

1.2.1.1.4 The use of Simple Sequence Repeats (SSR markers) in Fungal Diversity Studies

Simple sequence repeats (SSR markers) are repetitive nucleotide sequences 1-9 bases long (also known as microsatellites) that are repeated in tandem (Buschiazzo and Gemmell, 2006; Schlotterer and Tautz, 1992). They are useful genetic markers as they are distributed throughout the genomes of all organisms in coding and non-coding sequences (Burgess et al, 2004). SSRs possess a high mutation rate (10^{-4}) and are multi-allelic so therefore they are highly polymorphic (Atallah and Subbarao, 2012). They are easy to score statistically as they display co-dominance that can be detected, and results are simple to interpret as they are selectively neutral (Buschiazzo and Gemmell, 2006; Dutech et al, 2007; Burgess et al, 2004; Atallah and Subbarao, 2012). SSR markers are among the most variable of DNA sequences and are amplified by PCR using specific primers designed to conserved regions flanking SSRs. These markers are highly variable and are scored according to the length of these repeat units rather than by nature of the sequence itself. Therefore variability is scored according to the size of the amplicon which reflects repeats of the SSR motif (Burnett, 2003; Ellegren, 2004).

Microsatellite markers are robust as they can be used for many molecular projects in different fields of study. They can be used in genome mapping studies, chromosome mapping, association mapping, population diversity studies, cultivar identification and marker-assisted breeding (Dutech et al, 2007; Ellegren, 2004; Richard et al, 2008). In addition evolutionary studies, particularly kinship studies, have been limited by the availability of segregating genetic marker loci, which is overcome by using SSR markers (Queller et al, 1993).

SSR markers have been used in population studies of many fungal species. Table 1 summarises SSR markers developed from enrichment protocols and genome sequence data for fungal species over the period 2001-2009. The next part of the review will focus on on population studies of a few species that belong to the Dothidiomycetes viz. *Cercospora zeae-maydis*, *C. beticola*, *Diplodia pinea, Dothistroma septosporum* and *Mycosphaerella graminicola* (Dunkle et al, 2009; Groenewald et al, 2007; Burgess et al, 2004; Barnes et al, 2008; Gurung et al, 2011).

Cercospora zeae-maydis is the closest relative to *C. zeina*, a pathogen that also causes grey leaf spot on maize (Crous et al, 2006; Dunkle et al, 2009). Dunkle and colleagues developed ten microsatellite markers from expressed sequence tag (EST) databases of *C. zeae-maydis* and determined that there was great genetic variability within the population of *C. zeae-maydis* in Indiana, USA (Dunkle et al, 2009). These markers were found to be species specific and thus did not amplify in samples of *C. zeina* (Dunkle et al, 2009).

Table 1. A summary of articles published in Molecular Ecology Notes on the development of SSR markers in fungal species over the nine-year period 2001-2009.

Year of Publication	Fungal Organism	Citation	SSR markers	
			Developed from genome sequence or ESTγ library	Developed from enrichment protocols (i.e. RAMSα, ISSR-PCR, FIASCOβ)
2001	*Mycosphaerella musicola*	Molina et al, 2001		RAMS
2001	*Armillaria ostoyae*	Langrell et al, 2001		RAMS
2002	*Microbotryum violaceum*	Giraud et al, 2002		FIASCO
2003	*Beauveria bassiana*	Rehner et al, 2003		RAMS
2003	*Cryphonectria cubensis*	van der Merwe et al, 2003		ISSR-PCR
2003	*Hebeloma cylindrosporum*	Jany et al, 2003	EST	
2004	*Thielaviopsus basicola*	Geldenhuis et al, 2004		ISSR-PCR
2004	*Ceratocystis fimbriata*	Steimel et al, 2004		RAMS
2004	*Tuber magnatum*	Rubini et al, 2004		FIASCO
2004	*Venturia inaequalis*	Guerin et al, 2004		FIASCO
2005	*Aspergillus niger*	Esteban et al, 2005	GENOME	
2005	*Metarhizum anisopliae*	Enkerli et al, 2005		FIASCO
2005	*Melampsora medusae*	Steimel et al, 2005		RAMS
2005	*Melampsora larici-populina*	Steimel et al, 2005		RAMS

2005	*Paecilomyces fumosoroseus*	Dalleau-Clouet et al, 2005		FIASCO
2005	*Rhynchosporium secalis*	Linde et al, 2005		RAMS
2005	*Emericella nidulans*	Hosid et al, 2005	GENOME	
2005	*Phaesphaeria nodorum*	Stukenbrock et al, 2005	EST	
2006	*Colletogloeopsis zuluensis*	Cornitas et al, 2006		FIASCO
2006	*Laccaria amethystina*	Wadud et al, 2006		RAMS
2006	*Laccaria laccata*	Wadud et al, 2006		RAMS
2006	*Mycosphaerella nubilosa*	Hunter et al, 2006		FIASCO
2006	*Serpula lacrymans*	Hogberg et al, 2006		FIASCO
2006	*Ustilago maydis*	Munkacsi et al, 2006	GENOME	
2006	*Plasmopara viticola*	Delmotte et al, 2006		FIASCO
2006	*Rhynchosporium secalis*	Kepier et al, 2006		RAMS
2006	*Melampsora larici-populina*	Barres et al, 2006		RAMS & FIASCO

Year of Publication	**Fungal Organism**	**Citation**	**SSR markers**	
			Developed from genome sequence or ESTγ library	Developed from enrichment protocols (i.e. RAMSα, ISSR-PCR, FIASCOβ)
2007	*Melampsora medusae f. sp. Deltoidae*	Feau et al, 2007	EST	
2007	*Crinipellis perniciosa*	Gramacho et al, 2007	GENOME	
2007	*Cercospora beticola*	Groenewald et al, 2007		FIASCO
2007	*Cryphonectria parasitica*	Kubisiak et al, 2007	EST	
2007	*Magnaporthe grisea*	Adreit et al, 2007	GENOME	
2007	*Magnaporthe grisea*	Li et al, 2007	GENOME	
2007	*Puccinia triticina*	Szabo et al, 2007		FIASCO
2008	*Dothistroma septosporum*	Barnes et al, 2008		FIASCO
2008	*Phaeomoniella chlamydospora*	Smetham et al, 2008		FIASCO
2008	*Mycosphaerella fijiensis*	Zapater et al, 2008		RAMS
2008	*Mycosphaerella musicola*	Zapater et al, 2009		RAMS
2008	*Mycosphaerella eumusae*	Zapater et al, 2010		RAMS
2008	*Cryptococcus neoformans*	Karaoglu et al, 2008	GENOME	
2008	*Microbotryum dianthorum*	Giraud et al, 2008	EST	
2008	*Microbotryum lagerheimii*	Giraud et al, 2008	EST	
2008	*Microbotryum lychnis-dioicae*	Giraud et al, 2008	EST	
2008	*Microbotryum silenes-inflatae*	Giraud et al, 2008	EST	
2008	*Phialocephala subalpine*	Queloz et al, 2008		FIASCO
2008	*Phialocephala fortinii s.s.*	Queloz et al, 2008		FIASCO
2008	*Microbotryum violaceum*	Giraud et al, 2008	EST	
2008	*Kirramyces gauchensis*	Cortinas et al, 2008		FIASCO
2008	*Aspergillus sydowii*	Rypien et al, 2008		FIASCO
2008	*Fusarium pseudograminearum*	Scott & Chakraborty, 2008	GENOME	

2008	*Rhizontonia solani*	Zala et al, 2008		FIASCO
2008	*Moniliophthora perniciosa*	Silva et al, 2008	GENOME	
2008	*Mycosphaerella fijiensis*	Yang et al, 2008	GENOME	
2008	*Lactarius mammosus*	Hogberg et al, 2008		FIASCO
2008	*Rhizoctonia solani*	Ferrucho et al, 2008	GENOME	
2008	*Colletotrichum capsici*	Ranathunge et al, 2008	EST	
2008	*Ascophaera apis*	Rehner et al, 2008	GENOME	
2008	*Armillaria mellea*	Baumgartner et al, 2008		FIASCO
2008	*Macrophomina phaseolina*	Baird et al, 2008		FIASCO
2009	*Aspergillus flavus*	Grubisha et al, 2009	GENOME	
2009	*Alternaria dauci*	Benichou et al, 2009		FIASCO
2009	*Cercospora zeae-maydis*	Dunkle et al, 2009	EST	
2009	*Ophiostoma quercus*	Grobbelaar et al, 2009		FIASCO
2009	*Magnaporthe grisea*	Suzuki et al, 2009	GENOME	

α RAMS = random amplified microsatellites (Hantula et al, 1996)
β FIASCO = fast isolation by AFLP of GENOMEs containing repeats (Zane et al, 2002)
γ EST = expressed GENOME tags (Adams et al, 1991)

Cercospora beticola is the causal agent of sugar beet *Cercospora* leaf spot (Groenewald et al, 2007). Groenewald and colleagues employed RFLP, SSR and Single Nucleotide Polymorphism (SNP) markers in an attempt to fully understand the population diversity of *C. beticola* (Groenewald et al, 2007). They concluded that the use of five microsatellite markers were informative and enabled them to distinguish isolates from geographical regions and within regions (Groenewald et al, 2007). SNP markers are the most abundant marker within a genome, however possess a lower mutation rate (10^{-9}) than SSR markers (10^{-4}), thus currently, SNPs are used mainly for genotyping and linkage mapping (Atallah and Subbarao, 2012).

Diplodia pinea is a fungal pathogen of economically important forest *Pinus sp.* 12 SSR markers were used to determine the variability and origin of *D. pinea* collected in the southern hemisphere (Burgess et al, 2004). Results showed that isolates from the northern and southern hemispheres were clonal, but the pathogen ancestry could not be determined (Burgess et al, 2004). Bihon and colleagues subsequently designed additional microsatellites that revealed high genetic diversity of *D. pinea* within an infected *Pinus patula* tree. These markers are potentially useful for a global population study of *D. pinea* (Bihon et al, 2011).

Seventeen polymorphic microsatellite markers developed from an Expressed Sequence Tag (EST) database were used to determine the genetic diversity within populations of the Dothideomycete *Mycosphaerella graminicola*. These markers displayed great variability amongst isolates collected from 11 populations from different geographical regions within the United States (Goodwin et al, 2007; Gurung et al, 2011).

1.2.1.1.5 SSR marker development

Three methods used to develop microsatellite markers are discussed. These include random amplified microsatellites (RAMS) and fast isolation by AFLP of sequences containing repeats (FIASCO), and data mining microsatellite sequences from a readily available genome sequence, followed by experimental validation in the laboratory.

RAMS is a simple technique that is based on the principles of a RAPD PCR, using a primer that contains a microsatellite sequence with a degenerate 5' end (Hantula et al, 1996; Longato and Bonfante, 1997). The method is highly reproducible and has been developed for fungal species, and enables detection of interspecific and intraspecific DNA polymorphisms (Hantula et al, 1996). Although, RAMS is time-consuming and often requires genome walking to obtain the entire microsatellite sequence, it is effective for obtaining microsatellite markers (Cortinas et al, 2006; Hantula et al, 1996). This technique was used to develop eight microsatellites to elucidate the population diversity of *Colletogloeopsis zuluensis*, of which only two markers were polymorphic and informative (Cortinas et al, 2006).

Fast isolation by AFLP of sequences containing repeats (FIASCO) is an enrichment technique that captures microsatellite rich regions from AFLP PCR products using biotinylated primers and streptavidin magnetic beads (Cortinas et al, 2006; Barnes et al, 2008; Zane et al, 2002). DNA of interest is digested by a particular enzyme, followed by the ligation of adaptors. Primers specific to the adaptors are then used for PCR amplification of digested products (Zane et al,

2002). PCR amplification is then followed by microsatellite capturing using biotinylated primers (specific to the targeted microsatellite) and streptavidin beads (Zane et al, 2002). After the products have been purified, the DNA fragments are run on an agarose gel or PAGE to visualise polymorphisms, followed by sequencing to confirm microsatellite quality and polymorphism potential (Zane et al, 2002; Cortinas et al, 2006; Barnes et al, 2008). Primers are then designed to the flanking regions of the microsatellite, and fluorescently labelled. Fragments lengths generated by the primers in PCR are analysed by a high-throughput genetic analyser (ABI Genetic Analyser 3500xl) using the program GENESCAN™ (Cortinas et al, 2006; Barnes et al, 2008; Zane et al, 2002). This technique was used to develop 20 microsatellite markers, of which seven were polymorphic and used to elucidate the population diversity of *Colletogloeopsis zuluensis* (Cortinas et al, 2006). Additionally, FIASCO has been used to develop microsatellite markers that differentiate between two *Dothistroma* species (*D. pini* and *D. septosporum*) and has been used to determine the diversity within D. septosporum populations (Barnes et al, 2008).

In contrast to molecular-based microsatellite discovery, whole genome sequence data provides an unbiased look at microsatellites discovery (Ellegren, 2004). Microsatellites can be found both in coding and non-coding regions, giving rise to EST/gene SSR markers and genomic SSR markers, respectively (Goodwin et al, 2007; Torriani et al, 2008; Ellegren et al, 2004). Microsatellites have successfully been developed from EST libraries, for *Hebeloma cylindrosporum, Phaesphaeria nodorum, Melampsora medusae f. sp. deltoidae, Cryphonectria parasitica, Microbotryum sp.* and *Colletotrichum capsici* as observed in Table 1 (Jany et al, 2003; Stukenbrock et al, 2005; Feau et al, 2007; Kubisiak et al, 2007; Giraud et al, 2008; Ranathunge et al, 2008). EST and genomic data can be obtained from online databases such as NCBI (http://www.ncbi.nlm.nih.gov/), Tandem Repeats Database (Gelfand et al, 2006) or from the organism of interest's genome sequence. Primers are designed to the flanking regions of SSR

markers identified from the genome sequence, with the use of software packages such as CLC Bio and Primer3, for the amplification of the microsatellite by PCR (Rozen and Skaletsky, 2000).

Microsatellite markers developed from EST libraries are more conserved as they are found within gene regions. The microsatellite length found within a gene is required to be a multiple of three owing to selection pressures to maintain the correct reading frame. Although not restricted to, gene microsatellites are most commonly trinucleotide repeats (Metzgar et al, 2000). Due to their conserved nature gene microsatellites are valuable when comparing populations of sibling species such as *Microbotryum* sp. as there is sufficient conservation amongst genes to develop conserved primer sequences, and sufficient polymorphism amongst microsatellites within the genomes (Giraud et al, 2008). For example, 60 polymorphic markers were used to distinguish ten species of the phytopathogenic fungal complex *Microbotryum* populations (Giraud et al, 2008).

Presented here is a case of *Puccinia triticum* to illustrate which molecular marker technique is most useful for a fungal population study. In 1995, Kolmer and colleagues used RAPDs to clarify the population structure in relation to virulence of *P. triticum*. Two geographical clades with some evidence of virulence could be identified (Kolmer et al, 1995). In 2001, Kolmer realised that due to the shortcomings RAPDs pose, AFLPs were perhaps a better technique to pursue, and was able to distinguish between virulent and avirulent isolates on a resistant cultivar of wheat (Kolmer, 2001). Yet again, in 2007 Kolmer and colleagues realised that the dominant RAPD and AFLP markers could not distinguish genotypes due to the dikaryotic biology of *Puccinia*, and they finally developed 18 polymorphic SSR markers that were able to distinguish between different genotypes of *Puccinia triticum* (Szabo et al, 2007). SSR markers are thus a powerful tool that are more easily developed with the availability of a genome sequence.

SSR markers are useful molecular markers that will be employed in this study to determine the variability of *C. zeina* isolates within South Africa. RAMS and FIASCO are valuable molecular

tools that could be used to discover, design and analyse SSR markers. However the *Cercospora zeina* genome sequence is available to the MPPI research group and will subsequently be used to design SSR markers for this pathogen.

The availability of a genome sequence is a valuable resource. However a large amount of data is required to be processed before acquiring information on the distribution and lengths of microsatellites. MSATCOMMANDER is a publicly available platform-independent software package written in Python and designed for the purpose of rapidly and automatically identifying microsatellites (Faircloth, 2008). It is user-friendly and produces an output text file in a locus specific manner, as well as an option to design primers to the target region using PRIMER3 software (Faircloth, 2008; Rozen and Skaletsky, 2000). There are also other online packages that are able to identify microsatellite markers, such as Simple Sequence Repeat Identification Tool (SSRIT) that identifies perfect microsatellites in a given sequence (Temnykh et al, 2001). BatchPrimer3 is another online software package that can identify microsatellites in a given sequence with the advantage of identifying primers according to your preferences and desired fragment size (You et al, 2008).

Once the primers have been designed they are tested for amplification success and to determine if they are polymorphic in a given population. This is performed by PCR amplification, followed by in gel size visualization of bands. It is possible to determine the relative size of the SSR markers on a polyacrylamide gel, metaphor agarose gel, high concentration agarose gel, or by sequencing the samples (Li et al, 2007). The relative size of an SSR marker can also be interpreted using a fluorescently labelled primer that is detected by means of capillary electrophoresis using GENESCAN™ and viewed using software packages such as GeneMapper v4.1 (Life Technologies) or GeneMarker (Bihon et al, 2011; Bihon et al, 2012; Hulce et al, 2011).

Capillary electrophoresis technology is an accurate detection system used to study SSR markers. The ABI Genetic Analyzer 3500xl (Life Technologies) is an instrument available at the

University of Pretoria that detects the relative size of the fluorescing amplicon by a laser. Data is interpreted by software packages such as GeneMapper v4.1 (Life Technologies) or GeneMarker. This data is presented as an electropherogram, which represents polymorphisms as fragment lengths, which are read as alleles (Hulce et al, 2011). The allelic data is used to execute population genetics statistics to determine the diversity and structure of the sample population. Many software packages are used to analyse such microsatellite data, for example CLC Bio, BioEdit, PHYLIP, MEGA, POPGENE, Arlequin, STRUCTURE and MULTILOCUS (Abd-Elsalam, 2003; Felsenstein, 1989; Tamura et al, 2013; Yeh and Boyle, 1997; Schneider et al, 1997; Evanno et al, 2005).

1.3 Population Genetics

Plant disease is characterised by the identifiable group of symptoms that causes significant deterioration of the plant, caused by a population of a pathogen through multiple infections on more than one host individual (McDonald, 2004). Epidemiology is the study of the progression and movement of disease in a field and/or across continents, but it fails to recognise the effect of genetically distinct individuals within a population on disease progression and severity (McDonald, 1993). Consequently population studies provide valuable information for developing control strategies to managing disease spread and severity (McDermott and McDonald, 1993).

A population can be defined as: a group of organisms inhabiting a particular region, or a group of organisms that is defined by the boundaries of speciation (Xu, 2005). Population genetics studies the processes that lead to evolution in a pathogen population over generations and seasons focused on mutation, genetic drift, gene flow, mating systems and natural selection (McDonald, 2004). The process of mutation alters genetic material at a locus and gives rise to genetic variation (the amplicon of these variable fragments are termed alleles) within a population (Burnett, 2003). Natural selection and genetic drift are forces that result in altered

allelic frequencies within a population (Burnett, 2003; McDonald, 2004). Thus evolutionary information gathered from studying phytopathogen populations is informative to breeders and farmers so that they can manage disease more effectively. For example, if there is selection on fungicide resistant fungi within a field due to the excessive spraying of fungicides, it is possible to detect this through the decline in most genotypes and rise in a resistant genotype (McDonald, 2004). In addition, if a microsatellite is linked to a fungicide resistance gene, variability in this marker may indicate that the resistance gene is evolving (Ellegren, 2004; Torriani et al, 2008). This information is invaluable to farmers in order to make use of an alternative fungicide the following season to limit disease resistance progression.

Mutations arise randomly and are the source of new alleles within a population. Fungi possess a typical mutation rate (μ) of between 10^{-6} and 10^{-7} (Burnett, 2003; McDonald, 2004). Mutations within microsatellite regions occur more often, up to 10^{-4}, resulting in variable genetic fragments that are valuable to study (Atallah and Subbarao, 2012).

It is often observed that smaller and younger populations have fewer alleles than larger and older populations, which is attributable to gene flow, genetic drift and sexual reproduction in the older populations (McDonald, 2004). Gene flow introduces genetic variability into new populations from an outside population through mechanisms such as dispersal (Burnett, 2003; McDermott and McDonald, 1993). Genetic drift is the progression of a population over time, where bottlenecks (diminishing population) and founder events (starting off with a small population) are generated (McDonald, 2004). Alleles in a population become fixed when selected by genetic drift, thus individuals within a population are likely to be clonal (McDonald, 2004). Sexual reproduction may give rise to greater genotypic variability through the recombination of alleles rather than clonal populations in asexual reproduction systems that display little genotypic variation (McDonald and McDermott, 1993; Burnett, 2003; McDonald, 2004; Xu, 2005). In asexual reproductive systems, genetic drift results in fixed allelic frequencies within the population, and this influences the level of clonality due to the already

limited genotypic variability available (Burnett, 2003). Genotypic variability is also influenced by natural selection by the selection of a particular genotype (allelic combination) over another genotype to increase the fitness of the pathogen population (McDonald, 2004).

Analysing agricultural processes puts the host-pathogen interaction into perspective. Agronomical control strategies have a selective effect on population genetics and the evolution of a pathogen, as they unintentionally select for unique alleles through the exploitation of specific mono-cultivars, fungicide applications, crop rotation and tillage practices. Thus understanding what population we have inadvertently selected for may influence our strategy in the next season and the seasons after that.

1.3.1 Population genetics statistics

Alleles provided by scored microsatellite data are used as the input data for various population analysis programs. There are numerous population study software programs such as POPGENE, MULTILOCUS, GenAlEx, Arlequin and MEGA that can be used to analyse microsatellite data, however each program is concentrated towards the speciality the package was designed for. Population statistics are necessary to describe the population diversity, which includes the genotypic diversity, gene diversity, allelic richness, private alleles, linkage-disequilibrium, as well as determining the genetic distance between populations.

Population structure can be measured using Wright's F-statistics, a mathematical measurement dependent on defined genotypes to compute the allelic frequency variation (Wright, 1931). Wright's statistics are based on Mendelian equilibrium, used to determine the variation of gene frequency influenced by selection pressures of mutation and migration (Wright, 1931). Population structure is not necessarily defined by the geographical distribution of isolates, but by the genotypes of isolates from various regions (Evanno et al, 2005).

Table 2. A summary of population genetic statistics software packages that can be used for population analysis.

Software Package	**Structure**	**POPGENE**	**Arlequin**	**GDA**	**GenePop**	**EasyPop**	**GENALEX6**
Citation:	*Evanno et al, 2005*	*Yeh and Boyle, 1997*	*Schneider et al, 1997*	*Lewis and Zaykin, 1999*	*Raymond and Rousset, 1995*	*Balloux, 2001*	*Peakall and Smouse, 2006*
Data Types:							
Haploid	X	X	X	X	X	X	X
Diploid	X	X	X	X	X	X	X
Dominant Marker	X	X	X				X
Co-dominant Marker	X	X	X	X	X	X	X
Diversity:							
Heterozygosity	X	X	X	X		X	X
No. alleles/loci	X	X	X	X			X
Percentage polymorphic loci	X	X	X				X
Shannon-Weaver		X	X	X			X
Population Structure:							
F-statistics		X	X	X	X		X
AMOVA			X	X	X		X
Homogeneity		X			X		
Migration			X		X		
Isolation by distance					X		
Equilibrium:							
Hardy-Weinburg	X	X	X	X	X		X
Two-Loci	X	X	X	X	X		X
Multiloci	X	X		X			X
U-test					X		
Genetic Distance:							
Nei's		X	X	X		X	X
Rogers'							
Pairwise F_{ST}			X	X			X
Neutrality Test		X	X			X	
Clustering:							
Neighbour-joining				X			
UPGMA		X		X			

The program STRUCTURE v2.3.4 utilises a Bayesian analysis method that uses specified prior models to analyse data based on allelic frequencies while predicting the population structure by means of groupings that are not in disequilibrium (Pritchard et al, 2000; Falush et al, 2003;

Evanno et al, 2005). These simulations are subjected to a Markov chain Monte Carlo (MCMC) method, which uses values from a probability distribution to ensure the reliability of the simulation (Pritchard et al, 2000). STRUCTURE v2.3.4 (Table 2) allows the user to perform these simulations according to admixture, no-admixture and linkage models, (Falush et al, 2003). No-admixture models assume that individuals are derived from one lineage and thus each individual will represent one of the "k" populations which will possess its own alleles, where "k" represents a predicted number of populations (Pritchard et al, 2000; Falush et al, 2003). Admixture models assume that individuals are derived from a mixed or complex ancestry where individuals will possess genomic similarities with other individuals within a population of different origins (Falush et al, 2003). The Admixture model introduces a vector, Q, which provides the admixture proportion for each individual (Pritchard et al, 2000). Linkage models are admixture models that have taken linked loci into consideration during the simulation of population structure (Falush et al, 2003). A probability of K is determined to predict the most likely group individuals belong to; thus an estimate of the number of structured populations within the population data set provided, and the individuals to which they belong (Evanno et al, 2005).

POPGENE v1.31 (Table 2) is a multi-purpose software package developed by Francis Yeh (University of Alberta), Rong-cai Yang (University of Alberta) and Tim Boyle (Centre for International Forestry Research) for population genetic analysis which can compute genetic diversity, neutrality testing, linkage disequilibrium and population sub-division (Yeh and Boyle, 1997; Yeh et al, 1997). POPGENE v1.31 uses a complex set of statistical analyses to determine the descriptive statistics for a population. The effective number of alleles (ne) is determined by $ne = 2Ne\mu+1$, where Ne is the effective population size (the number of individuals that can contribute to the next generation) and μ is the mutation rate (Kimura and Crow, 1964; Hartl and Clark, 1989). Polymorphic loci are determined by the percentage of each allele at each locus. Gene diversity (Nei's heterozygosity, H) is a measure of the allelic frequency for each locus,

which describes the significance of the marker being used. Gene diversity can be calculated by $H = 1-\Sigma_k-\omega_{k^2}$ where ω_k is the frequency of the k^{th} genotype (Nei, 1972). A value for h that tends to 1 is indicative of high diversity, likewise a value tending to 0 is indicative of clonality.

Analysing allelic data can also provide valuable information regarding smaller populations, such as private alleles. Private alleles are alleles that are not shared amongst populations and are thus restricted to a particular population. This is valuable as it can be used as a geographical population specific marker and its distribution over time can indicate a possible direction of gene flow (Wolf and Soltis, 1992; Bash and Sujatha, 2007). HP-RARE, a software package that determines rarefaction within populations can identify private alleles in addition to the distribution of alleles in defined regions (Kalinowski, 2005).

Statistics that describe an individual isolate's genotype and the diversity of genotypes within a population includes calculating estimates of genotypic diversity, gene flow and genetic distance (Yeh et al, 1997). The Shannon Index is the measure of genotypic diversity in a population and provides information about the distribution of a genotype within a population (Yeh et al, 1997; Lewontin, 1972). The Shannon Index (SI) is a measure of diversity calculated by the proportion of isolates belonging to the i^{th} population (*pi*), multiplied by its natural logarithm (ln*pi*), where $SI=-\Sigma pi \times \ln pi$ such that as the number of genotypes increase and the evenness of its distribution, the measure of SI increases as well (Yeh et al, 1997; Lewontin, 1972).

Genetic differentiation (G_{ST}) can be determined from Nei's (1972) gene diversity where $G_{ST} = (h_T - h_S)/ h_T$, such that h_T is the genetic diversity of the population and H_S is the mean diversity of the population (Yeh et al, 1997; Slatkin and Barton 1989; Nei, 1972; Nei, 1978; Xu, 2005). An estimate of gene flow (Nm) between populations is determined using G_{ST}, whereby $Nm = 0.5(1-G_{ST})/G_{ST}$ (Yeh et al, 1997; Slatkin and Barton 1989; Nei, 1972; Nei, 1978; Xu, 2005). A population that has little genetic difference will exhibit no gene flow between individuals and thus Nm would be zero, and G_{ST} would be one. Similarly Wright's statistic F_{ST} is a measure of population

differentiation where F_{ST} = 1/ (1+4N_m), such that a value tending to one will exhibit high differentiation within a population (Wright, 1931; Yeh et al, 1997; Nei, 1972; Nei, 1978; Xu, 2005).

Genetic distance is determined through Nei's genetic identities and genetic distance (Nei, 1972; Nei, 1978). An illustration of the distance between populations is performed using the hierarchical clustering method UPGMA (Unweighted Pair Group Method with Arithmetic Mean) or NJ neighbour joining tree to construct the distance dendogram based on Nei's genetic distances between the predefined populations (Nei, 1972).

MULTILOCUS v1.3b can perform a myriad of analyses; these include defining linkage groups, population groups, calculating genotypic diversity, population differentiation and linkage disequilibrium (Agapow and Burt, 2001). Determining genotypic diversity and plotting the genotypic diversity against the number of microsatellite loci, is useful to determine whether a sufficient amount of microsatellite markers have been employed in a population study. Linkage disequilibrium is determined by calculating the Index of Association (I_A) and $\bar{r}_d$, these values are used to indicate whether there is potentially sexual recombination occurring within the population in the absence of mating type analyses (Brown et al, 1980; Maynard Smith et al, 1993).

GENALEX 6 (Table 2) is a Microsoft Excel based program Add-In that is able to perform a myriad of genetic statistics, and is used to perform an analysis of molecular variance (AMOVA), which determines where population variation occurs and validates the population structure observed (Peakall and Smouse, 2006). It measures the level of diversity between individuals and within a population and determines where the variance comes in (Peakall and Smouse, 2006).

PHYLIP (Phylogenetic Inference Package) is a free software package that has many uses, but is useful in particular for its ability to infer evolutionary relationships between organisms in the form of a phylogenetic tree (Felsentein, 1989). PHYLIP is employed to construct trees based on parsimony, genetic distance and maximum likelihood (Felsenstein, 1989). MEGA6 (Molecular Evolutionary Genetics Analysis) is a software package that is able to build alignments, infer phylogenetic relationships, and conduct molecular evolutionary analysis (Tamura et al, 2013). It also has the added ability to construct related trees and thus can be used to visualise and edit the genetic distance trees constructed by PHYLIP (Felsentein, 1989; Tamura et al, 2013).

These software packages are useful tools that will be used to reject or validate the following hypotheses:

1. Fifteen microsatellite markers will be sufficient to elucidate the genetic diversity of *Cercospora zeina* in South Africa.
2. The expected population structure of *Cercospora zeina* in South Africa is clonal.
3. There is no evidence for sexual recombination of *Cercospora zeina* occurring within South Africa.

Concluding remarks

Cercospora zeina is a destructive foliar pathogen of maize, belonging to the genus *Cercospora* which comprises over 5'000 species and is characterised by host specificity and devastating crop pathogens (Goodwin et al, 2001; Carson and Goodman, 2006; Crous et al, 2006). It was first discovered in Natal in 1988, and has since spread to the foremost maize growing areas in South Africa (Ward et al, 1997). In order to manage the development and spread of GLS, it is important to understand the biology of infection and the pathogen population diversity, which is poorly understood for *Cercospora zeina*. Molecular markers are useful tools used to investigate the population structure of an organism (McDonald, 1997). Molecular tools such as AFLPs have been able to discriminate between the sibling species *Cercospora zeina* and *Cercospora zeae-maydis,* which cause the same disease on maize, but have as yet been unable to

determine the population structure and diversity for either pathogen (Dunkle and Levy, 2000). Thus the aim of this project will be to develop microsatellite markers for *Cercospora zeina* in order to resolve the population diversity and structure within South Africa.

Materials and Methods

2.1 Fungal Isolates and DNA Extraction

Collections were made of maize material displaying GLS symptoms from various geographical regions within South Africa over the years 2011-2013. There were seven collection sites for the seasons 2011-2013, from Kwa-Zulu Natal (Greytown, Cedara, Baynesfield, Winterton Bergville and Empangeni), Mpumalanga (Machadodorp) and the North West Province (Klerksdorp) (Figure 5). Two leaves displaying GLS lesions were collected from fifty plants per site. Collections undertaken in KZN and MP over the periods 2011 to 2013 were performed in this study. Isolates from the year 2007 were obtained from collections performed by Dr Barbara Meisel, a former MPPI lab member (Meisel et al, 2009). Maize material obtained from Winterton and Bergville in 2012 were kindly collected by Rene Jacobs at Syngenta. Furthermore, collaborators at PANNAR collected maize material obtained from the North West Province in 2012. Additional material displaying GLS symptoms on sorghum (*Sorghum bicolor*) was collected during the 2013 season in this study, from a field in Greytown.

White acicular hyaline conidia attached to dark melanised conidiophores protruding from the plant stomata were identified under a dissecting microscope. A hypodermic insulin needle was used to carefully pick up the conidia, which were streaked onto V8 agar plates containing 100mg/ml carbenicillin to restrict bacterial growth. Single-spore isolates were made by transferring single germinating conidia to new V8 agar plates. All isolates were grown at 25°C for 4 months.

For DNA extractions, mycelia from 4-month-old cultures were scrapped off plates with a scalpel and freeze-dried. The mycelia were homogenised using metal beads and a homogeniser. A modified CTAB DNA extraction was then performed (Stewart and Via, 1993), which included adding 125µl β-mercaptoethanol (BME) (Sigma-Aldrich) and 1g of polyvinylpyrroilidone (Sigma-Aldrich) to 25ml CTAB (Hexadecyltrimethyl ammonium bromide) (Sigma-Aldrich)

buffer in a fume-hood, and set in a water-bath at 65°C overnight. One spatula of ground mycelia was transferred to a 2ml Eppendorf tube, to which 1ml CTAB:BME:PVP and 2µl RNase A (Qiagen) was added and incubated at 37°C for 30 min. One microlitre of Proteinase K (Sigma-Aldrich) was added to the mycelial mixture and incubated at 65°C for 60 minutes. In a fume-hood, 1ml chloroform (Merck univAR) was added to the mycelial mixture and then vortexed thoroughly for 1 min. The Eppendorf tube was centrifuged for 10 min at 13400 rpm. The aqueous phase was then transferred to a new 2ml Eppendorf tube with care, to ensure no transfer of chloroform. An additional chloroform clean-up step was performed for samples that were discoloured. A 0.8 volume of 7.5M ammonium acetate (Merck univAR) at 4°C and 0.5 volume 100% isopropanol at -20°C was added to an estimated volume of the aqueous phase and incubated at -20°C overnight. Thereafter, samples were centrifuged for 10 min at 13400 rpm. The supernatant was discarded. To the pellet, 1ml 100% isopropanol at -20°C was added and vortexed for one minute. After centrifugation for 10 min at 13400 rpm, the supernatant was discarded. One millilitre of 70% ethanol (Illovo, Durban, SA) at 4°C was added to the pellet and vortexed. The sample was centrifuged for 10 min at 13400 rpm and the supernatant was discarded. Any residual ethanol was allowed to evaporate. The pellet was then re-suspended in 100µl of MilliQ ddH_2O.

Control isolates of *Cercospora zeina, C. zeae-maydis* and *Cercospora spp*. were obtained from the Forestry Agricultural Biotechnology Institute (FABI), at the University of Pretoria. These isolates are designated by their collection codes, which correspond to the Centraalbureau voor Schimmelcultures (CBS) Fungal Biodiversity Centre codes and are as follows: *C. zeina* (CPC11998), *C. zeae-maydis* (CBS1177556, CBS117762) and *Cercospora spp.* (CPC12062).

Figure 5: A map illustrating the eight collection sites for GLS infected maize in South Africa. Isolates were obtained from the three provinces Kwa-Zulu Natal, Mpumalanga and the North West province. Furthermore there were six collection sites within the Kwa-Zulu Natal province namely, Greytown, Karkloof, Cedara, Baynesfield, Winterton and Empangeni. The purple line outlines the major maize growing areas, courtesy of the National Oceanic Atmospheric Association (NOAA) and USDA.

2.2 Histone PCR

A histone H3 specific diagnostic PCR test was used on all isolates to confirm the identity of the *Cercospora spp*. isolated (Crous et al, 2006). The first primer pair, CYLH3F/CYLH3R served as the histone H3 positive control amplifying a fragment size of 389bp to indicate that the isolate was of the genus *Cercospora*. The second primer pair, CzeinaHIST/CYLH3R confirmed the identity of *Cercospora zeina* by amplifying a fragment of 284bp. The third primer pair, CzeaeHIST/CYLH3R served to identify *Cercospora zeae-maydis* and the fourth, CmaizeHIST/CYLH3R confirmed the presence of a previously identified *Cercospora* species found on maize, which has subsequently not been characterised since 2006, both 284bp in length (Crous et al, 2006). The primer sequences are shown in Table 3. The following type isolates, obtained from Dr Meisel, were used as positive controls: CPC11998 = *Cercospora zeina* and CBS1177556 = *Cercospora zeae-maydis.*

The histone diagnostic PCR was set up for each isolate in a reaction volume of 25µl that consisted of 1x NH_4 PCR reaction buffer, 2.5mM $MgCl_2$, 0.5µM of dNTP mixture, 0.3µM of each primer, 1U of BIOTAQ™ DNA polymerase (Bioline), 20ng gDNA and distilled MilliQ water. The cycling conditions for primer pair CYLH3F/CYLH3R were 5 min at 94°C followed by 30 cycles of 20s at 94°C, 30s at 55°C and 40s at 72°C, followed by a final elongation step of 5 min at 72°C. The cycling conditions for primer pairs CzeinaHIST/CYLH3R, CzeaeHIST/CYLH3R and CmaizeHIST/CYLH3R were 5 min at 94°C followed by 30 cycles of 20s at 94°C, 30s at 58°C and 40s at 72°C, followed by a final elongation step of 5 min at 72°C. All PCR products were visualised by gel electrophoresis through 2% agarose containing 0.5µg/ml ethidium bromide.

Table 3. Diagnostic histone H3 PCR primer names and sequences used in this study to confirm the identity of the causal agent of GLS from the collected maize material (Crous et al., 2006).

	Primer name	Primer sequence	*Cercospora* species identified
1	CYLH3F	5'- AGGTCCACTGGTGGCAAG -3'	All *Cercospora* species
	CYLH3R	5'- AGCTGGATGTCCTGGACTG -3'	
2	CzeinaHIST	5'- TCGAGTGGCCCTCACCGT -3'	*Cercospora zeina*
	CYLH3R	5'- AGCTGGATGTCCTGGACTG -3'	
3	CzeaeHIST	5'- TCGACTCGTCTTTCACTTG -3'	*Cercospora zeae-maydis*
	CYLH3R	5'- AGCTGGATGTCCTGGACTG -3'	
4	CmaizeHIST	5'- TCGAGTCACTTCGACTTCC -3'	*Cercospora spp.*
	CYLH3R	5'- AGCTGGATGTCCTGGACTG -3'	

2.3 Microsatellite Mining and Primer Design

In collaboration between the University of Arkansas under the supervision of Prof Burton Bluhm and the University of Pretoria under the supervision of Prof Dave Berger, the genome sequences of a two characterised *Cercospora zeina* isolates from the United States (OYPA) and Zambia (Mkushi) respectively, were used to screen for SSR markers. In association with Phillip San Miguel, Purdue Genomics Core Facility at Purdue University, United States; the genome of the Zambian *Cercospora zeina* isolate (Mkushi) was sequenced using the Illumina platform. A former colleague, Fred van Staden, used the Velvet assembler to assemble the *C. zeina* Mkushi genome into contigs for SSR screening (Zerbino and Birney, 2008; van Staden et al, 2013).

Microsatellite repeats were mined using two strategies for each *C. zeina* genome sequence. Contigs from the United States isolate OYPA were run through the program Simple Sequence Repeat Identification Tool (SSRIT) tool (Temnykh et al, 2001). Dinucleotide, trinucleotide and tetranucleotide repeats that possessed 10 motifs or more were selected for further study. Primer3 was used to design primers flanking the microsatellite/SSR regions identified. Primers were designed to have the following properties: 19 to 23 nucleotides in length, a guanine or cytosine at the 3' end, possess a GC content higher than 50% and a melting temperature of 58°C

(Rozen and Skaletsky, 2000; Abd-Elsalam, 2003).

Contig files from the Mkushi genome sequence were mined for microsatellite repeats using MSATCOMMANDER (Faircloth, 2008; http://code.google.com/p/MSATCOMMANDER/). MSATCOMMANDER is a publicly available software package, designed for the purpose of rapidly and automatically identifying microsatellites and outputting the data in a locus specific manner with an added option of designing primers to that region by utilising PRIMER3 software (Faircloth, 2008; Rozen and Skaletsky, 2000). The length of each type of microsatellite was user-selected where dinucleotide repeats were selected with a minimum of 8 repeats, trinucleotides, tetranucleotides and pentanucleotides with a minimum of 6 repeats, and hexanucleotides with a minimum of four repeats. A total of 6389 potential microsatellite regions were identified.

Microsatellite repeats were selected from large contigs in the region of 500Mb in length, to reduce the probability of linkage. Pure microsatellites consisting of tri-nucleotide repeats were primarily selected followed by di-, tetra-, penta- and hexanucleotide repeats respectively. Sequences displaying more than five mononucleotide repeats in the flanking regions of the microsatellites were discarded. Primers were designed with a minimum of 30bp up and downstream of the microsatellite region using CLCBio and Primer3 with an annealing temperature of 58°C for the purpose of multiplexing (Singh and Nait, 1998; CLC Bio Main Workbench; Rozen and Skaletsky, 2000; You et al, 2008). Primers were designed to amplify fragments of between 120bp and 450bp in length to ensure sufficient multiplexing.

2.4 Amplification, Polymorphism and Sequencing of Microsatellites for *Cercospora zeina*

Primers were tested for amplification success using the Zambian (Mkushi) and American strains (OYPA). PCR reactions of volume 20µl consisted of 5X MyTaq™ reaction buffer (Bioline, Cape Town), 10µM of each primer (Integrated DNA Technologies), 1U of MyTaq™ polymerase

(Bioline, Cape Town), 10ng gDNA and MilliQ water. The cycling parameters were constant for all steps but the annealing step was varied to determine optimal primer annealing temperature. Initial denaturation was performed at 94°C for 1 minute followed by 30 cycles of 15s denaturation at 94°C, 15s annealing of primers at 54°C, 56°C or 58°C and 20s elongation at 72°C, followed by a final elongation step at 72°C for 1 minute. All PCR products were electrophoresed on 3% agarose gels stained with ethidium bromide and visualised under ultraviolet light using the Geldoc station (BioRad, South Africa) and viewed using Image Lab v3.0 (BioRad Laboratories, 2010). Primers that required further optimisation with magnesium chloride were later discarded to reduce laboratory complexity.

To test for polymorphisms, five isolates from a wide geographic area were used to screen all the microsatellite primers. These isolates included one from Zambia (Mkushi) an isolate from the United States (OYPA) and three South African isolates from Greytown, Cedara and Mpumalanga.

The PCR cycling parameters were carried out as described above with the annealing temperature optimised to 58°C. PCR amplicons were analysed by means of gel electrophoresis and those that displayed variation on gels were selected for sequencing to confirm the polymorphism was in the microsatellite region and not in the flanking regions.

Prior to sequencing, PCR products were cleaned using ethanol-salt purification and precipitation. Five microlitres 125mM EDTA and 60µl cold 70% ethanol was added to the PCR product and incubated at 25°C for 15 minutes, followed by centrifugation at 13400rpm for 15 minutes. The supernatant was discarded and the pellet washed with 70% ethanol, followed by centrifugation at 13400 rpm for 15 minutes. After removing the supernatant and allowing the excess ethanol to evaporate, the pellet was re-suspended in 10µl MilliQ water.

Sequencing reactions were set up in total volumes of 10µl as follows: 2µl BigDye® (Life Technologies), 1µl BigDye® buffer, 2µl PCR product, 1.6µl Primer (Forward or Reverse) and

3.4µl MilliQ water. Sequencing cycling parameters started with initial denaturation at 95°C for 10 minutes followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 20 seconds and elongation at 72°C for 30 seconds. A final elongation step at 72°C for 7 minutes completed the PCR cycle.

Microsatellite sequencing reactions were cleaned using the ethanol-salt purification and precipitation protocol described above. The products were sent to the University of Pretoria Sequencing Facility and sequenced on an ABI3500xl (Applied Biosystems). Sequences were viewed, aligned and analysed using CLC Main Workbench v6.0 (CLCbio). Isolates that displayed polymorphism in the microsatellite region between at least one pair of the five isolates were selected for fluorescent labelling of primers. These sequences are summarised in Annexure D, Mkushi sequences for all markers were deposited in the Genbank database, and accession numbers are in viewed Annexure D.

Polymorphic microsatellite primers were fluorescently labelled (Life technologies) with one of four available dyes on the 5' end of the forward primer, viz. 6-FAM™ (blue), VIC® (green), NED™ (yellow) and PET® (Red). Primers that could be pooled together into one run were arranged into a panel. A panel is the collection of microsatellites that can be analysed together. Each panel was set up such that primers that amplified fragments of the same size were labeled with different dyes.

To test whether the fluorescently labeled primers were able to amplify the correct microsatellite regions, three isolates (Mkushi, OYPA and Greytown) that displayed polymorphism in microsatellite sequences were used. PCR reactions with total volume of 10µl consisted of 10µM fluorescently labelled forward primer (Life Technologies), 10µM non-labelled reverse primer (Life Technologies), 5x MyTaq reaction buffer (Bioline, Cape Town), 1U of MyTaq™ DNA polymerase (Bioline, Cape Town), 10ng gDNA and distilled water. The cycling conditions were 1 min initial denaturation at 94°C followed by 30 cycles of 15s denaturation at

94°C, 15s annealing of primers at 58°C, and 20s elongation at 72°C, followed by a final elongation step of 1 min at 72°C. Amplification of PCR products were verified as described above. A dilution series for each microsatellite marker was performed to test the optimal dilution of product with water at 1:10, 1:25, 1:50 and 1:100 for ABI3500xl Genetic Analyzer (Life Technologies) analysis. The fragment data was analysed by GeneScan™ software and viewed using GeneMapper® v4.1 software (Life Technologies).

All *Cercospora* isolates were included for analysis on the Genetic Analyser (*Cercospora spp., Cercospora zeina, Cercospora zeae-maydis* and *Cercospora* species from sorghum), the Mkushi isolate was used as the positive control in each run. PCR reactions were performed in 96 well plates for each marker and later pooled into panels. PCR reactions of volume 10µl per well consisted of 5x MyTaq reaction buffer (Bioline), 10µM of each primer (Life Technologies), 1U of MyTaq™ DNA polymerase (Bioline), 10ng gDNA and distilled water. The cycling conditions were 1 min initial denaturation at 94°C followed by 30 cycles of 15s denaturation at 94°C, 15s annealing of primers at 58°C (for all but CzSSR10 and CzSSR12) and 59°C (for CzSSR10 and CzSSR12), and 20s elongation at 72°C, followed by a final elongation step of 1 min at 72°C. A dilution series was performed for each marker with respect to its optimal dilution concentration and then pooled into its respective panel (Table 7).

After preparing dilutions and pooling PCR products, samples were added to a formamide mixture containing LIZ®500 size standard (Life Technologies). A master mix of 1000:14 ratio of Formamide:LIZ®500 Size Standard (Life Technologies) was prepared and 10µl were aliquoted into each well of a 96 well plate. One microlitre for each pooled panel per sample was aliquoted into each well containing Formamide-LIZ®500 Size Standard. Following preparation, the 96 well plates were denatured at 95°C for 3 minutes prior to analysis on the ABI3500xl Genetic Analyzer (Life technologies).

2.5 GeneScan™ Analysis

The ABI Genetic Analyzer 3500xl (Life Technologies) uses the G5 Filter to detect the relative size of the fluorescing amplicons using GeneScan™ analysis software. The data obtained from GeneScan™ analysis is interpreted by the software package GeneMapper® v4.1 (Life Technologies). The electropherograms displayed in GeneMapper® v4.1 (Life Technologies) represent the microsatellite fragment lengths as peaks which are interpreted as alleles (Hulce et al, 2011).

Using the dilution test as a platform from which to analyse the population, using GeneMapper® v4.1 parameters were set to score specified markers into bins in each panel. Although the program allocated alleles to the assigned bins, each marker was individually analysed manually to ensure scoring was performed correctly. Fragment lengths were scored as alleles. Every allele per marker for each isolate was captured in an excel spreadsheet and used for data analysis.

2.6 Data Analysis and Statistical Inference

To validate whether the sample size and number of microsatellite markers used were sufficient and statistically significant, genotypic diversity was plotted against the number of loci. Multilocus was used to perform this analysis for 1000 repetitions (Agapow and Burt, 2001).

2.6.1 Alleles, Gene Diversity and Linkage Disequilibrium

Allelic frequency and gene diversity were calculated in POPGENE v1.31 (Nei, 1973; Burnett, 2003). Allelic frequency is the number of times an allele occurs at a specific locus in a population (McDonald, 1994). Gene diversity is subsequently calculated by $h = 1-\Sigma_k-\omega_{k^2}$ where ω_k is the frequency of the k^{th} genotype (Nei, 1973). Samples were organised into population subsets as follows: all isolates, isolates from South Africa only,

isolates grouped by their province Kwa-Zulu Natal, Mpumalanga or the North West, and isolates from the geographical sites (example: Greytown, Cedara) collected during each season (2011, 2012 and 2013). Allelic frequencies and gene diversities were calculated for all these populations.

The genetic differentiation (G_{ST}) between the population subsets by region was calculated using POPGENE version 3.1 (Yeh and Boyle, 1997; Wright, 1978). Gene flow (Nm) between populations was determined using the estimate of G_{ST}, whereby $Nm = 0.5(1-G_{ST})/G_{ST}$ (Yeh and Boyle, 1997; McDermott and McDonald, 1993).

To obtain a broader perspective of the relationship between each geographical population (United States, Zambian, Zimbabwean, from Kwa-Zulu Natal, Mpumalanga and North West in South Africa), POPGENE v3.1 was used to generate a UPGMA tree using Nei's (1972) genetic distance. A neighbour-joining tree was generated for all isolates using Populations v1.2.32 using Nei's (1983) distance (Da). Furthermore Populations v1.2.32 was used to analyse individuals in their corresponding population.

Private alleles were identified using HP-RARE, a software package that determines rarefaction within populations (Kalinowski, 2005). A private allele is an allele restricted to the distribution of a particular population. Although a private allele may be an artefact of poor sampling, an allele that is not shared amongst populations can be used as region specific marker (Wolf and Soltis, 1992).

2.6.2 Measures of Multilocus Linkage Disequilibrium

The Shannon Index (SI) is the measure of genotypic diversity distribution within a population, and was determined using POPGENE version 3.1 (Yeh et al, 1997; Lewontin, 1972). Shannon's Index is calculated by $SI = -\Sigma pi \times \ln pi$, where *pi* represents the

proportional value of isolates belonging to the i^{th} population. As the number of genotypes along with the evenness of genotypic distribution increases, SI will also increase (Yeh et al, 1997; Lewontin, 1972).

Multilocus linkage disequilibrium (the effect of sexual recombination) on genetic diversity was measured by calculating the Index of Association (I_A) and $\bar{r}_d$ using Multilocus v1.3b (Brown et al, 1980; Agapow and Burt, 2001; Maynard Smith et al, 1993). A particular allele at a locus that is observed with another particular allele at a different locus repeatedly in individuals was investigated. If these alleles are repeatedly identified in multiple individuals, it would indicate that the loci are genetically linked. I_A is determined by the variance of loci that are shared between individuals as a measure against the expected variance to determine linkage disequilibrium (Brown et al. 1980). A modified statistic ($\bar{r}_d$) makes allowance for the dependency of I_A upon the number of markers included within the study. This allows comparisons to be made between studies using different number of loci. Both I_A and $\bar{r}_d$ were calculated for 1000 projected randomisations. The observed I_A and $\bar{r}_d$ value for the population should fall within the range produced by 1000 randomisations at a p-value > 0.05 to validate whether sexual recombination is occurring in addition to determining whether alleles are linked.

2.6.3 Population structure

To determine the population structure the software program STRUCTURE v2.3.4, which determines the probability distribution (Pr) of an individual was used. The probability of Pr($X|Z,P$) was determined, where X denotes the isolates haplotype, Z denotes the population of origin and P denotes the allelic frequencies in all the populations (Evanno et al, 2005). The number of optimal populations (K) was investigated by forecasting a

value for *K* and validating the value of *K* through statistical inference following the simulation distribution (Pritchard et al, 2000; Evanno et al, 2005). Parameters were defined as *K* number of values between 1 and 10 (populations predicted between 1 and 10), with a 50 000 burnin and 200 000 Markov chain Monte Carlo (MCMC) method, subsequent to 10 iterations (Pritchard et al, 2000). Both Admixture and No-admixture models were tested with and without LOCPRIOR (the geographical location of the individual is taken into consideration). In addition, correlated and independent allelic frequencies were both tested (Falush et al, 2003).

GenAlEx6 was used to determine from where variability ensues between isolates in South Africa when grouped geographically and temporally. An analysis of molecular variance (AMOVA) was performed with 10000 permutations with confidence at p-value <0.002 (Peakall and Smouse, 2006).

Additionally, population differentiation was demonstrated using Weir's θ statistics. Theta (θ) was determined for 1000 randomisations by $\frac{A-B}{1-B}$, where *A* is the probability that two alleles from the same population are the same, and *B* is the probability that two alleles from different populations are the same (Agapow and Burt, 2001).

Results

3.1 Fungal Isolates and DNA Extraction

The quantity of isolates of *Cercospora zeina* and a *Cercospora* species identified on sorghum, collected from each geographical region and year (2007-2013, used in this study is summarised in Table 4. The total inumber of solates used for comparative population analysis per region from Cedara, Baynesfield, Greytown and Machadodorp over the 2011-2013 seasons amounted to 55, 36, 131 and 110 respectively. In total, 398 isolates of *C. zeina* were used for the population analysis of GLS of maize in South Africa (397 RSA isolates collected in 2007 to 2013, as well as the type culture of *C. zeina* CPC11998 an isolate from KZN).

Table 4. Isolates of *Cercospora zeina* included in this study isolated from maize material collected in southern Africa, during the 2007 and 2011-2013 seasons.

Year	Collection Region	Province/Country	Collector	*Cercospora* species	No. of isolates	Total
2007	Greytown	KZN/RSA	Barbara Meisel	*C. zeina*	13	34
	Cedara	KZN/RSA	Barbara Meisel	*C. zeina*	1	
	Karkloof	KZN/RSA	Barbara Meisel	*C. zeina*	8	
	Empangeni	KZN/RSA	Barbara Meisel	*C. zeina*	2	
	Winterton	KZN/RSA	Barbara Meisel	*C. zeina*	4	
	Zimbabwe	Zimbabwe	Barbara Meisel	*C. zeina*	1	
	Zambia	Zambia	Barbara Meisel	*C. zeina*	5	
2011	Greytown	KZN/RSA	Mischa Muller	*C. zeina*	29	91
	Cedara	KZN/RSA	Mischa Muller	*C. zeina*	15	
	Machadodorp	MP/RSA	Mischa Muller	*C. zeina*	47	
2012	Baynesfield	KZN/RSA	Mischa Muller	*C. zeina*	26	170
	Cedara	KZN/RSA	Mischa Muller	*C. zeina*	11	
	Greytown	KZN/RSA	Mischa Muller	*C. zeina*	64	
	Winterton	KZN/RSA	Syngenta	*C. zeina*	20	
	Machadodorp	MP/RSA	Mischa Muller	*C. zeina*	29	
	Klerksdorp	NW/RSA	PANNAR	*C. zeina*	20	
2013	Baynesfield	KZN/RSA	Mischa Muller	*C. zeina*	10	115
	Greytown	KZN/RSA	Mischa Muller	*C. zeina*	35	
	Greytown	KZN/RSA	Mischa Muller	*C. species*	7	
	Cedara	KZN/RSA	Mischa Muller	*C. zeina*	29	
	Machadodorp	MP/RSA	Mischa Muller	*C. zeina*	34	
						410

KZN - Kwa-Zulu Natal; MP - Mpumalanga; NW - North West; RSA - Republic of South Africa

A total of 413 isolates were used in this study. Seven isolates of the 413 were a *Cercospora* species isolated from sorghum and collected in Greytown. Five isolates from Zambia and one isolate from Zimbabwe were also included. One isolate, OYPA, from the United States was also included in this study. Furthermore, CPC12062, the type culture for *Cercospora spp.* was also included in this study.

3.2 Histone PCR

All isolates from maize material that displayed GLS symptoms produced a band of 284bp using primer pair CzeinaHIST/CYLH3R (Crous et al, 2006) confirming their identity as *Cercospora zeina.* Isolate CPC11998, the type culture for *Cercospora zeina,* and isolates CBS1177556 and CBS117762 of *Cercospora zeae-maydis* were used as positive controls (Figure 6).

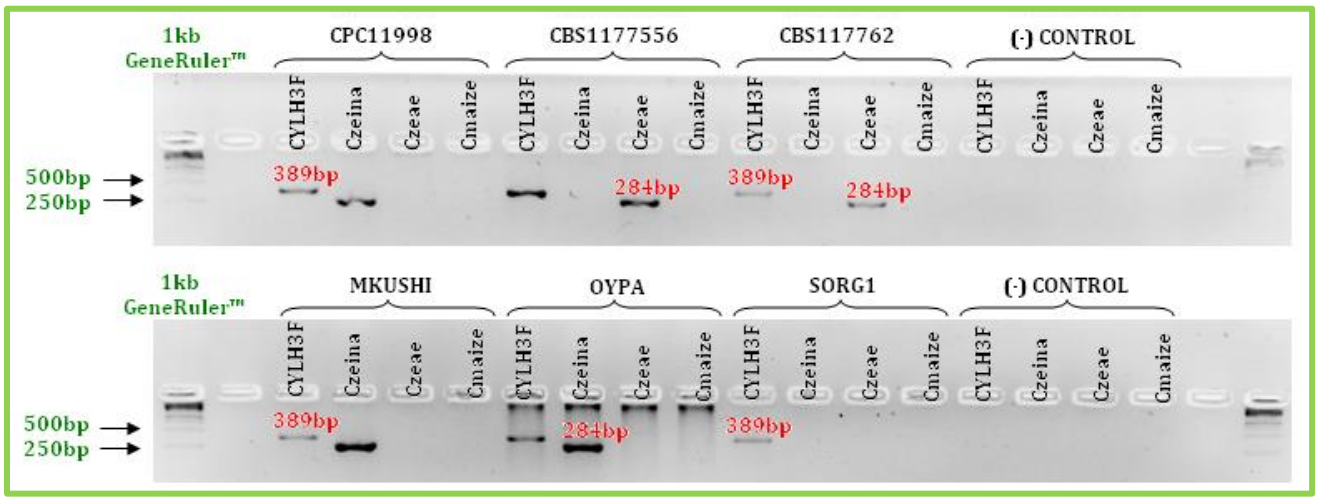


Figure 6. A 2% agarose gel illustrating the use of the diagnostic histone PCR designed for *Cercospora sp.* identification using the type cultures for *Cercospora zeina* and *Cercospora zeae-maydis* as positive controls. Primers CYLH3F, Czeina, Czeae and Cmaize were used. A 1kb GeneRuler™ (Thermo Scientific) was used for sizing. CPC11998, Mkushi (Zambian isolate) and OYPA (American isolate) are confirmed to be *Cercospora zeina*. Similarly, isolates CBS1177556 and CBS117762 are confirmed to be *Cercospora zeae-maydis* isolates.

Although Mkushi and OYPA could be positively identified as *Cercospora zeina* (Figure 6), SORG1

could not be determined at a species level. SORG1 is an isolate cultured from GLS diseased Sorghum leaves from Greytown and was shown to be neither *C. zeina* nor *C. zeae-maydis* (Figure 6). An additional seven isolates made from infected sorghum showing GLS symptoms could not be identified at a species level either by means of the diagnostic histone PCR. The generic histone PCR primers amplified the Histone H3 region of 389bp, indicating that the isolate belongs to the genus *Cercospora* (Crous et al, 2006). To correctly identify the isolates, the ITS region was amplified using primers ITS 1 and ITS 4 and sequenced (White et al, 1990). The sequence was subjected to a blast search against the NCBI database and aligned closely to the Genbank accessions AF291707 (*Cercospora sorghi*) and AF297233 (*C. sorghi f. maydis*) in CLC Main Workbench v6.0 (Figure 12, Annexure A). The *Cercospora* species from sorghum were most closely related to *C. sorghi f. maydis* as indicated in the neighbour joining tree (Figure 13) in Annexure A.

3.3 Microsatellite Mining and Primer Design

In previous work, 36 microsatellites were designed using SSRIT and Primer3 (Muller, 2011). These primers were further considered and utilised in this study. The draft *C. zeina* Mkushi genome sequence contained 48969 contigs with N50 value of 145745 (van Staden, 2013). Using MSATCOMMANDER, 6389 microsatellites were discovered on 1295 contigs and 39 primer sets were designed flanking the microsatellite sequences of interest using CLC Main Workbench v6.0 and Primer3 (CLC Bio; Rozen and Skaletsky, 2000). These microsatellites are listed in Table 5.

Table 5: List of microsatellite primers designed for *Cercospora zeina*, indicating repeat motifs and software programs used for the identification and design of each marker.

SSR Name	Primer sequences 5' to 3'		Motif	Identification/ Design Program
	Forward	Reverse		
2011SSR01	CTACTGCCGGCTCCTACTTG	TGCTGCTTCTGCTACTGTGG	GA^23*	SSRIT/Primer3
2011SSR02	AATTAATCGTAAGCACGACGA	CTCCCTCCACAACCACAACT	TGT^15	SSRIT/Primer3
2011SSR03	GTTGCTGCTGTCATCCTCAG	TGGGATCTGGAACCCAAATA	TGT^23	SSRIT/Primer3
2011SSR04	TCCACGTCTGGTTCTGGAC	TTCGCATCACAACAACAACA	TGT^22	SSRIT/Primer3
2011SSR05	TCGAGAGGAGGAGAATTTCG	TTGATGAGATTGAGCTGACGA	TCA^15	SSRIT/Primer3
2011SSR06	CGTACCGGTAGGAGGTACGA	CGTGAAGGAAACAACGGATT	GT^25	SSRIT/Primer3
2011SSR07	ACAACCATGCAGCAACAATG	ATTCGCGCATACGGAACTAT	GAT^15	SSRIT/Primer3
2011SSR08	GGTTAGCGTGTAGCCGAGTT	ACGAAACCGAAACACAACAA	GTT^20	SSRIT/Primer3
2011SSR09	CCGCGTACTTCGCTGAAC	AGTGCACTGCCCAACGAC	GTGCT^20	SSRIT/Primer3
2011SSR10	CTTCGACTACGTTGCGTTGA	AGCCCTTGACAGCACTGACT	TAC^17	SSRIT/Primer3
2011SSR11	CGAAAGTATTGTCCGCGACT	GCAGGTTTCTGCTTCTGCTT	GAA^19	SSRIT/Primer3
2011SSR12	TCCCTCCAGCTCTTGTGTG	GCAGAGGCAATGGCACAG	CA^17	SSRIT/Primer3
2011SSR13	CAGAAAGAAGGCACCAAAGC	GAGCAGGTTTAGTCGGAGGA	TCT^15	SSRIT/Primer3
2011SSR14	CAAGAATGCCAATGATGCTG	GTCTCCTTTCTGGCGAAGTG	ATG^23	SSRIT/Primer3
2011SSR15	GTAACTCCGCGAGATTCCTG	AGCAGCAGCAGCAGTAACAA	TGT^17	SSRIT/Primer3
2011SSR16	TTCTGGCATTTCCTTGAACC	CCTTGCAAAGGAGGAGAAGA	TCA^19	SSRIT/Primer3
2011SSR17	ATGCAGCAACTGCAACAAC	GAGTCCTCTTCGGTGGTGTC	CAA^18	SSRIT/Primer3
2011SSR18	TCGAGAGAATAAGGCGAAGC	CCCCTACCCAACTCTTCCTC	CTT^17	SSRIT/Primer3
2011SSR19	GCGTTACTTCGAAGGTGCTT	GTTGGTCGTTTGTTTTGTCCT	ATG^16	SSRIT/Primer3
2011SSR20	TGATAATCGAGACCGCACAA	GTCGATGGTTGGTGTTGTTG	TCA^18	SSRIT/Primer3
2011SSR21	GACGACAACGACAAAGACGA	ATCATCATCACCGGTTGGTT	GAT^18	SSRIT/Primer3
2011SSR22	ATTCCGAGTCATCTGCGACT	CGGTCGGCCTCGTCTTAAT	ACT^35	SSRIT/Primer3
2011SSR23	AAGTTGGTCGGTTGATGAGG	ACAAAATGCCACGGAAAAAG	GT^20	SSRIT/Primer3
2011SSR24	GCCTAGAAAGCTTTTCTTTACGA	ACCCGTCCCGACCTATTCTA	AATA^15	SSRIT/Primer3
2011SSR25	TCCGGCTGTAAATTGTGTTG	CATTTGCGTCATTCCTTTCA	CAT^19	SSRIT/Primer3
2011SSR26	CGTCTGGCCATCTTCACCATC	CGCCGTAGGCGTAAAGAATA	TAG^36	SSRIT/Primer3
2011SSR27	GTACCGTCGGAACGTAGAGG	GCCGCCGTAGTAGTAGCAAT	CTATTA^19	SSRIT/Primer3
2011SSR28	CGACGAGAGTAATAGCGACGA	GAGGCGTTCGGTAGCTTAAA	TAC^18	SSRIT/Primer3
2011SSR29	TTTTAGCCGGTACTTTTCTAGC	GGTCGAGCTCGTCTCGATAA	CTA^16	SSRIT/Primer3
2011SSR30	ACGTACTATTTGTTCCGTTATTT	GGAGTTTCGAGTCGTATGCAG	CTA^42	SSRIT/Primer3
2011SSR31	CTAGGGCGTCTACCGAAACA	TTACCCGATACGCTCTACGG	GTA^15	SSRIT/Primer3
2011SSR32	GGTCCTCGTATACTCGCCTAA	ATAACTCGCCGTAGGCGTAA	TAG^48	SSRIT/Primer3
2011SSR33	AGAAGCTCGCTTACTAAATAGCC	GAGGCTTTTCTCTCGCAAAT	TTTA^23	SSRIT/Primer3
2011SSR34	CGACCGGTTAGGTATTAGGC	GAGATAGATCGCGAGGGATAA	ACT^25	SSRIT/Primer3
2011SSR35	CGCTTACTAAATAGCGCGTAAGA	ACTCCGCTACTATTTATTCCGATA	TAG^15	SSRIT/Primer3
2011SSR36	GAAGGCTTTTCTCTCGCAAA	TTGTCCCTCGGTCGCTTAT	AATA^19	SSRIT/Primer3
2012SSRA1	GAGAGATAGTTGCGGCGT	GATGATGATTTGAGGAGTGTTG	ACAGC^9	MSATCOMMANDER/CLCBio
2012SSRA2	GAGCAACAAGCAAGAGAAA	GAGGAAACTGTGGAAAGGA	AG^19	MSATCOMMANDER/CLCBio
2012SSRA3	CATTCTTTGTCCGCGTTC	CACTCACTTCCCACATAC	CGG^9	MSATCOMMANDER/CLCBio
2012SSRA4	ATTTGATTAGGGCTTCGG	GATGGAGATTGGGAAGTG	GAT^12	MSATCOMMANDER/CLCBio
2012SSRA5	TGAGAGGAGGAGGAGAATATG	CGGCCCTAAGAAACCAAA	GATT^16	MSATCOMMANDER/CLCBio
2012SSRA6	CACACATTCCTCAGCACA	AGCAACGACGACATGAAA	AATAC^4	MSATCOMMANDER/CLCBio
2012SSRA7	CCTCTCTCTCTTTCTCTCT	CTTACTTACTTGCCCTGTT	CTT^9	MSATCOMMANDER/CLCBio
2012SSRA8	GTGTCTTGGAGTGGTTTG	GAGAGGAGAGAAGACGAA	CGG^8	MSATCOMMANDER/CLCBio
2012SSRA9	CCCTCTCTCTCTTTCTCTCT	ACTTACTTACTTGCCCTGTT	AG^17	MSATCOMMANDER/CLCBio
2012SSRB1	ATCTTGGATATTCCCGTACTC	ATTCATTCATTCAACATCGTC	AGT^7	MSATCOMMANDER/CLCBio
2012SSRB2	TTTCTTTTCTACCCCTCAAAC	CGGATAAGGACCTCATACTTC	TCA^11	MSATCOMMANDER/CLCBio
2012SSRB3	TATTTGATATCGTGTCCTGCT	GTTTTGATCTAGGCTGATTCC	TTC^18	MSATCOMMANDER/CLCBio
2012SSRB4	TTTGTGTTCTTCTTCTGCTTC	TTACGAACAGTTCCATATGCT	CGA^10	MSATCOMMANDER/CLCBio
2012SSRB5	GCACGCTAAGAGGTGTGTATT	GACATGACGACATGACTGTTG	TGT^11	MSATCOMMANDER/CLCBio
2012SSRB6	CACGAGGCCAACATACAGTAG	GAGGGATGGTGGTGAGTATG	ACC^7	MSATCOMMANDER/CLCBio
2012SSRB7	GGTGTTTGTCGATAACCTGTG	CAAAATTCCAAAGAACCTCGT	CCT^9	MSATCOMMANDER/CLCBio
2012SSRB8	GTTACCGGAAGGTTGTGTTTT	ACGTTCGAAGACACTTTGATG	TC^17	MSATCOMMANDER/CLCBio
2012SSRB9	AGCCTACAGTGACCTCTCCTC	ATGACATGGTGGTGTGAGC	CT^17	MSATCOMMANDER/CLCBio
2012SSRB10	GACTCTTCTGGCTCATCTTCC	CATTGTATAACCAACGCCATT	CT^18	MSATCOMMANDER/CLCBio

* Motif GA^23 means the microsatellite motif GA is repeated 23 times for an isolate E.g. OYPA.

SSR Name	Primer sequences 5' to 3'		Motif	Identification/ Design Program
	Forward	Reverse		
2013SSR01	CAGTCGGACTCTTTCAGAACA	TGCTCTCCATTCGTCTCTCTA	TGT^10	MSATCOMMANDER/CLCBio
2013SSR02	GCATAATCCTCAGTGTCATCG	TGAGTGGAATGAACGAATGAT	ATC^12	MSATCOMMANDER/CLCBio
2013SSR03	ACGTAGAATTTGCAGTTGGTG	CATCGCAATCATTTGAAAAAC	CA^21	MSATCOMMANDER/CLCBio
2013SSR04	TTGTTGATGCTGATGTTGATG	AATGACAGAAAGGATGGAAGG	GA^29	MSATCOMMANDER/CLCBio
2013SSR05	GAGCTTCAGCAAAACAACAGA	AAGATGACCTTGGTGTCCTTG	CTC^7	MSATCOMMANDER/CLCBio
2013SSR06	ACACAATGTACCACCTCCAAA	ACGATGACGATGACCAAATAG	TC^9	MSATCOMMANDER/CLCBio
2013SSR07	GAACATGTCTTCCTTCCCTTC	CTACTGCCGACTCTTTTGATG	TAT^10	MSATCOMMANDER/CLCBio
2013SSR08	TGATCTTGCTGGAAATTCTTG	TTGACAATTGTTACGCCAAGT	TCAA^7	MSATCOMMANDER/CLCBio
2013SSR09	ATACCCAGCAGAAGGAAAATG	GAAACCTATTGGAGCTTTTCG	CTC^6	MSATCOMMANDER/CLCBio
2013SSR10	ATGCGTCAAAATCACACTTTC	AAAGCGTCTCCTCATCGATAC	CTC^9	MSATCOMMANDER/CLCBio
2013SSR11	GCGCATAAGATGTTGAATGTT	TGGTCGATAGATGACTGGAAG	GAT^9	MSATCOMMANDER/CLCBio
2013SSR12	AAGAACTCTCCAGTCGTCGTT	AAATGGGAAGAAGAAATCGTG	TAG^21	MSATCOMMANDER/CLCBio
2013SSR13	ACTTTGGACTTTGGACTTTGG	GATTGATTGACCGACTGATTG	CA^9	MSATCOMMANDER/CLCBio
2013SSR14	TCCAGACACTGGAAAGCATAC	TCAGCATCTCGCTTACTGAAC	AG^11	MSATCOMMANDER/CLCBio
2013SSR15	GAAGGGGTAGGTGATTGTGAG	CAACTTCTGCTGAATGGACTT	TTG^17	MSATCOMMANDER/CLCBio
2013SSR16	TATGCGCTGGCACTAGACTAC	CCTCGAACAACTTTCCATCTC	GA^30	MSATCOMMANDER/CLCBio
2013SSR17	TAGTCGTGATCATGTGCAAAG	ATCCGCCTCGAAGACATC	CCG^11	MSATCOMMANDER/CLCBio
2013SSR18	CCGTCTTTCCCATCTATTTTC	AGAGTAGTCGAAGGAGGCAAA	TC^14	MSATCOMMANDER/CLCBio

* Motif GA^23 means the microsatellite motif GA is repeated 23 times for an isolate E.g. OYPA.

3.4 Amplification, Polymorphism and Sequencing of Microsatellites for *Cercospora zeina*.

Nine of the 75 microsatellite primer pairs designed (Table 5) did not amplify either the Mkushi and OYPA isolates. Of the 66 primers that did amplify, 15 displayed multiple bands and were discarded. The remaining 51 primers presented clean bands, and were further tested for polymorphisms on a larger subset of isolates from three different geographical regions in South Africa. Amplified products for twenty-four primers showed polymorphism on a 3% agarose gel (data not shown). These fragments were sequenced to confirm the regions were polymorphic (Annexure D).

After sequencing, microsatellite regions amplified by 19 primer pairs showed polymorphism (these microsatellite markers are underlined in Table 5). The 19 polymorphic markers were further tested on the *Cercospora zeae-maydis* isolates and did not amplify microsatellites in any of the isolates (CBS1177556 and CBS117762) (data not shown). These markers were then tested on the *Cercospora sorghi* isolates and all produced amplification products (data not shown

Table 6. Details of fluorescently labelled polymorphic SSR markers used for genetic analysis of *Cercospora zeina* in South Africa, indicating primer sequences, microsatellite repeat motif, panel, fluorescent tags (attached to the end of the forward primer), marker allelic range and number of alleles.

New Marker Name	Forward Primer	Reverse Primer	Motif	Flourescent Tag	No. of Alleles	Alleles	Genbank Accession No.
CzSSR01	AATTAATCGTAAGCACGACGA	CTCCCTCCACAACCACACAACT	TGT^	6-FAM	4	152, 155, 158, 161	KP015832
CzSSR02	ACGTCTCGGAATCTCTTTCT	TGGGATCTGGAACCCAAATA	TGT^	6-FAM	4	342, 345, 348, 379	KP015833
CzSSR04	GGTTAGCGTGTAGCCGAGTT	CGACCAAGTGCTTGTCAAC	GTT^	VIC	4	459, 462, 465, 468	KP015834
CzSSR05	CTTCGACTACGTTGCGTTGA	AGCCCTTGACAGCACTGACT	ATG^	VIC	4	239, 242, 245, 248	KP015835
CzSSR06	CAGAAAGAAGGCACCAAAGC	GAGCAGGTTTAGTCGGAGGA	TCT^	6-FAM	4	220, 223, 233, 236	KP015836
CzSSR07	CAAGAATGCCAATGATGCTG	GTCTCCTTTCTGGCGAAGTG	ATG^	PET®	12	206, 209, 212, 214, 217, 220, 223, 226, 230, 233, 236, 239	KP015837
CzSSR08	GTAACTCCGCGAGATTCCTG	AGCAGCAGCAGCAGTAACAA	TGT^	VIC	4	190, 196, 199, 202	KP015838
CzSSR10	GCGTTACTTCGAAGGTGCTT	GTTGGTCGTTTGTTTTGTCCT	ATG^	6-FAM	4	175, 178, 191, 194	KP015839
CzSSR11	CGAGACTAATAGCGACGAGAG	GAGGCTTTTCTCTCGCAAAT	TTTA^	PET®	10	331, 335, 339, 345, 348, 352, 356. 360, 364, 368	KP015840
CzSSR12	GAAGGCTTTTCTCTCGCAAA	TTGTCCCTCGGTCGCTTAT	AATA^	NED	5	232, 236, 240, 244, 248	KP015841
CzSSR13	GAGAGATAGTTGCGGCGT	GATGATGATTTGAGGAGTGTTG	ACAGC^	NED	2	329, 334	KP015842
CzSSR14	GAGCAACAAGCAAGAGAAA	GAGGAAACTGTGGAAAGGA	AG^	NED	4	239, 241, 243, 245	KP015843
CzSSR15	CATTCTTTGTCCGCGTTC	CACTCACTTCCCACATAC	GGC^	PET®	2	245, 251	KP015844
CzSSR16	ATTTGATTAGGGCTTCGG	GATGGAGATTGGGAAGTG	GAT^	VIC	14	383, 386, 398, 401, 404, 407, 414, 436, 439, 442, 445, 448, 451, 454	KP015845
CzSSR17	GGTGTTTGTCGATAACCTGTG	CAAAATTCCAAAGAACCTCGT	CCT^	6-FAM	3	474, 480. 483	KP015846
CzSSR18	ATGCGTCAAAATCACACTTTC	AAAGCGTCTCCTCATCGATAC	CTC^	VIC	2	134-137	KP015847

The 19 primers were labelled with fluorescent dyes according to the G5 ABI platform and arranged into panels (see Table 6 and Figure 7). The primer pairs were further screened, optimised and analysed by the ABI3500xl genetic analyser using GeneScan™ software. Three markers (CzSSR03, CzSSR09 and CzSSR19) were excluded from further analysis due to the absence of peaks or peak patterns that could not be scored.

The final 16 fluorescently labelled primer pairs (Annexure D) were divided into two panels of eight primers each to be run on the Genetic Analyser ABI3500xl (Table 7). Each primer pair was amplified separately in a 96-well plate, and aliquots for each primer pair were pooled into their respective panel (Table 7). The dilution concentrations for each microsatellite marker in their pooled panel are indicated in Table 7. A diagrammatic representation of the 16 fluorescently labelled microsatellite markers and their expected fragment length range is shown in Figure 7.

Table 7. The optimised pooling of *Cercospora zeina* microsatellite marker PCR products into two panels prior to capillary electrophoresis and microsatellite fragment size analysis.

Panel 1 *			**Panel 2 ***		
Marker name §	**Volume #**		**Marker name §**	**Volume #**	
CzSSR10	1	µl	CzSSR01	1	µl
CzSSR17	1.5	µl	CzSSR06	1	µl
CzSSR18	1	µl	CzSSR02	1.5	µl
CzSSR05	1	µl	CzSSR08	1	µl
CzSSR16	1.5	µl	CzSSR04	1.5	µl
CzSSR12	1	µl	CzSSR14	1	µl
CzSSR13	1	µl	CzSSR07	1	µl
CzSSR15	2	µl	CzSSR11	2	µl
Water:	90	ul		90	µl
Total volume:	100	µl		100	µl

* Eight markers were pooled together for each panel to a total volume of 100µl. One microlitre of the pooled products was run on the ABI 3500xl for fragment analysis.
§ Marker names corresponding to Table 6
# The volume of PCR product for each marker, to be pooled. 1µl of the total 100µl volume is used to run on the ABI genetic analyser

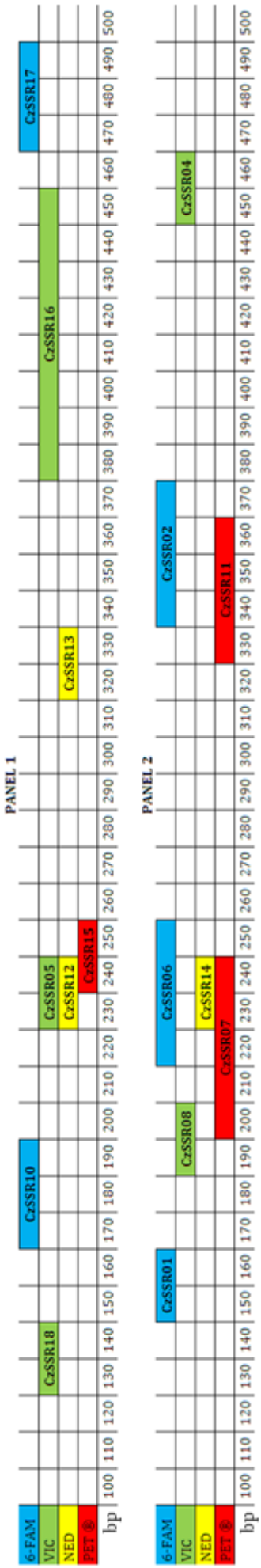


Figure 7. Diagrammatic representation of 16 microsatellite markers for *Cercospora zeina* organised into two pooling panels preceding capillary electrophoresis. Each panel consists of eight microsatellite markers, arranged according to fluorescent tags 6-FAM (blue), VIC (green), NED (yellow) and PET® (red) respectively, and expected fragment length range (in base pairs).

3.5 GeneScan™ Analysis

GeneScan™ analysis was performed for all 16 polymorphic SSR markers across 413 isolates (397 RSA *C. zeina*, 1 RSA *C. zeina* type (CPC11998), 5 Zambian *C. zeina*, 1 Zimbabwe *C. zeina*, 1 United States *C. zeina*, 7 *Cercospora* species on sorghum, 1 *Cercospora spp*. type (CPC12062)). Sixty-six alleles were identified across the entire population. The most polymorphic marker was CzSSR16, which had 14 alleles. CzSSR07 closely followed this with 12 alleles and CzSSR11 with 10 alleles (Table 6).

Twelve microsatellite markers were straightforward to analyse. Two markers (CzSSR07 and CzSSR12) required closer inspection due to stutter bands, and two markers (CzSSR14 and CzSSR16) necessitated re-amplification and re-analysis.

3.6 Data Analysis and Statistical Inference

The mean genotypic diversity against microsatellite loci in this study plateaued at 12 loci, it can be extrapolated that 16 microsatellite markers are sufficient to use in this study (Figure 8).

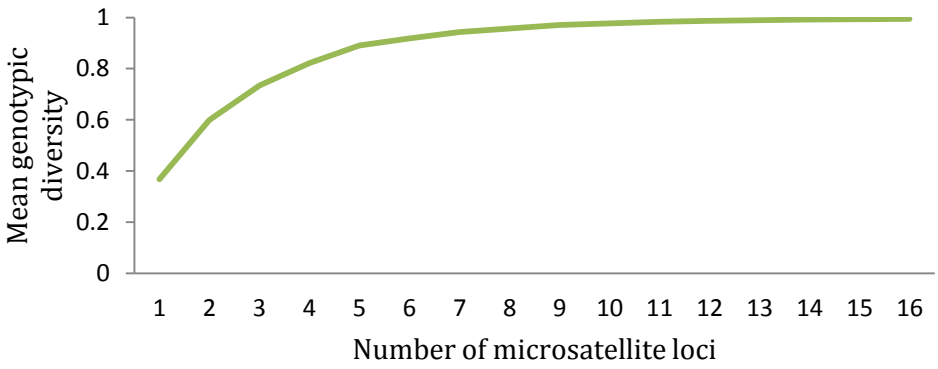


Figure 8. A line graph depicting the mean genotypic diversity against the number of microsatellite markers employed for the population study of *Cercospora zeina*. The graph reached a plateau at 12 microsatellite loci, which indicates an adequate number of

microsatellite markers have been developed in this study.

3.6.1 Alleles, Gene Diversity and Linkage Disequilibrium

The US isolate OYPA possessed a number of private alleles for markers CzSSR02, CzSSR07, CzSSR08, CzSSR13, CzSSR16 and CzSSR17 as indicated in Table 8. Eight private alleles were identified for marker CzSSR16 in isolates from Kwa-Zulu Natal and the North-West Province. No private alleles were detected for marker CzSSR16 in isolates from Mpumalanga. In contrast, private alleles were observed in isolates from Mpumalanga for markers CzSSR06, CzSSR07, CzSSR08 and CzSSR12.

Private alleles constrained to the Kwa-Zulu Natal province, were further restricted to the geographical regions from which samples were collected. For example, private alleles for marker CzSSR16 for Kwa-Zulu Natal were obtained in Cedara, Greytown and Baynesfield. Alleles 407, 445 and 454 were obtained from Cedara, 383 from Greytown and 414 from Baynesfield. No private alleles were identified for the *Cercospora* species isolated from sorghum.

Table 8. A summary of all private alleles, indicating its allele size observed for microsatellite markers designated to geographical populations of *Cercospora zeina*. Alleles highlighted in yellow indicate that there are more than one isolate with this allele within a field.

Marker	Alleles restricted to geographical region					
	KZN	**MP**	**NW**	**Zam**	**Zim**	**USA**
CzSSR01	152					
CzSSR02	348				342	379
CzSSR05	248			239		
CzSSR06		220				
CzSSR07	220	214, 223, 239				217
CzSSR08		202				190
CzSSR10	194	178				
CzSSR11	331, 339, 345, 368				335	
CzSSR12	248	232				
CzSSR14	239					
CzSSR16	383, 407, 414, 445, 454		386, 448			451
CzSSR17						483

* There were no private alleles identified for markers CzSSR04, CzSSR13, CzSSR15 and CzSSR18

Allelic richness indicated in Table 9, describes the level of allelic diversity across a population of isolates. The data shown in Table 9 indicates that there is only one allele per marker for the seven isolates of the *Cercospora* species obtained from sorghum samples in Greytown, Kwa-Zulu Natal. This indicates that all isolates belonging to the *Cercospora* species isolated from sorghum are most likely clones. This haplotype was also identified in *C. zeina* isolates, in three isolates collected in 2007 from Greytown, two isolates collected from Greytown in 2012 and one isolate collected in 2012 from the North West province. Collectively this haplotype has been labelled haplotype G01 in Annexure B.

The *Cercospora spp*. (CPC 12062) isolate does not share any haplotypes with *C. zeina* nor isolates from sorghum. It does, however, share a unique allele 334 for marker CzSSR13, which is common to OYPA. Although it is a different species to *C. zeina*, it was included in the analysis to obtain a general idea of where its haplotype may group, and so grouped with isolates obtained from Winterton in 2007 (Annexure B).

Kwa-Zulu Natal possesses the highest average allelic richness over Mpumalanga and the North West province, which could be attributed to the larger sample size. There is a lower allelic richness observed for North West isolates when comparing markers CzSSR01, 2, 5, 6, 8 and 12, to isolates in Kwa-Zulu Natal and Mpumalanga. In contrast marker CzSSR16 displays a higher allelic richness for isolates from the North West province than any other region.

Nei's (1972) gene diversity was determined for all isolates studied, with a mean value of 0.375 observed (Nei, 1972), (Table 10). Gene diversities were further calculated for South African isolates with h = 0.364. Kwa-Zulu Natal, Mpumalanga and the North West province possess gene diversities of 0.346, 0.349 and 0.219 respectively.

Table 9. A summary of the calculated allelic richness observed for microsatellite markers designated to geographical populations of *C. zeina*.

		Geographic region					
		KZN	MP	NW	Zambia	*C. Sorgi* (KZN)	
	No. isolates	270	110	20	5	7	
Microsatellite markers	CzSSR 01	2.33	2.26	1.00	2.00	1.00	2.22
	CzSSR 02	1.12	1.00	1.00	1.00	1.00	1.08
	CzSSR 04	2.67	2.37	2.00	3.00	1.00	2.53
	CzSSR 05	2.22	1.99	1.00	3.00	1.00	2.08
	CzSSR 06	2.00	2.25	1.00	2.00	1.00	2.00
	CzSSR 07	2.85	3.31	2.53	1.00	1.00	2.90
	CzSSR 08	1.99	2.46	1.00	1.00	1.00	2.04
	CzSSR 10	2.24	1.90	1.88	2.00	1.00	2.11
	CzSSR 11	3.71	2.98	1.88	2.00	1.00	3.35
	CzSSR 12	1.68	1.81	1.00	2.00	1.00	1.67
	CzSSR 13	1.00	1.00	1.65	1.00	1.00	1.03
	CzSSR 14	2.87	2.37	3.00	2.00	1.00	2.69
	CzSSR 15	2.00	2.00	2.00	2.00	1.00	1.98
	CzSSR 16	3.52	2.56	4.38	2.00	1.00	3.24
	CzSSR 17	2.00	1.95	2.00	2.00	1.00	1.97
	CzSSR 18	2.00	2.00	1.97	2.00	1.00	1.98
							Average allelic richness

Two hundred and sixty five multilocus haplotypes were identified from the clone corrected data set with a clonal fraction of 0.35. Clones with the same multilocus haplotype could be identified in the same field during a season. Furthermore, multilocus haplotype were identified in the same field over more than one season. Moreover mutilocus haplotypes were shared over different seasons from different provinces. One haplotype in particular was identified from an isolate collected in Greytown in 2007 and again detected in 2012. This isolate was again detected in 2012 in the North West province.

Nei's (1972) gene diversities are measures of the allelic diversity for each SSR marker. The allelic frequencies impact on the allelic diversity as a measure of the distribution of an allele. Therefore the allelic diversity for each marker will impact on the level of gene diversity observed. While Nei's (1972) mean gene diversity (H) was relatively high for most markers, it compares to the level of allelic diversity observed for each marker.

Table 10. Overall summary of allelic statistics, Nei's gene diversity and Shannon's Index statistics for all 16 microsatellite markers for clone corrected and non-clone corrected data for all *Cercospora zeina* isolates.

			Gene diversity				
SSR Marker Name	**Not clone corrected data**				**Clone corrected data**		
	Na[α]	N[β]	H[γ]	I[δ]	N[β]	H[γ]	I[δ]
CzSSR01	5	405	0.446	0.723	293	0.497	0.794
CzSSR02	3	405	0.019	0.06	293	0.02	0.064
CzSSR04	4	405	0.52	0.854	293	0.548	0.885
CzSSR05	5	405	0.434	0.697	293	0.459	0.699
CzSSR06	3	405	0.38	0.597	293	0.416	0.635
CzSSR07	12	405	0.488	0.95	293	0.544	1.036
CzSSR08	4	405	0.439	0.68	293	0.483	0.742
CzSSR10	4	405	0.305	0.535	293	0.371	0.62
CzSSR11	10	390	0.483	1.007	293	0.606	1.29
CzSSR12	5	405	0.107	0.286	293	0.118	0.309
CzSSR13	2	405	0.014	0.043	293	0.02	0.057
CzSSR14	5	405	0.553	0.926	293	0.506	0.795
CzSSR15	3	405	0.47	0.675	293	0.488	0.681
CzSSR16	14	405	0.511	1.058	293	0.569	1.181
CzSSR17	3	405	0.39	0.591	293	0.425	0.633
CzSSR18	2	405	0.437	0.628	293	0.456	0.648
Mean	5.25	404	0.375	0.644	293	0.408	0.692
St. Dev	3.5684		0.174	0.303		0.187	0.34

[α] Na = Observed number of alleles
[β] N = Sample size
[γ] H = Nei's (1972) gene diversity
[δ] I = Shannon's Information index [Lewontin (1972)]

Table 11. Overall summary of allelic frequencies for 16 microsatellite markers analysed for clone-corrected and non clone-corrected data.

Dominant alleles are bolded below.

Non clone corrected data

Locus	CzSSR01	CzSSR02	CzSSR04	CzSSR05	CzSSR06	CzSSR07	CzSSR08	CzSSR10	CzSSR11	CzSSR12	CzSSR13	CzSSR14	CzSSR15	CzSSR16	CzSSR17	CzSSR18
A	0.005	0.002	0.007	**0.695**	0.005	0.007	0.002	**0.818**	0.003	0.017	**0.993**	0.002	**0.630**	0.002	**0.736**	**0.678**
B	**0.685**	**0.990**	**0.598**	0.291	0.243	0.264	**0.683**	0.167	0.003	0.012	0.007	**0.569**	0.371	0.005	0.262	0.322
C	0.295	0.005	0.351	0.010	**0.751**	0.010	0.305	0.012	0.003	**0.947**		0.085		0.007	0.002	
D	0.015	0.002	0.044	0.005	0.002	0.002	0.010	0.002	0.021	0.019		0.344		**0.656**		
E						0.002			0.180	0.005				0.017		
F						0.005			**0.692**					0.002		
G						0.002			0.069					0.002		
H						0.010			0.021					0.010		
I						**0.666**			0.005					0.240		
J						0.024			0.005					0.046		
K						0.005								0.005		
L						0.002								0.002		
M														0.002		
N														0.002		

Clone corrected data

Locus	CzSSR01	CzSSR02	CzSSR04	CzSSR05	CzSSR06	CzSSR07	CzSSR08	CzSSR10	CzSSR11	CzSSR12	CzSSR13	CzSSR14	CzSSR15	CzSSR16	CzSSR17	CzSSR18
A	0.007	0.000	0.007	**0.659**	0.007	0.010	0.003	**0.761**	0.003	0.021	**0.990**	0.003	**0.577**	0.003	**0.696**	**0.649**
B	0.003	**0.990**	**0.509**	0.328	0.280	**0.345**	0.621	0.222	0.000	0.010	0.010	**0.594**	0.423	0.007	0.300	0.352
C	**0.608**	0.007	0.437	0.014	**0.710**	0.014	0.362	0.014	0.003	**0.939**		0.027		0.007	0.003	
D	0.365	0.003	0.048	0.000	0.003	0.003	0.014	0.003	0.021	0.024		0.375		**0.594**		
E	0.017					0.003			0.184	0.007				0.024		
F						0.003			**0.587**					0.003		
G						0.003			0.089					0.003		
H						0.010			0.027					0.014		
I						0.580			0.079					0.273		
J						0.017			0.007					0.058		
K						0.007								0.003		
L						0.003								0.003		
M														0.003		
N														0.003		

Microsatellite markers CzSSR02 and CzSSR13 exhibited low gene diversities with Nei's gene diversities of 0.02 and 0.01 respectively, for non-clone corrected data. Although CzSSR02 consists of four alleles, 99% of the isolates possess allele 345, with allele 342 belonging to the isolate from Zimbabwe, allele 348 belonging to two isolates from Baynesfield, and allele 379 belonging to OYPA (Table 11). Similarly, CzSSR13 comprises two alleles, 334 and 329, 99% of all isolates possess allele 329, while the *Cercospora spp*. and the OYPA isolate possessing allele 334 (Table 11). In contrast to the low gene diversities observed for CzSSR02 and CzSSR13, marker CzSSR16 has 14 alleles and has a high gene diversity with h = 0.51 for non-clone corrected data (Table 10). Similarly, microsatellite marker CzSSR14 possesses a gene diversity with h = 0.55 for non-clone corrected data (Table 10).

For all markers, a dominant allele could be identified, with over 50% of the isolates possessing the dominant allele (Table 11).

Table 12. A summary of statistics for non-clone corrected data of all *C. zeina* isolates used in this study, divided into their population groups. Nei's (1972) gene diversity, measures of genotypic diversity and estimates of geneflow for all isolates are shown (Nei, 1973; Lewontin, 1972; Nei, 1987; McDermott and McDonald, 1993).

Location	N^{α}	H^{β}	I^{γ}	$G_{ST}{}^{\delta}$	Nm^{ε}
All isolates*	405	0.375	0.644	0.439	0.638
South African	397	0.398	0.679	0.048	9.869
Kwa-Zulu Natal	267	0.406	0.682	0.053	8.987
Mpumalanga	110	0.358	0.592	0.109	4.092
North West	20	0.219	0.371	-	-

* All *C. zeina* isolates = RSA isolates + US isolate (OYPA) + 5 Zambian isolates + 1 Zimbabawean isolate + *C. zeina* Type culture (CPC11998)

N^{α} = Sample size

H^{β} = Nei's (1972) gene diversity

I^{γ} = Shannon's Index (Lewontin, 1972)

Nm^{ε} = estimate of gene flow from G_{ST} (genetic differentiation) such that $Nm = 0.5(1-G_{ST})/G_{ST}$ (McDermott and McDonald, 1993)

Genetic differentiation (G_{ST}) for all isolates was estimated at 0.439, this includes all the *C. zeina* isolates from South Africa and includes the isolate OYPA from the United States, and isolates from Zimbabwe and Zambia (Table 12). The G_{ST} estimate for South African isolates drops to 0.048. Although the differences between these two population groups are just eight isolates, they include unique and genetically diverse haplotypes, which causes a bias. Therefore there is evidence of population differentiation between the United States, Zambia, Zimbabwe and South Africa. Evidence for genetic differentiation within South Africa however is lacking. Similarly, isolates within Kwa-Zulu Natal and the Mpumalanga province also display a lack in genetic differentiation as indicated in Table 12. Although there is little evidence of genetic differentiation within Kwa-Zulu Natal and Mpumalanga, the Mpumalanga province exhibits a higher G_{ST} value of 0.109 than Kwa-Zulu Natal with 0.053. This is due to the geographic location bias, as the seasonal populations within Mpumalanga originate from the same farm in Machadodorp. Kwa-Zulu Natal isolates however, originate from geographically distinct areas within Kwa-Zulu Natal.

The estimate of geneflow (Nm) determined for all isolates, was relatively low, which is attributed to the difficulty of isolates from the United States and South Africa firectly exchanging genetic material (Table 12). There is evidence for high levels of gene flow between isolates within South Africa, as indicated by the estimate of geneflow (Nm) with a value of 9.87. The estimate of gene flow decreases to 8.99 and 4.09 in Kwa-Zulu Natal and Mpumalanga respectively. Although these values indicate that there is gene flow occurring within each province, it is more interesting to note that there is evidence of a high level of gene flow occurring between these provinces. While there is little data to elaborate on the level of gene flow within the North West province, the estimate of gene flow within South Africa evaluated the gene flow between Kwa-Zulu Natal, Mpumalanga and the North West province, thus it is possible to speculate that this pathogen in migrating between provinces.

3.6.2 Measures of Multilocus Linkage Disequilibrium

Multilocus disequilibrium is the occurance of a combination of alleles to make a particular haplotype more or less than would be expected in randomly proliferation populations. The observed I_A and $\bar{r}_d$ for all isolates did not fall within the predicted expected range for I_A (-0.209 to -0.0787) and $\bar{r}_d$ (-0.016 to -0.006) at a confidence value of P<0.001, thus the null hypothesis is rejected and there is no evidence for sexual recombination occurring within isolates from South Africa. f

Table 13. Multilocus linkage disequilibrium analysis (I_A and $\bar{r}_d$) for isolates of *Cercospora zeina* for designated populations.

Population	Observed I_A	Range	Observed	Range	P-value
RSA Only	0.516	-0.075 to 0.080	0.039	-0.006 to 0.006	P < 0.001
KZN Only	0.511	-0.097 to 0.120	0.039	-0.007 to 0.009	P < 0.001
KZN 2011	1.428	-0.362 to 0.514	0.113	-0.029 to 0.041	P < 0.001
KZN 2012	0.968	-0.186 to 0.286	0.076	-0.015 to 0.023	P < 0.001
KZN 2013	0.345	-0.127 to 0.181	0.027	-0.001 to 0.014	P < 0.001
GT Only	0.801	-0.130 to 0.201	0.067	-0.011 to 0.017	P < 0.001
GT 2011	1.469	-0.309 to 0.587	0.124	-0.026 to 0.050	P < 0.001
GT 2012	1.236	-0.204 to 0.322	0.104	-0.017 to 0.027	P < 0.001
GT 2013	0.237	-0.158 to 0.231	0.020	-0.013 to 0.019	P < 0.001
CD Only	0.279	-0.185 to 0.297	0.022	-0.014 to 0.023	P < 0.001
CD 2011	1.294	-0.423 to 0.762	0.100	-0.033 to 0.059	P < 0.001
CD 2012	1.449	-0.454 to 0.771	0.112	-0.035 to 0.060	P < 0.001
CD 2013	0.335	-0.253 to 0.465	0.028	-0.021 to 0.039	P < 0.001
BF Only	0.665	-0.256 to 0.455	0.048	-0.019 to 0.033	P < 0.001
BF 2012	1.127	-0.316 to 0.564	0.094	-0.026 to 0.047	P < 0.001
BF 2013	0.704	-0.513 to 1.170	0.050	-0.037 to 0.084	P < 0.007
MP Only	0.421	-0.139 to 0.183	0.033	-0.017 to 0.014	P < 0.001
MP 2011	0.283	-0.161 to 0.232	0.022	-0.012 to 0.018	P < 0.001
MP 2012	0.332	-0.289 to 0.501	0.031	-0.036 to 0.047	P < 0.013
MP 2013	0.425	-0.321 to 0.320	0.037	-0.028 to 0.028	P < 0.001
NW 2012	0.408	-0.359 to 0.831	0.046	-0.041 to 0.094	P < 0.025

* RSA = only isolates from South Africa were included. KZN = Isolates from Kwa-Zulu Natal. MP = Isolates from Mpumalanga. NW = Isolates from the North West Province. GT = Isolates from Greytown, KZN. CD = Isolates from Cedara, KZN. BF = Isolates from Baynesfield, KZN.

The observed I_A and $\bar{r}_d$ for KZN isolates were 0.345 and 0.027 respectively, which did not fall within the predicted range and therefore there is no evidence for sexual recombination (Table 13). I_A and $\bar{r}_d$ were evaluated for isolates divided into populations viz. South Africa, Kwa-Zulu Natal, Mpumalanga, North West and further into respective seasons, the statistical data suggested no evidence of sexual recombination occurring at geographical locations and between seasons (Table 13).

Seasonal populations for Cedara and Baynesfield potentially exhibited evidence for linkage equilibrium during the 2013 season as I_A and $\bar{r}_d$ fell within the range created by 1000 randomisations. Furthermore, at p-values at $p < 0.001$ and $p < 0.007$, the null hypothese are rejected, thus there is evidence for linkage equilibrium and no evidence for sexual recombination. During the 2012 season Mpumalanga and North West province populations indicated potential evidence for linkage equilibrium as the observed values fall within the expected range generated by 1000 randomisations. Therefore there is little evidence for sexual recombination occurring within isolates from South Africa

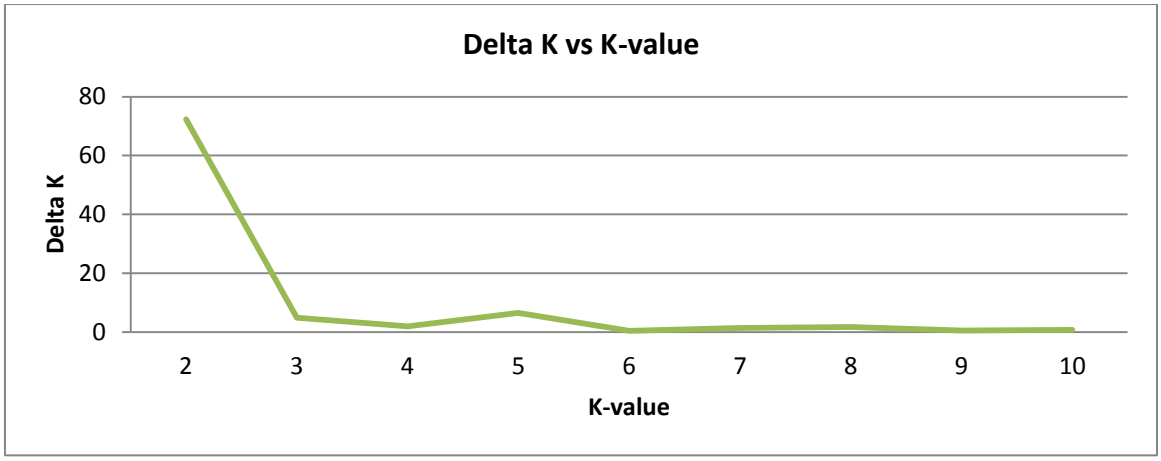
3.6.3 Population Structure

STRUCTURE v2.6.4 was not successful in identifying interpretable population structure within the *Cercospora zeina* data set. Clone-corrected data sets and non clone-corrected data sets assuming the no admixture and admixture model, exhibited a delta K value of 2 and 3 respectively. Figure 9 illustrates the Delta K value against K-value for clone-corrected data assuming the no admixture model where there are peaks at K = 2, K = 5 and K = 8. According to the graph there is greater evidence that there are two populations, rather than five or eight. The minimum number of populations STRUCTURE v2.6.4 is able to predict is 2. According to the no admixture model, there is no evidence for more than two populations; thus it is possible that there is only one highly diverse population. Figure 9 also illustrates the clone-corrected data

interpreted as bar graphs for K values 2 to 5. These graphs show isolates from each geographical region in South Africa with varying ancestry composition, suggesting that there is a lack of population structure within each geographical region in South Africa. If there are two population origins then isolates shown with K = 2 possess either green or red at a value of 1.00 or both green and red at 0.50 to indicate an isolate derived from sexual reproduction.

Two hundred and sixty five multilocus haplotypes were identified from the clone corrected data set with a clonal fraction of 0.35. Clones with the same multilocus haplotype could be identified in the same field during a season. Furthermore, multilocus haplotype were identified in the same field over more than one season. Moreover mutilocus haplotypes were shared over different seasons from different provinces. One haplotype in particular was identified from an isolate collected in Greytown in 2007 and again detected in 2012. This isolate was again detected in 2012 in the North West province.

Structure analysis for clone-corrected data sets assuming the admixture model suggests that there are three populations, as indicated by the peak in the graph of Figure 10. Similarly, the bar graphs show a lack of population structure observed within each geographical region (Figure 10).



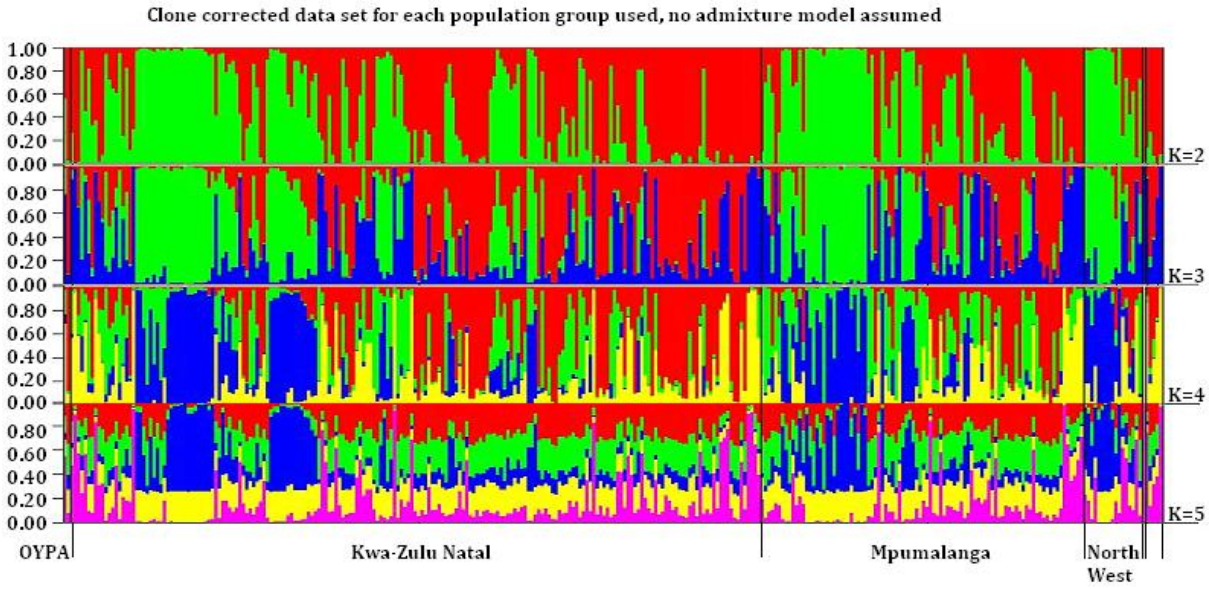
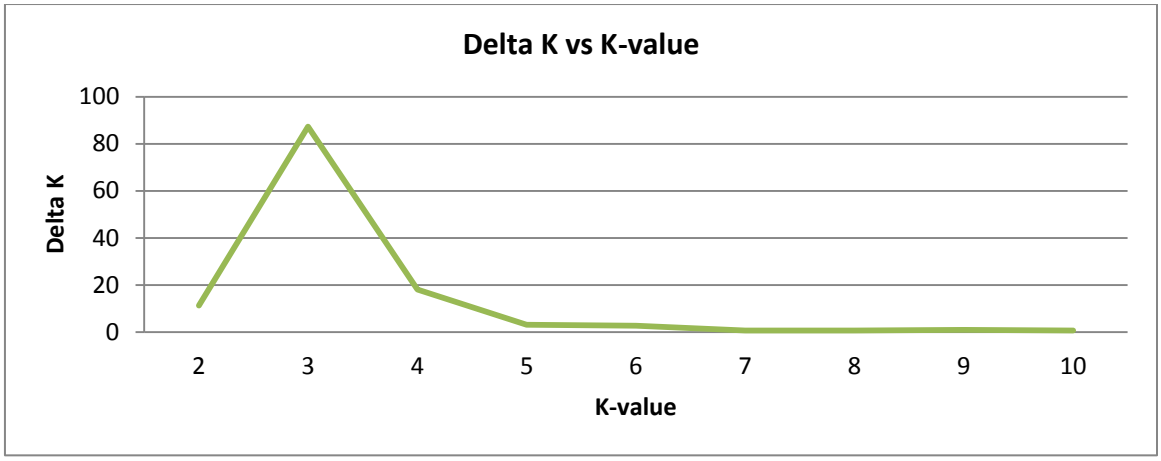


Figure 9. A graph above illustrating the Delta K value against the K-value (predicted populations) for clone corrected data assuming no admixture model. Below is a compilation of bar graphs showing clone-corrected data for the population study of *Cercospora zeina*. Isolates are grouped according to geographical region. Each bar graph indicates individual isolates as a measure of the number of predicted populations, which is indicated by the K value.



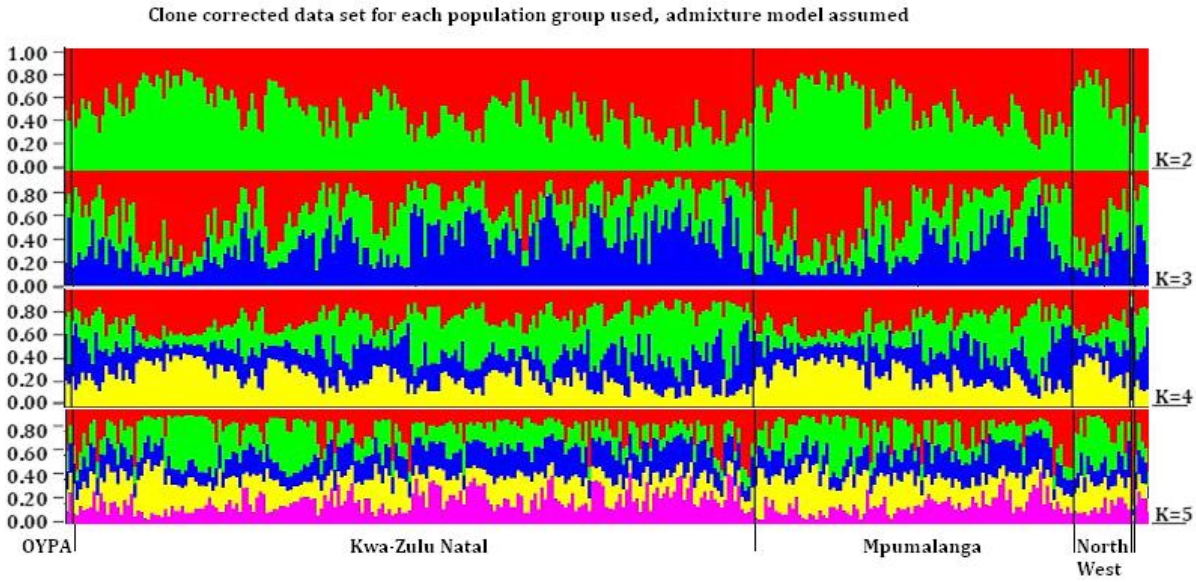


Figure 10. A graph illustrating the Delta K value against the K-value for clone-corrected data assuming the admixture model. A compilation of bar graphs showing clone-corrected data for the population study of *Cercospora zeina*, assuming admixture. Each bar graph represents individual isolates as a measure of the predicted population admixture as indicated by the K value.

The effect of geographic location (province) on the overall South African population variability was found to contribute to 14% of the overall population variability at a p-value < 0.001 (Table 14). The effect of seasonal populations (samples collected in 2007, 2011, 2012 and 2013) contributed to 18% of the population variability, while 82% was attributed to the isolates within the population at a p-value < 0.0001.

Table 14. A summary of AMOVA analysis for isolates of *Cercospora zeina* in South Africa (Peakall and Smouse, 2006).

Source	Df	Est. Var.	PhiRT	PhiPR	PhIPT	%
Testing the influence of seasons on population variability						
Among Pops	4	1375.304		0.184		18%
Within Pops	408	6087.351				82%
Total	412	7462.655				100%
P-value				0.0001		
Testing the influence of individual fields within provinces of South africa on population variability						
Among Regions	3	983.610	0.130	0.015	0.145	13%
Among Pops	5	114.239				2%
Within Pops	404	6488.509				86%
Total	412	7586.358				100%
P-value			0.002	0.109	0.001	
Testing the influence of geographic location (provinces) on population variability						
Among Pops	3	1063.018		0.140		14%
Within Pops	409	6538.284				86%
Total	412	7601.302				100%
				0.001		

Key:	
Est. Var. =	Estimated variability obtained from 10000 permutations
PhiRT =	Variability among regions/ Total variability
PhiPR =	Variability among Population/Total variability
PhiPT =	(Variability among populations + variability among regions)/Total variability
df =	Degrees of freedom

The effect of each collection field (Cedara, Greytown, Baynesfield, Winterton, Machadodorp, Klerksdorp) within each province (Kwa-Zulu Natal, Mpumalanga and North West) attributed to 2% overall variability at a confidence value of 0.109. Thus there is little variability arising from individual fields, and it is not of great consequence to the overall variability of *Cercospora zeina* in South Africa. Moreover, 13% variation was attributed to the provinces at a P-value < 0.002, while individual isolates contributed 86% of the overall variability at a p-value < 0.001. It is thus evident that variability is less influenced by where individuals come from, instead the individual isolates are the source of variability within South Africa.

The observed value of Weir's θ = 0.042 does not fall within the predicted range (-0.008 to 0.013) at a p-value < 0.001, subjected to 1000 randomisations. This suggests no population differentiation within South Africa.

According to STRUCTUREv2.6.4 analysis, AMOVA analysis and Weir's θ statistics there is no population structure for isolates of *Cercospora zeina* within South Africa. To illustrate graphically how isolates are distributed, a neighbour joining circular tree was constructed using Nei's (1983) distance (Da) method by means of Populations v1.2.32 as seen in Figure 11 (a linear version can be seen in Annexure B). Figure 11 illustrates that isolates from the same geographical locations did not automatically group together. Isolates from the Mpumalanga province are represented in green and are not clustered together. Furthermore, isolates from the North West province are highlighted in purple, and are also not clustered together. The isolate from the United States is highlighted in light blue and is separated from the southern African isolates, demonstrating their dissimilarity. The laboratory strain Mkushi as well as other Zambian isolates are highlighted in red, with the isolate from Zimbabwe highlighted in yellow. They too did not group together. This shows that isolates are diverse in their respective geographical populations, subsequently supporting no population structuring.

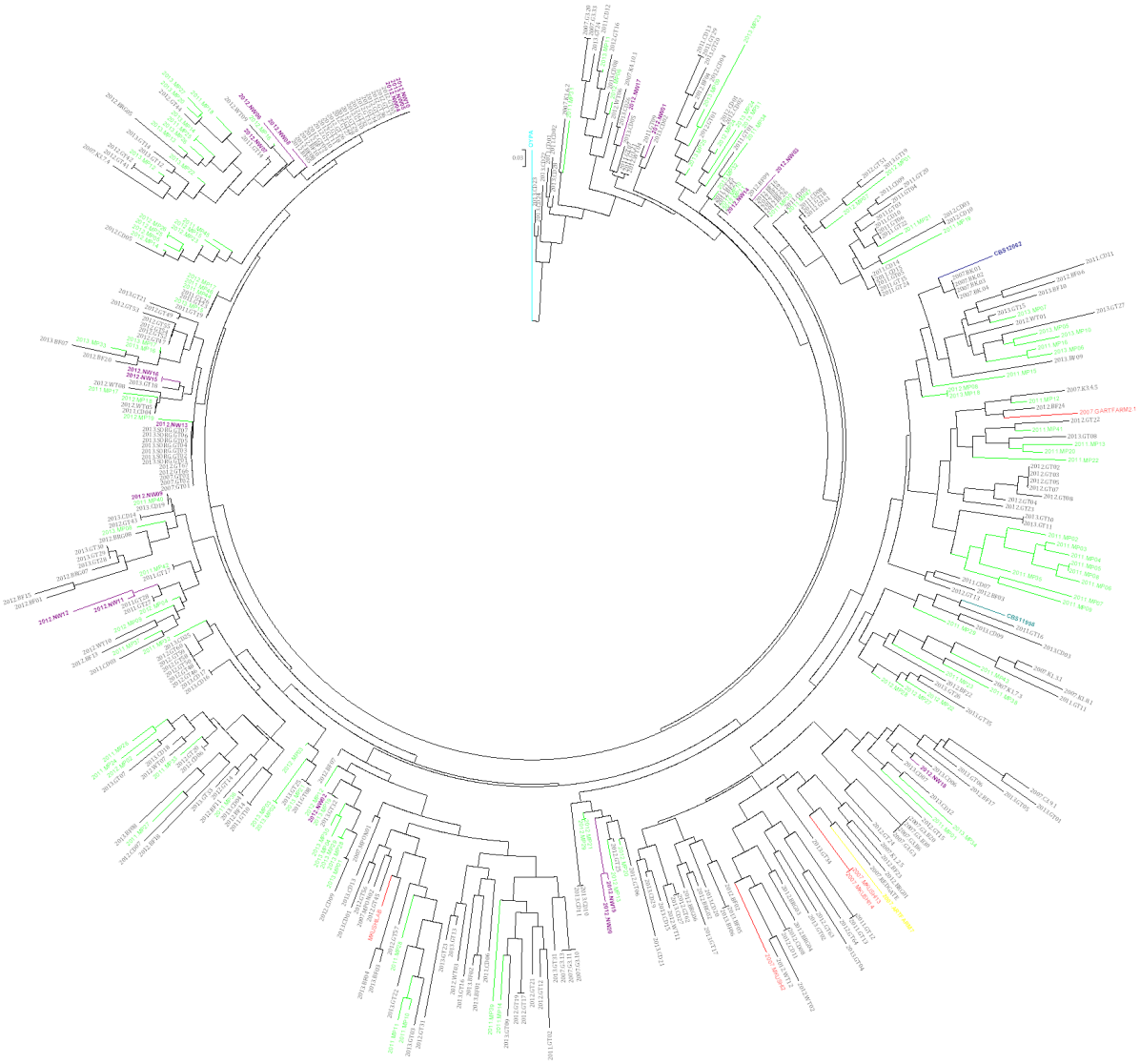


Figure 11. A neighbour joining circular tree for all non clone-corrected data sets and all markers constructed using Populations v1.2.32 and Nei's (1983) genetic distance (D_A) for 1000 bootstraps and edited in MEGA6 (Langela, 1999; Tamura et al, 2013). Isolates from Kwa-Zulu Natal, Mpumalanga, North West, Zambia and Zimbabwe are highlighted in black, green, purple, red and yellow respectively. OYPA (US isolate) is highlighted in light blue. The *Cercospora spp.* isolate is highlighted in navy blue.

A linear representation of Figure 11 is shown in Annexure B. In the tree, it is evident that although isolates in general do not group together, some isolates from the same geographical region do group together as indicated by cluster MP01 (Annexure B). In Annexure B, cluster MP01 shows isolate 2011.MP02, 2011.MP03, 2011.MP04, 2011.MP05, 2011.MP06, 2011.MP07, 2011.MP08, 2011.MP09 and 2011.MP35 from Mpumalanga grouping together. These isolates are not all clones. Isolates 2011.MP06, 2011.MP07, 2011.MP08 and 2011.MP09 are different haplotypes and were isolated from different lesions from the same maize leaf. Similarly, 2011.MP04 and 2011.MP05 are not clones, group together, and were isolated from the same lesion, but from a different maize plant to 2011.MP06, 2011.MP07, 2011.MP08 and 2011.MP09. Interestingly 2011.MP05 and 2011.MP08 are clones of the same haplotype, which indicates that there was movement of this haplotype in this field in 2011.

Discussion and Conclusion

The aim of this study was to determine the genetic diversity of *Cercospora zeina* within South Africa, determine its distribution and population structure. Additionally, the study aimed to verify whether there is genetic variability between geographic regions as well as over seasons to gain better understanding of this population and influence on disease development. Furthermore we aimed to determine whether the markers used in this study could be used in cross-species amplification.

Genetic diversity of *Cercospora zeina*

Due to the lack of physical evidence for sexual reproduction and apparent asexual biology of *Cercospora zeina*, we expected to discover a clonal population in South Africa (Brunelli, 2008). This was expected because the first discovery of GLS in South Africa occurred in 1988, 26 years ago (Ward et al, 1997). This suggests a founder effect, which would result in a population of clones (McDonald, 2004; Xu et al, 2005). Asexuality of *Cercospora* species however has been a topic of great interest. Previous studies have shown a uniform distribution of mating type genes among isolates within a small population indicating potential for sexual reproduction (Groenewald et al, 2006; Groenewald et al, 2007; Kück and Pöggeler, 2009). In this study, no mating types were measured, however sexual recombination was evaluated by analysing the linkage disequilibrium between markers using index of association (I_A) and $\bar{r}_d$ (Agapow and Burt, 2001), a lack of evidence for sexual recombination was observed. Although sexual recombination was not detected by means of (I_A) and $\bar{r}_d$ analysis, we cannot exclude the possibility of sexual recombination occurring, which may account for the high genetic diversity observed.

With the use of 16 polymorphic microsatellite markers, a moderately high degree of genetic diversity was observed for isolates of *C. zeina* within South Africa. Dunkle (2009) developed ten

polymorphic microsatellite markers from an EST library of *Cercospora zeae-maydis*, and discovered very high gene diversity per marker ranging from 0.61 to 0.91 for a population of *C. zeae-maydis* in Indiana. These gene diversities observed are very high with all the microsatellite markers possessing a Nei's (1972) gene diversity above 0.60. There were 80 isolates used in this study, obtained from different hybrids planted in eight fields in Indiana, United States of America (Dunkle et al, 2009). In contrast to such high genetic diversity observed in Indiana, the gene diversity for the clone-corrected data for *C. zeina* ranged between 0.02 and 0.60, with 13 of the 16 microsatellite markers possessing gene diversities above 0.40. As *C. zeae-maydis* and *C. zeina* are closely related species, it suggests that there are potentially many more haplotypes that can be identified from one field

Moderately high gene diversity for isolates of *Cercospora zeina* within South Africa, in particular Kwa-Zulu Natal and Mpumalanga were observed. Gene diversity is dependent on mutations; the more mutations that arise within a sequence, the higher the gene diversity (McDonald, 2004). Since *C. zeina* is a relatively new disease to South Africa (26 years from identification), the high level of gene diversity may indicate that this pathogen is trying to establish itself within this environment.

Measures of haplotypic diversity (G_{ST}) suggest no genetic differentiation between isolates within South Africa, however high levels of gene flow between geographical populations is indicated (Yeh and Boyle, 1997; McDermott and McDonald, 1993; Yeh et al, 1997).

A haplotype as defined for the purpose of this dissertation is an organism that possesses a set of alleles. Isolates that possess the same set of alleles as another possess the same haplotype. Two hundred and sixty unique haplotypes were identified from 397 *C. zeina* isolates in South Africa; therefore there are roughly 65% unique haplotypes within South Africa. Although factors such as natural selection and genetic drift influence the frequency of a particular allele of interest, they also influence the level of gene diversity within a population and the frequency of unique

haplotypes (Burnett, 2003). The abundance of unique haplotypes indicates that there is a great potential for genetic mutation and that natural selection has some influence on the population dynamics (McDonald, 2004).

In this study, the same haplotypes was observed for isolates from different geographical regions, which suggests the possibility of migration. Haplotype B01 (Annexure B) consists of three isolates, 2011.GT07, 2011.GT21 and 2012.WT04. Although all three isolates descend from Kwa-Zulu Natal province, 2011.GT07 and 2011.GT21 originate from Greytown during the 2011 season and 2012.WT04 from Winterton during the 2012 season. This indicates that haplotype B01 migrated from Greytown to Winterton. Similarly, six *C. zeina* isolates belong to Haplotype G01 (Annexure B). Haplotype G01 was first observed in Greytown during the 2007 seaon, again in 2012 and in 2013, it was additionally observed in the North West province during the 2012 season. This indicates that not only has this haplotype been established in Greytown since 2007, but it has also migrated to the North West Province. Seven *Cercospora* isolates from sorghum also share the same alleles as haplotype G01. Although the "pogo hypothesis" states that different *Cercospora* species may occupy the same lesion on a host, it does not mention whether there is horizontal gene flow, and this should be further investigated (Crous and Groenewald, 2005)

Migration of *C. zeina* from Kwa-Zulu Natal to Mpumalanga has been observed for haplotype F01, which consists of 7 isolates; 2011.GT19, 2011.GT23, 2011.GT26, 2011.MP44, 2011.MP46, 2012.MP17 and 2013.MP15 (Annexure B). Isolates 2011.GT19, 2011.GT23 and 2011.GT26 originate from Greytown and were isolated during the 2011 season and has since migrated to Mpumalanga, where this haplotype F01 has been observed for the 2011, 2012 and 2013 seasons.

The largest number of clones belongs to Haplotype H01, which consists of 23 isolates (Annexure B). Four isolates were identified during the 2012 season in Baynesfield, sixteen isolates

originate from Greytown (2012), and three isolates are from the North West province (2012) (Annexure B). The isolates 2012.GT09, 2012.GT10 and 2012.GT11 were isolated from different plants, which were collected from a different field to the isolates 2012.GT26 – 2012.GT30, which were also isolated from separate plants.

According to the allelic richness observed in Table 11, there is a prevalent allele for each marker. Although certain markers are more common, not one isolate possesses all these prominent alleles. All isolates possess a combination of more common alleles with some less common alleles.

Is there more than one Cercospora zeina haplotype observed on one maize plant?

There were 23 plants from which more than one isolate was obtained. These isolates were further analysed to determine whether more than one haplotype could be isolated from a plant. To elaborate on this topic, haplotype E01 and cluster MP01 will be discussed further (Annexure B).

Haplotype E01 consists of the following isolates, 2012.GT46, 2012.GT48, 2012.GT50, 2012.GT58, 2012.GT59 and 2012.GT60. Although all these isolates are clustered together, only 2012.GT59 and 2012.GT60 are from the same plant. Therefore it is possible to detect the same haplotype on the same plant. Isolate 2012.GT49 (which does not belong to haplotype E01, and is a unique haplotype) and 2012.GT50 are isolates obtained from different lesions of the same plant, which indicates that more than one unique haplotype can be obtained on a plant.

Cluster MP01 consists of isolates 2011.MP02, 2011.MP03, 2011.MP04, 2011.MP05, 2011.MP06, 2011.MP07, 2011.MP08, 2011.MP09 and 2011.MP35. Isolates 2011.MP06, 2011.MP07, and 2011.MP08 were isolated from separate lesions of the same leaf from the same plant, and although they clustered together, they are not the same haplotype. Similarly, 2011.MP02 and 2011.MP03 are clustered together, come from the same plant (different plant from which

isolates 2011.MP06, 2011.MP07, and 2011.MP08 were obtained), are not obtained from the same lesion and are unique haplotypes. In this cluster, 2011.MP04 and 2011.MP05 were isolated from the same lesion from a maize plant, but a different plant entirely than isolates 2011.MP02, 2011.MP03, 2011.MP06, 2011.MP07, and 2011.MP08. Isolates 2011.MP04 and 2011.MP05 cluster together, however are unique haplotypes. This challenges our conventional thought on disease development, which relates to one inoculum per lesion (Beckman and Payne, 1982; Dillard, 1989). The rationale behind different haplotypes from the same lesion may hint at sexual recombination occurring within the plant, which would account for cryptic sexual recombination and the absence of sexual structures, which have yet to be identified in the field (Groenewald et al, 2006; Groenewald et al, 2007). Alternatively this result could indicate that there is little competition between individuals for resources within a leaf lesion. It could also further substantiate the "pogo hypothesis" that states that a species of *Mycosphaerella* can colonise on a relative's lesion in the absence of its true host, sporulate and produce enough inoculum to see it through to the next season (Crous and Groenewald, 2005). Although the *Cercospora* species isolated from sorghum was not isolated from maize in this study, we cannot exclude the possibility that this may occur in the field.

Zhan and colleagues (2003) performed a population study on the wheat pathogen, *Mycosphaerella graminicola*. They found on average one genotype of *M. graminicola* per leaf (Zhan et al, 2003), which is congruent with results for *C. zeina*. However unlike *C. zeina,* the same genotype was observed from lesions from the same plant, which could be attributed to the physiology of the wheat plant (Zhan et al, 2003). Similarly a study on *Mycosphaerella nubilosa* performed by Pérez and colleagues (2010) showed that on a micro-spatial scale (one leaf of a plant), there was a high degree of selfing and therefore the same haplotype would be observed on the same plant. Furthermore, more than one haplotype was observed when comparing isolates on a macro-spatial scale (isolates form different geographical regions) and this was attributed to sexual recombination (Pérez et al, 2010).

Is there selection of haplotypes in the field by fungicidal sprays?

Maize leaf material that displayed GLS symptoms but had been exposed to fungicidal spraying was collected from the commercial field in Mpumalanga. The effect of fungicidal sprays on the population of *C. zeina*, for the 2011 to 2013 seasons in Mpumalanga could, therefore, be discussed. The commercial field for the above mentioned seasons as subjected to different fungicidal spraying strategies. The 2011 season marked the first severe outbreak of GLS in Mpumalanga, which stands to reason, as these maize plants were not sprayed with fungicides (Farmer, personal communication). Due to the previous year's severity of infection, a rigid fungicidal treatment program was implemented in 2012 and 2013. In contrast to over one hundred leaf samples collected in one field during the 2011 outbreak, 2012 and 2013 seasons were relatively free of infection and greater effort and time was required to identify and collect approximately 40 leaf samples from the farm per season. Thus the sampling strategy in 2012 and 2013 differed greatly from the 2011 season, where sampling was restricted to one field. Different maize cultivars were collected from in all seasons.

Although gene diversity may seem unhampered by fungicidal spray, the bias in different sampling methods may have underestimated the population diversity for the 2011 season. Furthermore, due to the high number of unique genotypes observed on the farm, it is difficult to extrapolate whether a haplotype was absent in the following season due to fungicidal sprays. There were haplotypes in 2011 that were not identified in 2012 and 2013 as indicated by the Clade M. Haplotype M1 (Annexure B) indicates that the same haplotype is observed in 2012 and 2013. Similarly, Haplotype F01 (Annexure B) was observed in 2011, 2012 and 2013.

Regardless of biased sampling strategies, sexual recombination in addition to random mutation may account for increased gene diversity within a population, and therefore I_A and $\bar{r}_d$ were determined for each seasonal population in Mpumalanga. Although these values are low and may indicate the possibility of sexual recombination, they did not fall within the projected

randomised distribution range, and thus the probability of sexual recombination occurring is low. Similarly for both the 2012 and 2013 seasons, the I_A and $\bar{r}_d$ did not fall within the 1000 randomised distributional range and so there is no evidence for sexual recombination.

Although there was no statistical evidence for sexual reproduction, mating type analysis needs to be performed to confidently dismiss the possibility of sexual reproduction. Sexual reproduction requires great investment from the organism, but occurs when variation is required for survival, such as selection against fungicides (Xu et al, 2005). The stress on the population within Mpumalanga from climate and fungicidal spraying would have resulted in the removal of less adept haplotypes from the population. The resultant haplotypes were unique and private alleles confined to this farm in Mpumalanga could further validate observations for haplotypic diversity. To validate this, pathogenicity and fungicidal resistance tests should be performed.

It is concerning that in spite of fungicidal treatments there is still great diversity of *C. zeina* in Mpumalanga – which is why it does not make sense that there is no sexual reproduction taking place. It is possible that new haplotypes were introduced by means of migration by way of wind dispersal of conidia, which is supported by the high estimate of gene flow values observed for Mpumalanga (Table 12) (Torriani et al, 2009). However some level of selection pressure due to fungicidal spraying may give rise to genetic diversity (Torriani et al, 2009). The fungicide applied contains both a triazole and strobilurin to inhibit germ tube development and conidial germination respectively, as was believed to be a good control strategy (Bartlett et al, 2002; Sierotzki et al, 2007). However the development of resistance towards strobilurins by *Mycosphaerella* species is an increasing occurrence as in the case of *Mycosphaerella graminicola* (Torriani et al, 2009). Since 2001, *M. graminicola* has developed resistance against strobilurins in Europe on four independent occasions (Torriani et al, 2009). In addition, *Cercospora* species such as *C. sojina* have developed strobilurin resistance in the United States (Faske, 2012).

Furthermore, the development of fungicidal resistance by *Mycosphaerella* and *Cercospora* species occurred within the first five years of the introduction of strobilurins as a fungicide in 1996 (Sierotzki et al, 2000; Fraaije et al, 2005; Sierotzki et al, 2005; Sierotzki et al, 2007).

The efficacy of fungicidal sprays for the inhibition of *Mycosphaerella* and *Cercospora* species should be investigated further. Certainly, fungicidal resistance assays along with a more in depth population study is required to make definitive conclusions regarding fungicidal selection of haplotypes and resistance development.

Could we identify the origin of introduction of *Cercospora zeina* into South Africa?

GLS was first identified in South Africa in the Kwa-Zulu Natal province (Ward et al, 1997). Kwa-Zulu Natal isolates possess the highest gene diversity, genotypic diversity and estimate of gene flow for each geographical population in addition to the highest hierarchy according to allelic richness and private allele richness. Thus it is probable that Kwa-Zulu Natal was the first introduction site, as previously described (Ward, 1997). However, the Mpumalanga population, although quite far in distance from the region of first occurrence of GLS in 1988, is highly diverse and exhibits unique haplotypes not found within KZN. As a result we can only infer that multiple *C. zeina* introductions into South Africa have occurred to account for such a high diversity in the absence of acknowledged sexual recombination.

Mycosphaerella species and relatives (like *Cercospora*) have displayed this trend of high genetic diversity with a cryptic sexuality (Groenewald et al, 2006). High genetic diversity has been observed in *Mycosphaerella graminicola* on a global scale along with evidence of gene flow and recombination of some form (Zhan et al, 2003).

The claim that *Cercospora zeina* arrived in South Africa from the America's cannot be verified without a detailed comparative study between isolates from the United States and South Africa. The isolate from the US, OYPA, was the only isolate used for comparison in this study. Private

alleles for OYPA were determined for markers CzSSR02, CzSSR07, CzSSR08, CzSSR16 and CzSSR17. These could potentially be used as geographical markers to distinguish African and North American isolates. These markers may also be informative regarding the distribution and origin of *Cercospora zeina* on a global scale. This however does not exclude the possibility that the alleles observed within South Africa could be observed within the United States and other parts of the world.

The Zambian and Zimbabwean isolates are completely dispersed among South African isolates as seen in Figure 11 (Annexure B). However there are alleles shared between the Zimbabwean isolate and OYPA. It is possible that isolates migrated from North America to Zimbabwe or vice versa. Therefore a more detailed population analysis is required to determine whether isolates from Zambia and Zimbabwe may have migrated south into South Africa or vice versa.

Evidence from this study suggests multiple introductions of *Cercospora zeina* into South Africa. These multiple introductions could be attributed to agents of migration such as the dispersal of conidia by means of wind or human intervention through trade, travel and machinery (Zhan and McDonald, 2010). Such a movement of isolates of *C. zeina* is evident throughout South African maize producing areas, and possibly with its host. However, mating type analysis would be invaluable in determining whether sexual recombination is a mechanism for which high genetic diversity and pathogen adaptability is attributed. Mating type ratio analyses would also clarify as to whether multiple introductions could be the foremost source of genetic diversity. However where these isolates were introduced from is a subject that should be further considered by performing a population study on *C. zeina* on a global scale. Similarly, isolates of *C. sojina* from the America's and China were found to display high genetic diversity from where the origin could not be determined without more in depth study (Bradley et al, 2012).

The application of *Cercospora zeina* microsatellite markers to *Cercospora* species from Sorghum.

The microsatellite markers designed for *Cercospora zeina* were successfully applied to elucidate the population diversity of *C. zeina* within South Africa. In addition, they were used to amplify microsatellites in *Cercospora species isolated from sorghum*. Although markers indicated clonality for the seven isolates obtained from one field in Kwa-Zulu Natal, they are potential microsatellites that could be used to study this species. Furthermore, the SSR markers successfully amplified the unknown *Cercospora spp*. found on maize in South Africa (Crous et al, 2006). However these markers failed to amplify in *C. zeae-maydis*, the sibling species of *C. zeina*. Correspondingly, microsatellite markers designed for *C. zeae-maydis* failed to amplify in *C. zeina* (Dunkle et al, 2009). It was previously suggested that *Cercospora zeina* and *C. zeae-maydis* are more closely related and that *Cercospora species isolated from sorghum* is more closely related to *C. zeae-maydis* than to *C. zeina* (Goodwin et al, 2001). It is common for microsatellites to be used for the analysis of sibling species due to their similarity in genetic material (Deitz et al, 2012). Often some microsatellites do not amplify in the sibling species due to the variability in the primer region (Deitz et al, 2012). As primers designed to *C. zeina* amplify in *Cercospora species isolated from sorghum* and not in *C. zeae-maydis*, and primers designed to *C. zeae-maydis* did not amplify in *C. zeina*, the phylogenetic relationship between these species as stipulated by Crous and colleagues in 2006, should be revisited.

Considerations for the future

GLS is a disease of global importance, as well as having immense importance to South African maize producers (Latterell and Rossi, 1980; Ward et al, 1998). With evidence of high genetic diversity and the potential for fungicide tolerance, it is important to make informed decisions on disease control to minimize yield losses.

Mating types for C. zeina isolates need to be determined and the potential for sexual

recombination needs to be investigated to assess whether it may be an important source of genetic variability. Fungicidal trial studies need to be implemented in order to evaluate fungicide efficacy on a myriad of haplotypes of *C. zeina* in addition to other foliar pathogens that occur simultaneously within the field (Sierotzki et al, 2007). Climatic conditions need to be re-evaluated for different geographical regions and projections of climate change need to be considered in order to effectively predict where this disease may spread to next (Jones and Thornton, 2003). Most importantly however, maize cultivars need to be bred with quantitative resistance and/or tolerant traits to ensure that all genotypes are selected against to minimize the development of resistant haplotypes (Berger et al, 2014; Zwonitzer et al, 2010). Furthermore, crop rotation with the legume soybean should be re-evaluated as *Cercospora zeina* may over-season on *Cercospora kikuchi* lesions, which will ensure the presence of *C. zeina* inoculum in the following season (Crous and Groenewald, 2005).

We now have an improved understanding of the diversity of *Cercospora zeina*, the causal agent of grey leaf spot on maize in South Africa. It is possible to deduce that the high genetic diversity, evidence of migration and gene flow between isolates in South Africa support the increased fitness of this pathogen in the field (Burnett, 2003; McDonald, 2004). Furthermore the potential of fungicidal resistance development due to this high diversity is of concern. The availability of microsatellite markers and a genome sequence for further microsatellite design may be useful in determining potential linkage between fungicide resistance development and a particular marker (Ellegren, 2004; Torriani et al, 2008).

The combined knowledge of pathogen diversity, mode of reproduction, cultivars, climate change, and fungicide efficacy would be instrumental in formulating an intelligent informed decision regarding the future of maize production and its relationship with this disease.

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Annexure A: Alignment and tree construction of *Cercospora* species.

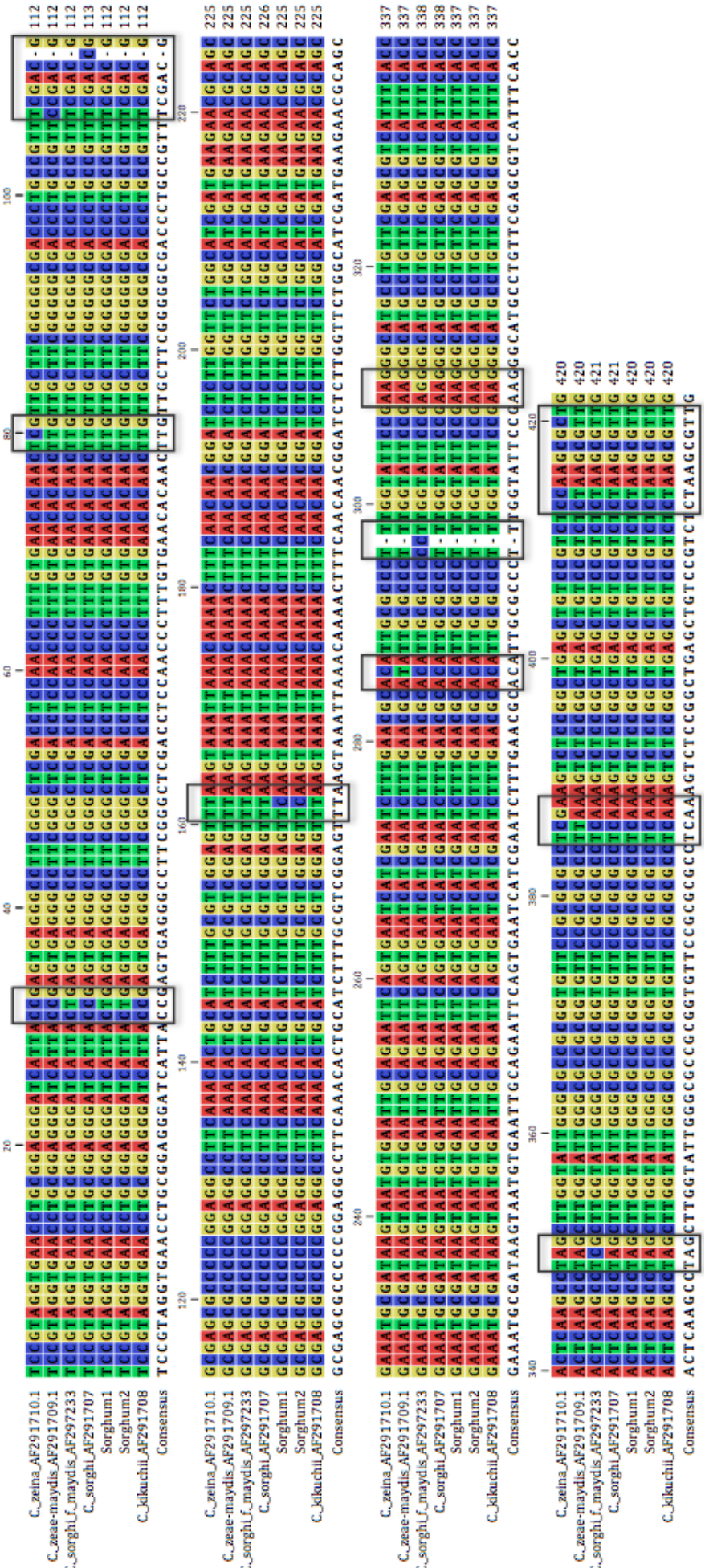
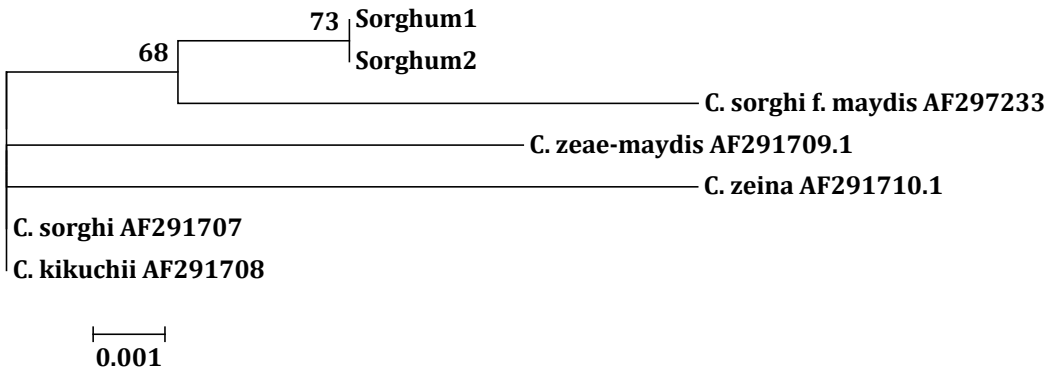


Figure 12. The alignment of ITS sequences using ITS 1 and ITS 4 primers for *Cercospora* species. The grey boxes encapsulate the informative sites. From this alignment it is evident that the *Cercospora* species isolates from sorghum is neither *C. sorghi* nor *C. sorghi f. maydis*.

Figure 13. Phylogenetic analysis of *Cercospora* species using ITS sequence data, represented by a maximum likelihood tree constructed using MEGA6. The tree is rooted using the type strain of *C. kikuchii* (AF291708). Bootstrap confidence estimates above 50% are shown.



Annexure B: Tabulated summary of all the haplotypes identified from the linear version of the neighbour joining tree in Figure 11

	HAPLOTYPE DESIGNATION	Samples	Date Collected	Collection Region	Comments
HAPLOTYPES CONSISTING OF TWO CLONES	HAPLOTYPE A01	2007.G3.20	2007	Greytown	Collected in Greytown by Dr Meisel
	HAPLOTYPE A01	2007.G3.33	2007	Greytown	
	HAPLOTYPE A02	2011.CD13	2011	Cedara	Different regions within Kwa-Zulu Natal
	HAPLOTYPE A02	2011.GT29	2011	Greytown	
	HAPLOTYPE A03	2012.CD01	2012	Cedara	Isolated from different plants (different cultivars)
	HAPLOTYPE A03	2012.CD02	2012	Cedara	
	HAPLOTYPE A04	2011.CD05	2011	Cedara	Different provinces within South Africa
	HAPLOTYPE A04	2011.MP47	2011	Mpumalanga	
	HAPLOTYPE A05	2012.CD03	2012	Cedara	Isolated from different plants
	HAPLOTYPE A05	2012.CD10	2012	Cedara	
	HAPLOTYPE A06	2013.MP18	2013	Mpumalanga	Same farm, different seasons
	HAPLOTYPE A06	2012.MP08	2012	Mpumalanga	
	HAPLOTYPE A07	2013.GT10	2013	Greytown	Isolated from different plants
	HAPLOTYPE A07	2013.GT11	2013	Greytown	
	HAPLOTYPE A08	2011.MP05	2011	Mpumalanga	Isolated from different plants (different cultivars)
	HAPLOTYPE A08	2011.MP08	2011	Mpumalanga	
	HAPLOTYPE A09	2007.MKUSHI13	2007	Zambia	Collected from the Mkushi region, Zambia
	HAPLOTYPE A09	2007.MKUSHI14	2007	Zambia	
	HAPLOTYPE A10	2011.GT12	2011	Greytown	Isolates obtained from same plant, different lesions
	HAPLOTYPE A10	2011.GT13	2011	Greytown	
	HAPLOTYPE A11	2012.CD08	2012	Cedara	Isolated from different plants
	HAPLOTYPE A11	2012.CD11	2012	Cedara	
	HAPLOTYPE A12	2013.BF05	2013	Baynesfield	Isolated from different plants
	HAPLOTYPE A12	2013.BF06	2013	Baynesfield	
	HAPLOTYPE A13	2012.GT62	2012	Greytown	Different regions within Kwa-Zulu Natal, different season
	HAPLOTYPE A13	2013.CD27	2013	Cedara	
	HAPLOTYPE A14	2012.MP21	2012	Mpumalanga	Isolated from different plants
	HAPLOTYPE A14	2012.MP29	2012	Mpumalanga	
	HAPLOTYPE A15	2013.CD10	2013	Cedara	Isolated from different plants
	HAPLOTYPE A15	2013.CD11	2013	Cedara	
	HAPLOTYPE A16	2012.GT17	2012	Greytown	Isolated from different plants
	HAPLOTYPE A16	2012.GT19	2012	Greytown	
	HAPLOTYPE A17	2013.MP29	2013	Mpumalanga	Isolated from different plants
	HAPLOTYPE A17	2013.MP28	2013	Mpumalanga	
	HAPLOTYPE A18	2013.MP02	2013	Mpumalanga	Isolated from different plants
	HAPLOTYPE A18	2013.MP03	2013	Mpumalanga	
	HAPLOTYPE A19	2011.GT10	2011	Greytown	Different regions within Kwa-Zulu Natal, different season
	HAPLOTYPE A19	2012.BF14	2012	Baynesfield	
	HAPLOTYPE A20	2013.CD04	2013	Cedara	Different provinces within South Africa, different season
	HAPLOTYPE A20	2011.MP36	2011	Mpumalanga	
	HAPLOTYPE A21	2012.CD06	2012	Cedara	Different regions within Kwa-Zulu Natal
	HAPLOTYPE A21	2012.GT20	2012	Greytown	
	HAPLOTYPE A22	2013.CD16	2013	Cedara	Isolated from different plants
	HAPLOTYPE A22	2013.CD17	2013	Cedara	
	HAPLOTYPE A23	2011.GT27	2011	Greytown	Isolates obtained from same plant, different lesions
	HAPLOTYPE A23	2011.GT28	2011	Greytown	
	HAPLOTYPE A24	2011.GT17	2011	Greytown	Different provinces within South Africa
	HAPLOTYPE A24	2011.MP42	2011	Mpumalanga	
	HAPLOTYPE A25	2012.GT43	2012	Greytown	Different regions within Kwa-Zulu Natal, different season
	HAPLOTYPE A25	2013.CD14	2013	Cedara	
	HAPLOTYPE A26	2012.NW15	2012	North West	Isolated from different plants
	HAPLOTYPE A26	2012.NW16	2012	North West	
	HAPLOTYPE A27	2013.MP16	2013	Mpumalanga	Isolates obtained from same plant, different lesions
	HAPLOTYPE A27	2013.MP17	2013	Mpumalanga	
	HAPLOTYPE A28	2012.MP25	2012	Mpumalanga	Isolated from different plants (different

	HAPLOTYPE DESIGNATION	Samples	Date Collected	Collection Region	Comments
HAPLOTYPES CONTAINING THREE CLONES	HAPLOTYPE A28	2012.MP26	2012	Mpumalanga	cultivars)
	HAPLOTYPE A29	2012.GT41	2012	Greytown	Isolated from different plants
	HAPLOTYPE A29	2012.GT42	2012	Greytown	
	HAPLOTYPE A30	2013.MP14	2013	Mpumalanga	Same farm, different seasons
	HAPLOTYPE A30	2011.MP25	2011	Mpumalanga	
	HAPLOTYPE A31	2011.GT14	2011	Greytown	Different provinces within South Africa, different season
	HAPLOTYPE A31	2012.NW07	2012	North West	
	HAPLOTYPE B1	2011.GT07	2011	Greytown	Greytown isolates from different plants. Different regions within KZN, different season
	HAPLOTYPE B1	2011.GT21	2011	Greytown	
	HAPLOTYPE B1	2012.WT04	2012	Winterton	
	HAPLOTYPE B2	2011.CD08	2011	Cedara	Different regions within Kwa-Zulu Natal, different season
	HAPLOTYPE B2	2011.GT18	2011	Greytown	
	HAPLOTYPE B2	2012.GT61	2012	Greytown	
	HAPLOTYPE B3	2011.CD10	2011	Cedara	Greytown isolates from different plants. Different regions within KZN, different season
	HAPLOTYPE B3	2011.GT06	2011	Greytown	
	HAPLOTYPE B3	2011.GT22	2011	Greytown	
	HAPLOTYPE B4	2007.G3.10	2007	Greytown	Collected in Greytown by Dr Meisel
	HAPLOTYPE B4	2007.G3.11	2007	Greytown	
	HAPLOTYPE B4	2007.G3.13	2007	Greytown	
	HAPLOTYPE B5	2013.MP19	2013	Mpumalanga	Different provinces within South Africa, different season
	HAPLOTYPE B5	2013.GT32	2013	Greytown	
	HAPLOTYPE B5	2012.NW02	2012	North West	
	HAPLOTYPE B6	2013.MP21	2013	Mpumalanga	Different provinces within South Africa, different season
	HAPLOTYPE B6	2011.GT08	2011	Greytown	
	HAPLOTYPE B6	2013.GT25	2013	Greytown	
	HAPLOTYPE B7	2013.GT28	2013	Greytown	Isolated from different plants (different cultivars)
	HAPLOTYPE B7	2013.GT29	2013	Greytown	
	HAPLOTYPE B7	2013.GT30	2013	Greytown	
	HAPLOTYPE B8	2013.CD19	2013	Cedara	Different provinces within South Africa, different season
	HAPLOTYPE B8	2011.MP40	2011	Mpumalanga	
	HAPLOTYPE B8	2012.NW09	2012	North West	
	HAPLOTYPE B9	2011.CD04	2011	Cedara	Different provinces within South Africa, different season
	HAPLOTYPE B9	2012.WT05	2012	Winterton	
	HAPLOTYPE B9	2012.MP18	2012	Mpumalanga	
HAPLOTYPES CONTAINING FOUR CLONES	HAPLOTYPE C1	2007.BK.01	2007	Winterton	Collected by Dr Meisel
	HAPLOTYPE C1	2007.BK.02	2007	Winterton	
	HAPLOTYPE C1	2007.BK.03	2007	Winterton	
	HAPLOTYPE C1	2007.BK.04	2007	Winterton	
	HAPLOTYPE C2	2012.GT02	2012	Greytown	Same farm, different plants (same cultivar)
	HAPLOTYPE C2	2012.GT03	2012	Greytown	
	HAPLOTYPE C2	2012.GT05	2012	Greytown	
	HAPLOTYPE C2	2012.GT07	2012	Greytown	
	HAPLOTYPE C3	2007.G3.B20	2007	Greytown	Collected in Greytown by Dr Meisel
	HAPLOTYPE C3	2007.G3.B30	2007	Greytown	
	HAPLOTYPE C3	2007.G3.B6	2007	Greytown	
	HAPLOTYPE C3	2007.G3.G3	2007	Greytown	
	HAPLOTYPE C4	2012.GT47	2012	Greytown	2012.GT47, 2012.GT52 were isolated from different plants. 2012.GT45, 2012.GT55 were isolates from the same plant, different lesions
	HAPLOTYPE C4	2012.GT52	2012	Greytown	
	HAPLOTYPE C4	2012.GT54	2012	Greytown	
	HAPLOTYPE C4	2012.GT55	2012	Greytown	
FIVE CLONES	HAPLOTYPE D1	2011.GT25	2011	Greytown	Different provinces within South Africa, different season
	HAPLOTYPE D1	2012.BF21	2012	Baynesfield	
	HAPLOTYPE D1	2012.MP10	2012	Mpumalanga	
	HAPLOTYPE D1	2012.MP11	2012	Mpumalanga	
	HAPLOTYPE D1	2012.NW14	2012	North West	

	HAPLOTYPE DESIGNATION	Samples	Date Collected	Collection Region	Comments
HAPLOTYPES CONTAINING	HAPLOTYPE D2	2012.BF16	2012	Baynesfield	Isolates from Baynesfield are obtained from different plants. Different provinces within South Africa, different season
	HAPLOTYPE D2	2012.BF19	2012	Baynesfield	
	HAPLOTYPE D2	2012.BF25	2012	Baynesfield	
	HAPLOTYPE D2	2012.BF26	2012	Baynesfield	
	HAPLOTYPE D2	2011.MP30	2011	Mpumalanga	
	HAPLOTYPE D3	2011.CD14	2011	Cedara	Isolated from different plants. Different regions within Kwa[Zulu Natal.
	HAPLOTYPE D3	2011.CD15	2011	Cedara	
	HAPLOTYPE D3	2011.GT05	2011	Greytown	
	HAPLOTYPE D3	2011.GT15	2011	Greytown	
	HAPLOTYPE D3	2011.GT24	2011	Greytown	
SIX CLONES	HAPLOTYPE E1	2012.GT46	2012	Greytown	2012.GT46, 2012.GT48, 2012.GT50 and 2012.GT58 were obtained from different plants. 2012.GT59 and 2012.GT60 were obtained from the same plant but different lesions.
	HAPLOTYPE E1	2012.GT48	2012	Greytown	
	HAPLOTYPE E1	2012.GT50	2012	Greytown	
	HAPLOTYPE E1	2012.GT58	2012	Greytown	
	HAPLOTYPE E1	2012.GT59	2012	Greytown	
	HAPLOTYPE E1	2012.GT60	2012	Greytown	
SEVEN CLONES	HAPLOTYPE F1	2013.MP15	2013	Mpumalanga	Isolated from different plants. Different provinces within South Africa, different season
	HAPLOTYPE F1	2011.GT19	2011	Greytown	
	HAPLOTYPE F1	2011.GT23	2011	Greytown	
	HAPLOTYPE F1	2011.GT26	2011	Greytown	
	HAPLOTYPE F1	2011.MP44	2011	Mpumalanga	
	HAPLOTYPE F1	2011.MP46	2011	Mpumalanga	
	HAPLOTYPE F1	2012.MP17	2012	Mpumalanga	
THIRTEEN CLONES	HAPLOTYPE G1	2007.GT01	2007	Greytown	Isolates obtained in 2007 were collected by Dr Meisel. *C. zeina* isolates from Greytown in 2012 were obtained from different plants. Sorghum isolates were isolated form different plants. Samples belong to different provinces in South Africa.
	HAPLOTYPE G1	2007.GT02	2007	Greytown	
	HAPLOTYPE G1	2007.GT03	2007	Greytown	
	HAPLOTYPE G1	2012.GT66	2012	Greytown	
	HAPLOTYPE G1	2012.GT67	2012	Greytown	
	HAPLOTYPE G1	2013.SORG.GT01	2013	Greytown	
	HAPLOTYPE G1	2013.SORG.GT02	2013	Greytown	
	HAPLOTYPE G1	2013.SORG.GT03	2013	Greytown	
	HAPLOTYPE G1	2013.SORG.GT04	2013	Greytown	
	HAPLOTYPE G1	2013.SORG.GT05	2013	Greytown	
	HAPLOTYPE G1	2013.SORG.GT06	2013	Greytown	
	HAPLOTYPE G1	2013.SORG.GT07	2013	Greytown	
	HAPLOTYPE G1	2012.NW13	2012	North West	
TWENTY THREE CLONES	HAPLOTYPE H1	2012.BF05	2012	Baynesfield	Baynesfield isolates were obtained from different plants. 2012.GT09 and 2012.GT10 were obtained from different plants from a different field to the rest of the Greytown isolates. 2012.GT18 to 2012.GT67 were obtained from different plants. These plants were exposed to fungicidal treatments. Isolates from the North West province were obtained from different plants. All isolates were obtained during the 2012 season.
	HAPLOTYPE H1	2012.BF08	2012	Baynesfield	
	HAPLOTYPE H1	2012.BF10	2012	Baynesfield	
	HAPLOTYPE H1	2012.BF12	2012	Baynesfield	
	HAPLOTYPE H1	2012.GT09	2012	Greytown	
	HAPLOTYPE H1	2012.GT10	2012	Greytown	
	HAPLOTYPE H1	2012.GT11	2012	Greytown	
	HAPLOTYPE H1	2012.GT18	2012	Greytown	
	HAPLOTYPE H1	2012.GT26	2012	Greytown	
	HAPLOTYPE H1	2012.GT27	2012	Greytown	
	HAPLOTYPE H1	2012.GT28	2012	Greytown	
	HAPLOTYPE H1	2012.GT29	2012	Greytown	
	HAPLOTYPE H1	2012.GT30	2012	Greytown	
	HAPLOTYPE H1	2012.GT32	2012	Greytown	
	HAPLOTYPE H1	2012.GT33	2012	Greytown	
	HAPLOTYPE H1	2012.GT34	2012	Greytown	
	HAPLOTYPE H1	2012.GT35	2012	Greytown	
	HAPLOTYPE H1	2012.GT36	2012	Greytown	
	HAPLOTYPE H1	2012.GT37	2012	Greytown	

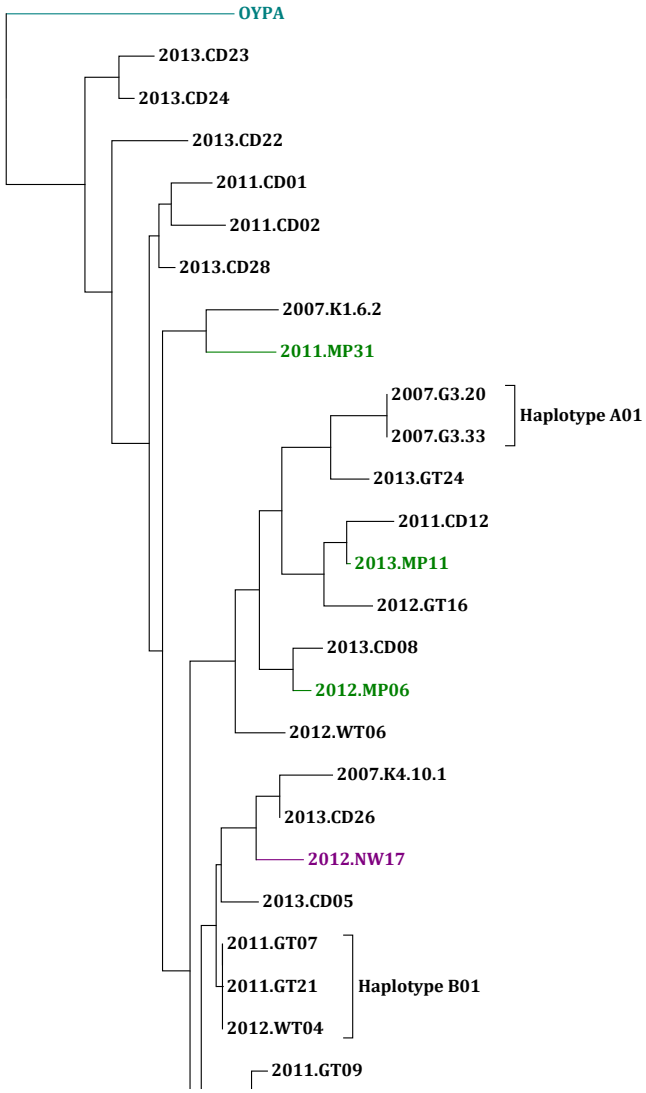
HAPLOTYPE) DESIGNATION	Samples	Date) Collected	Collection) Region	Comments
HAPLOTYPE)H1	2012.GT65	2012	Greytown	
HAPLOTYPE)H1	2012.NW04	2012	North)West	
HAPLOTYPE)H1	2012.NW05	2012	North)West	
HAPLOTYPE)H1	2012.NW10	2012	North)West	

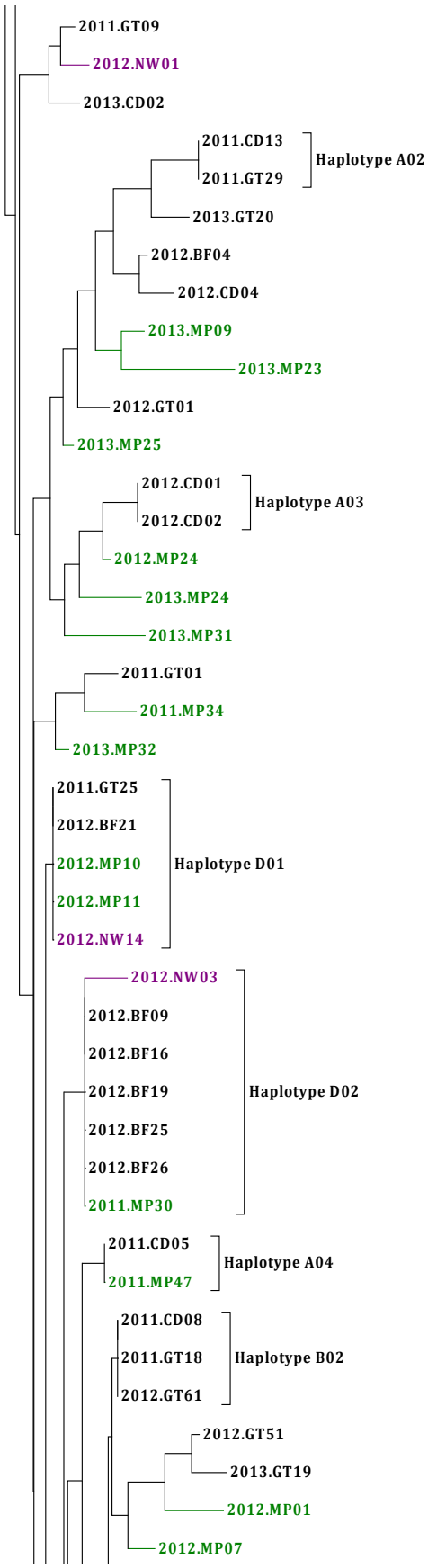
Unique haplotypes containing only one isoate were not given haplotypic designations.

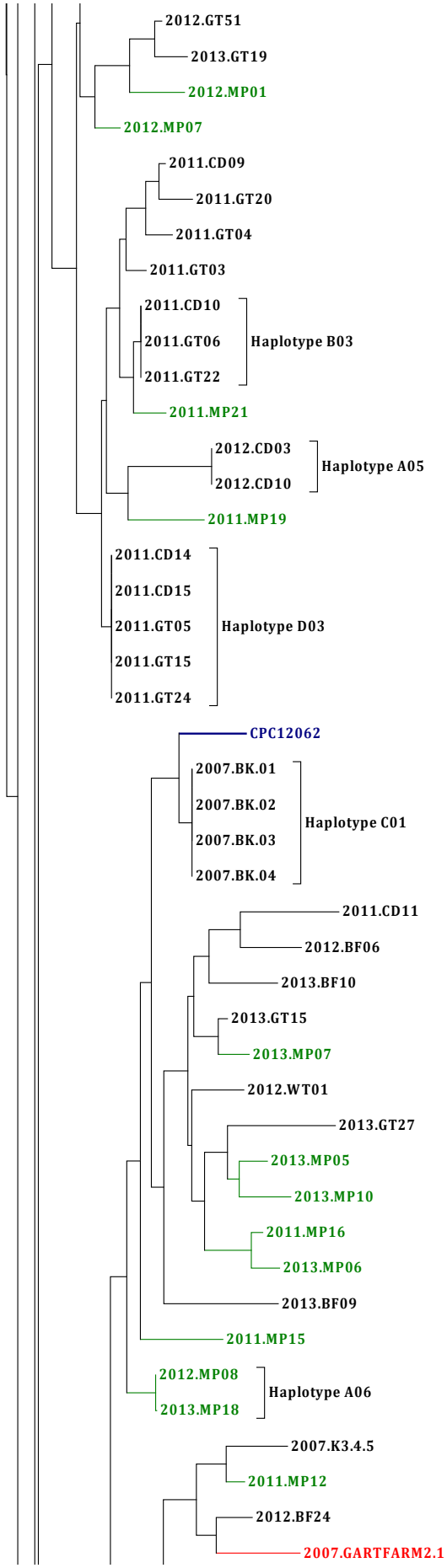
Annexure B: Linear version of the neighbour joining tree in Figure 11

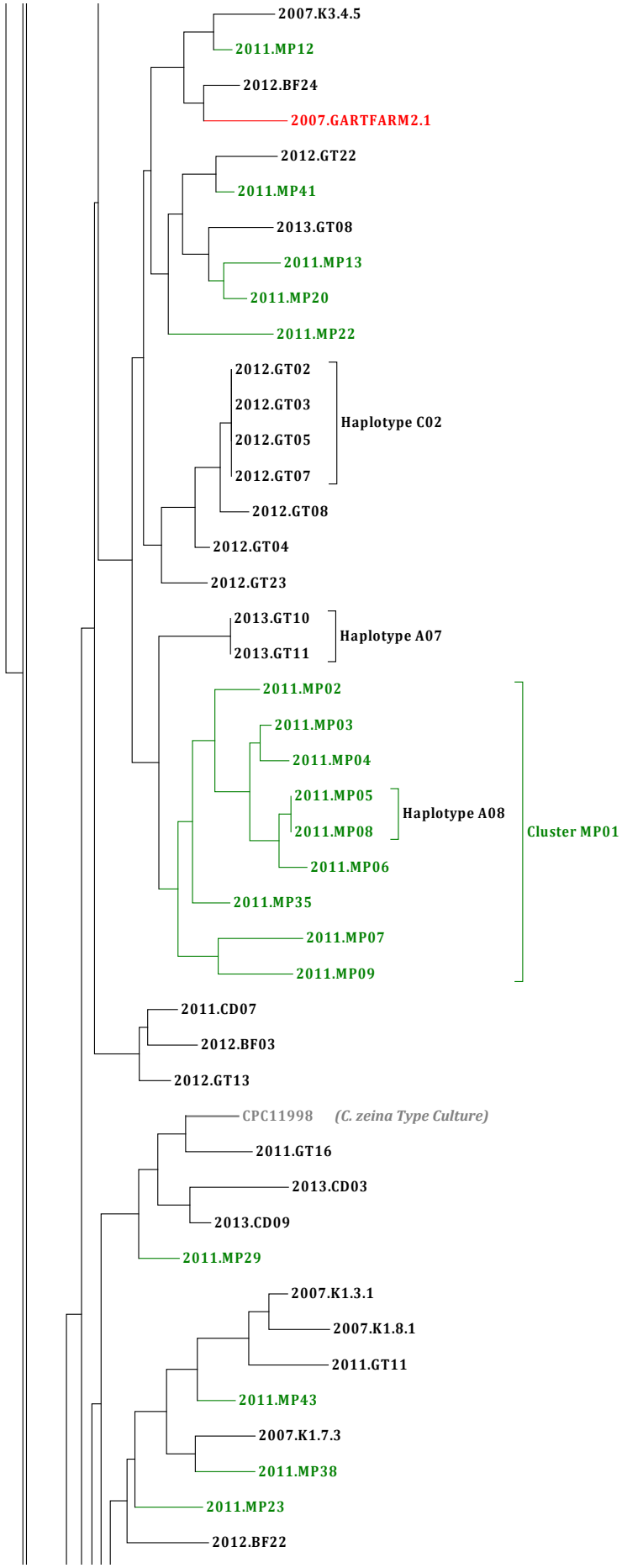
A neighbour joining tree constructed using Populations v1.2.32 and Nei's (1983) genetic distance (D_A) algorithm and edited in MEGA6. Isolates from Mpumalanga, North West, Zambia and Zimbabwe are highlighted in green, purple, red and yellow respectively. OYPA (US isolate) is highlighted in light blue.

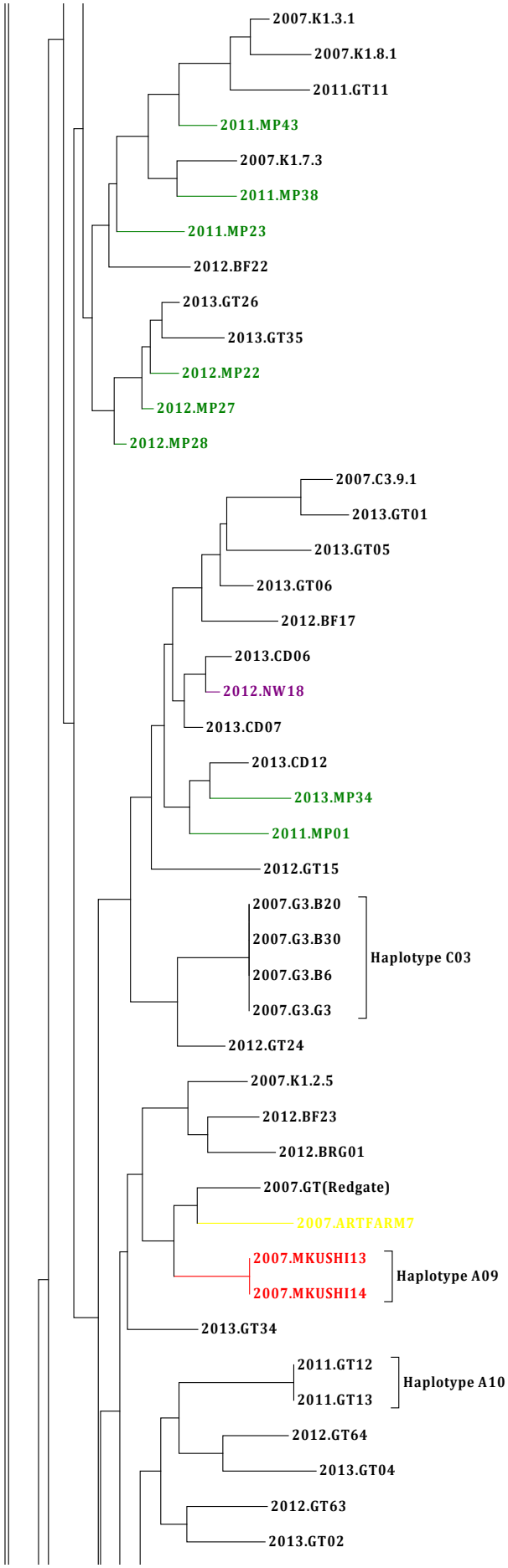
Naming of isolates are as follows: Example - 2013.CD23. 2013 refers to the year of collection, CD refers to the geographic region of collection (Cedara, Kwa-Zulu Natal) and the number following represents the isolate number. Likewise, BF – Baynesfield (KZN), K – Karkloof, G/GT – Greytown (KZN), MP – Mpumalanga, NW – North West Province, MPON – Empangeni (KZN), and WT/BRG/BK – Winterton/Bergville/Brandkraal (all regions were grouped as Winterton, Kwa-Zulu Natal).

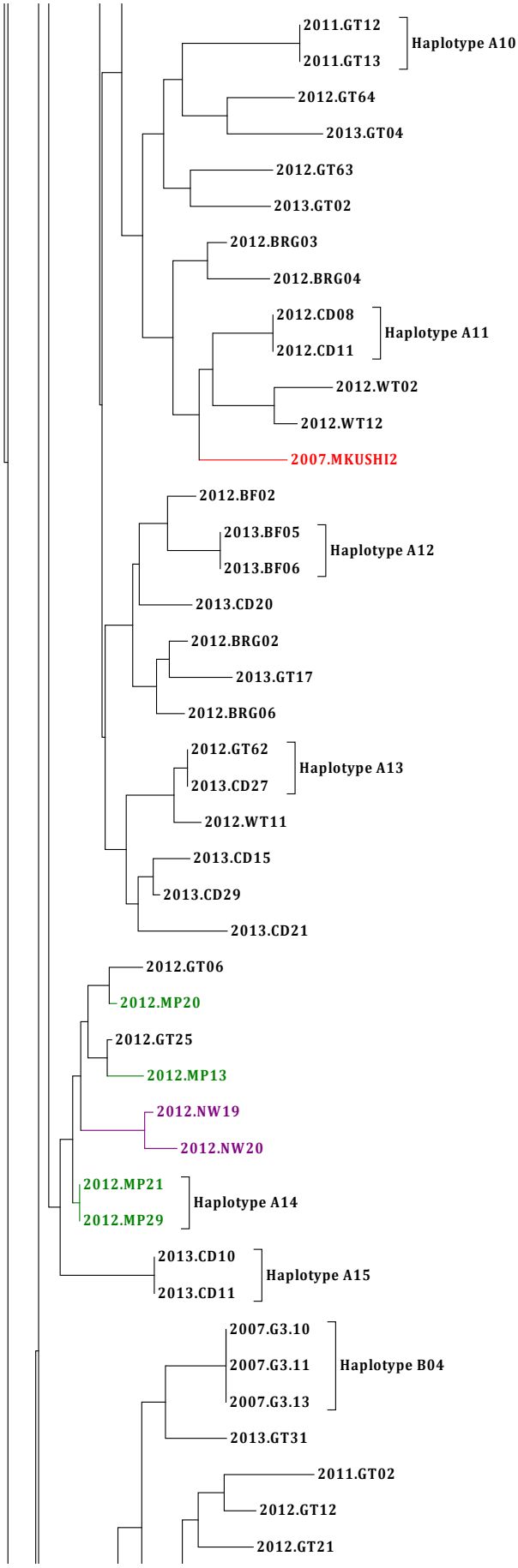


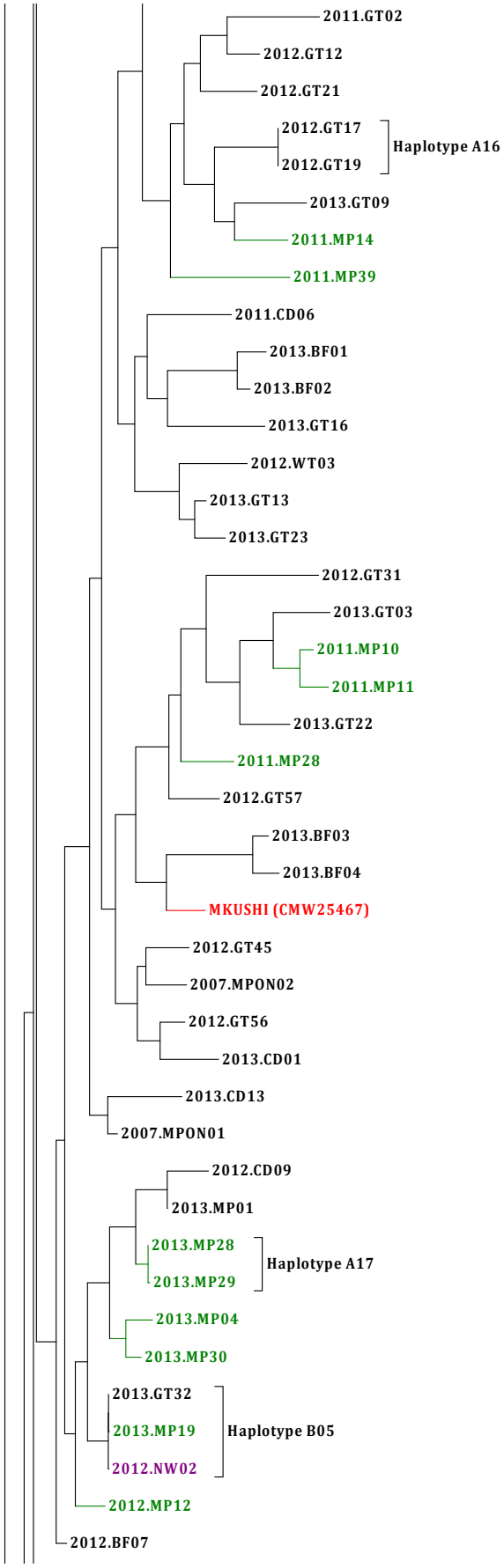


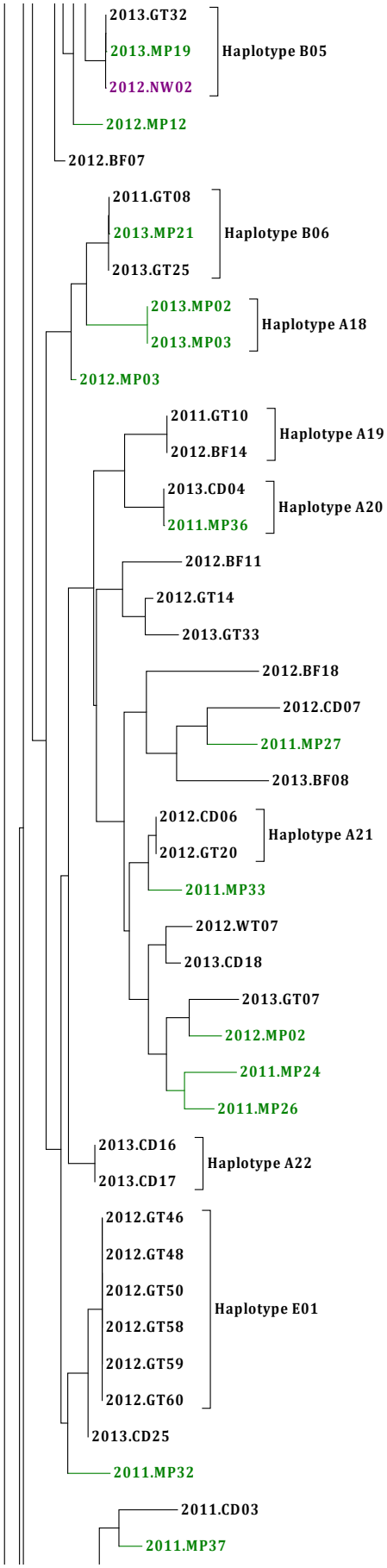


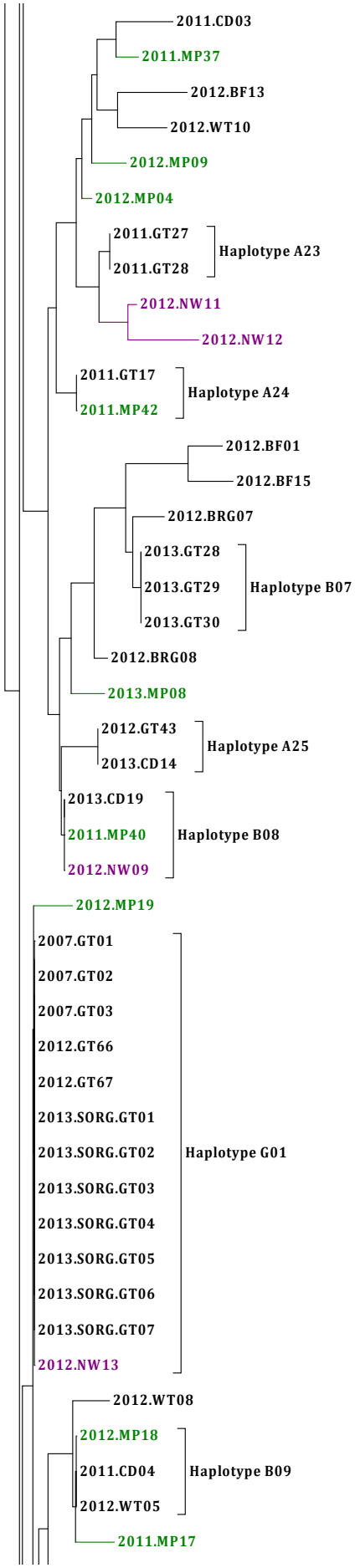


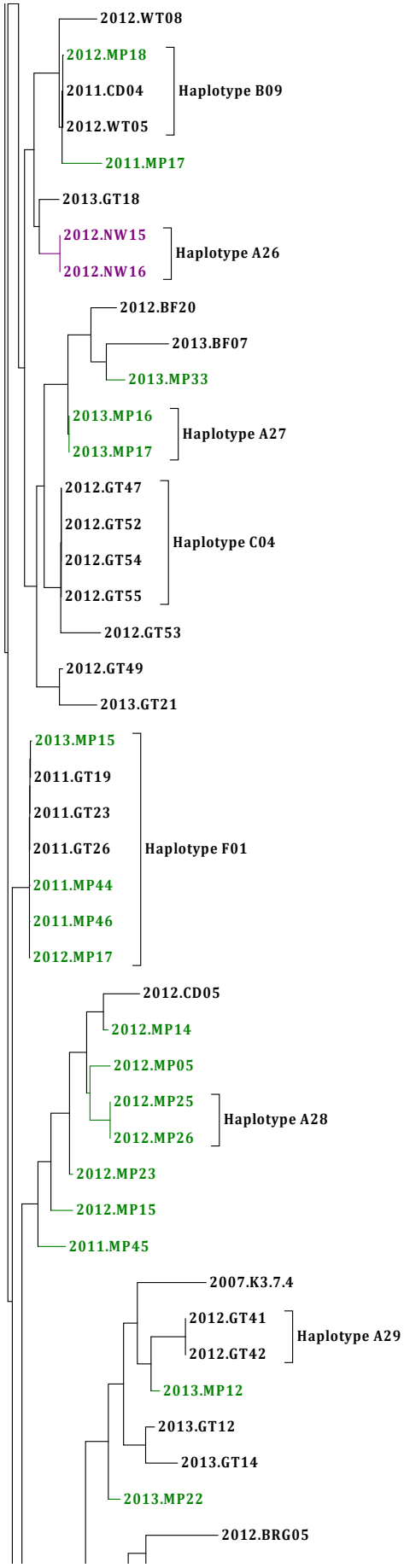


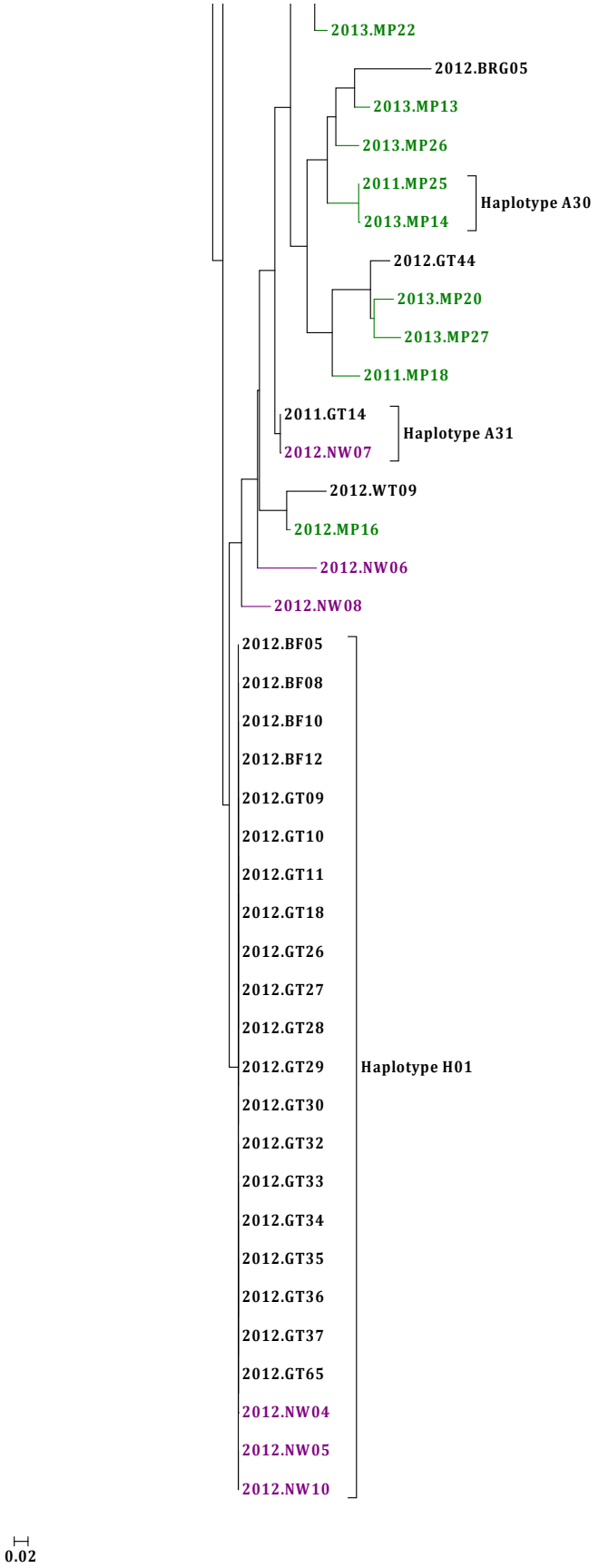






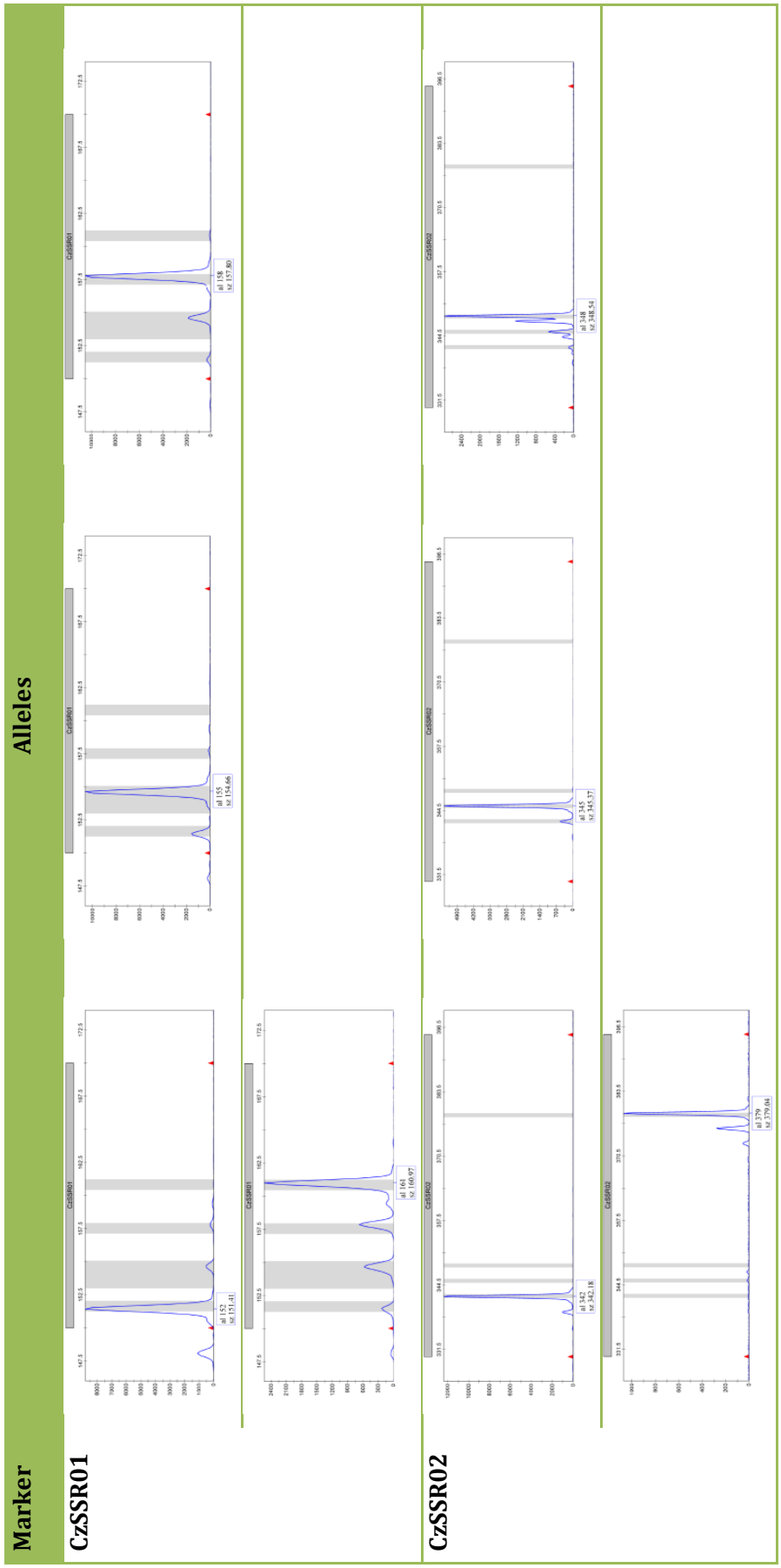


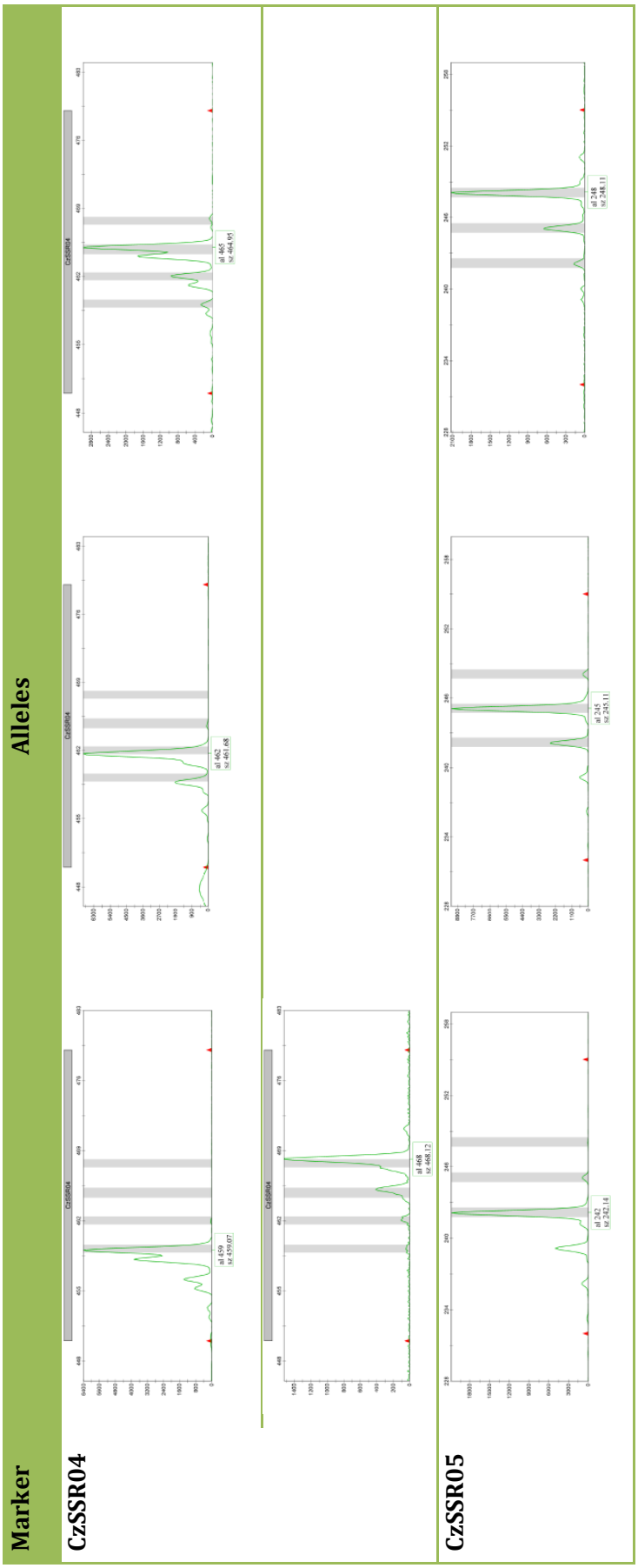


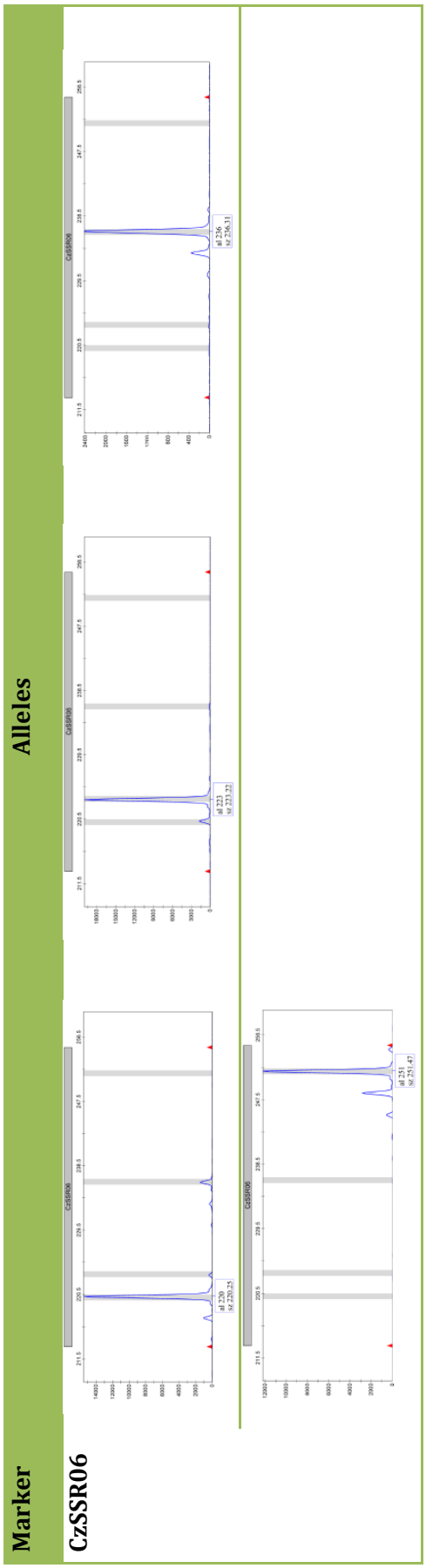


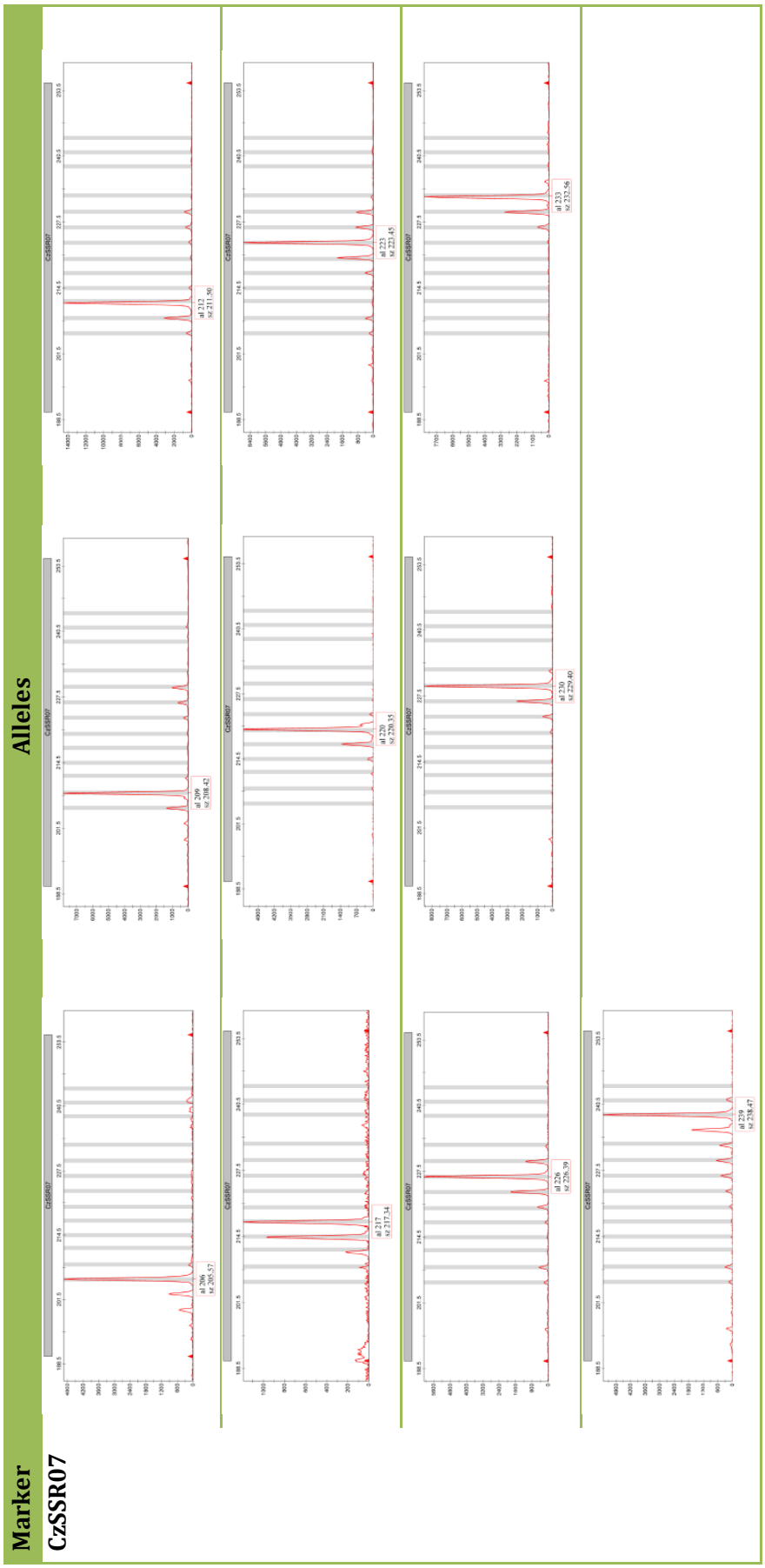
Annexure C: Microsatellite marker profiles

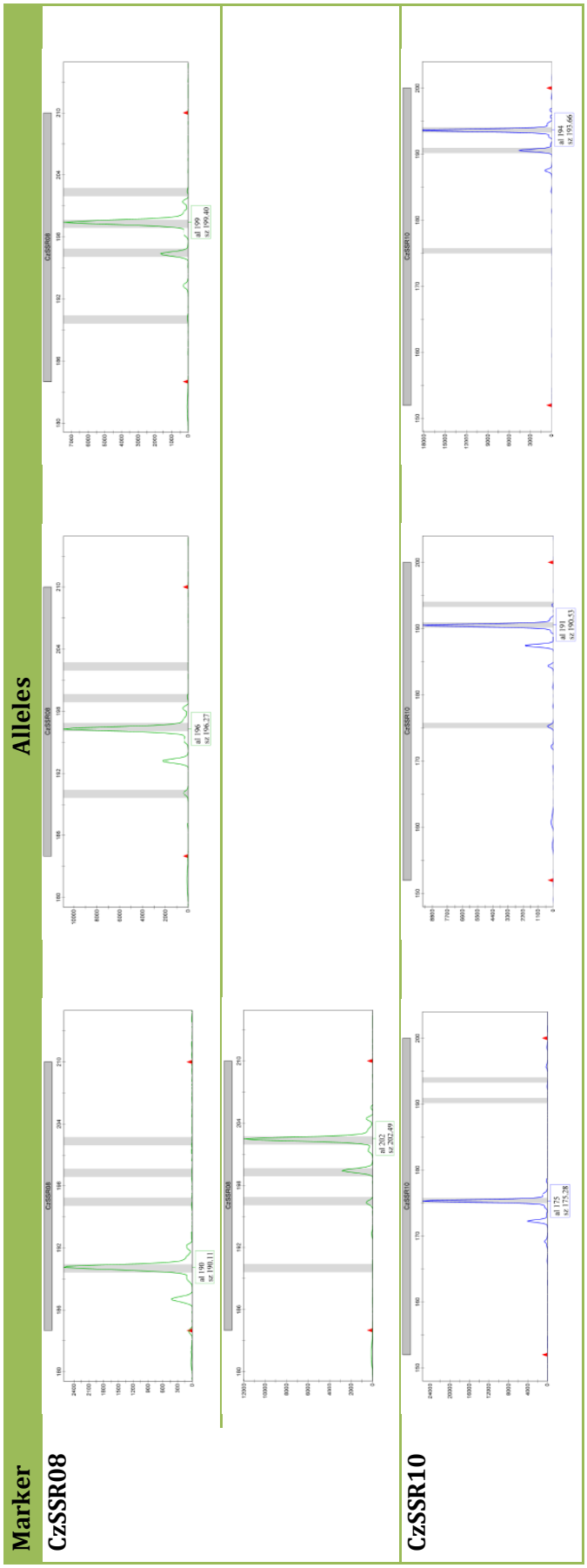
The pattern profiles of the microsatellite markers CzSSR01, CzSSR02, CzSSR04, CzSSR05, CzSSR06, CzSSR07, CzSSR08, CzSSR10, CzSSR11, CzSSR12, CzSSR13, CzSSR14, CzSSR15, CzSSR16, CzSSR17 and CzSSR18 designed to the genome of *Cercospora zeina* (CMW 25467).

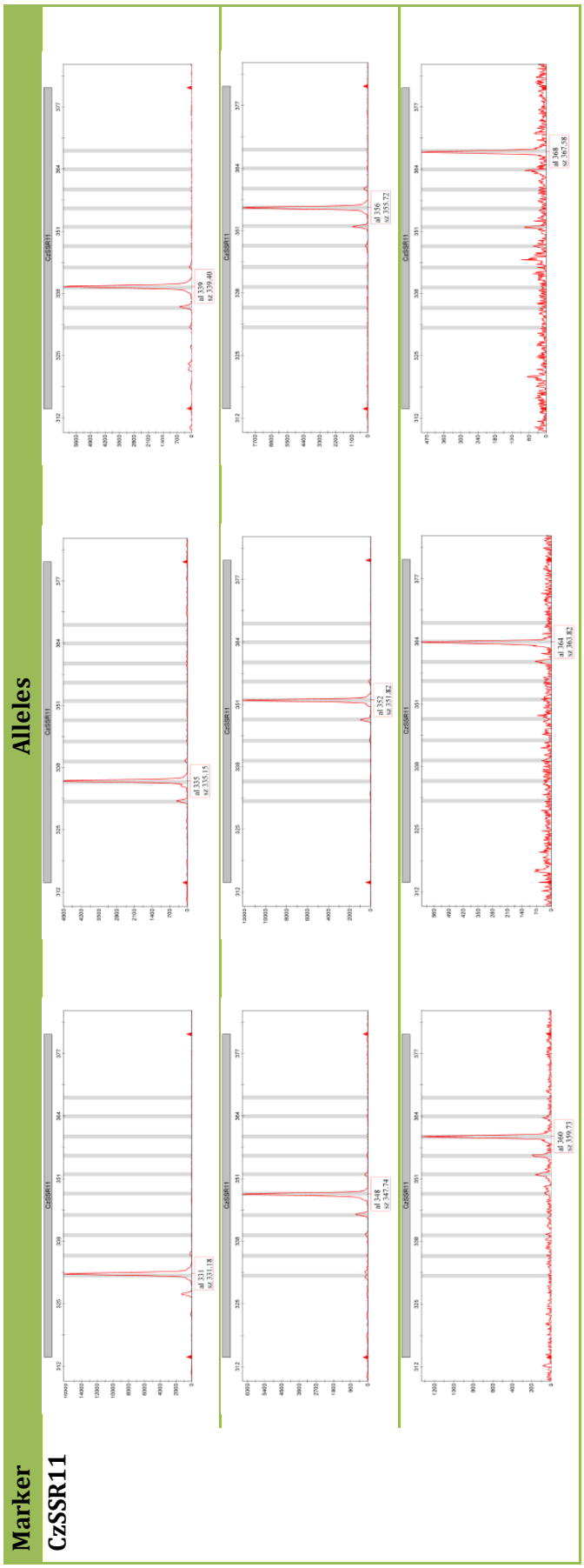


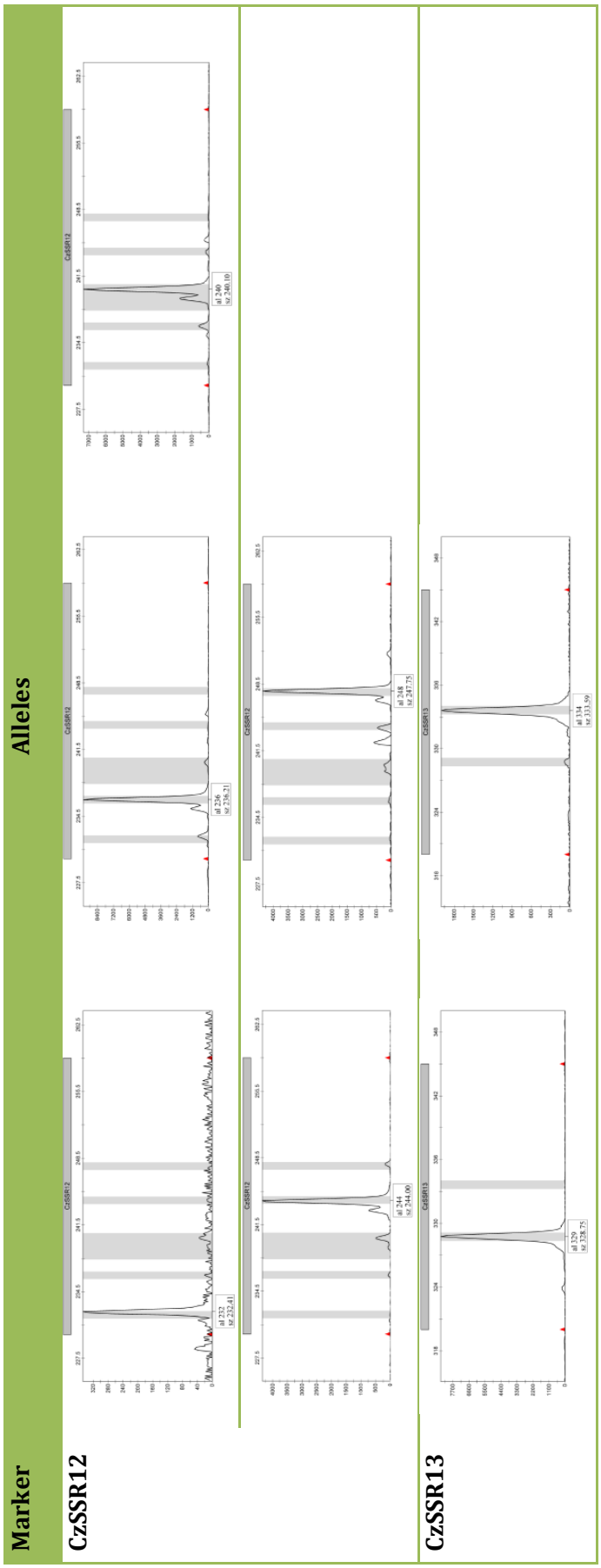


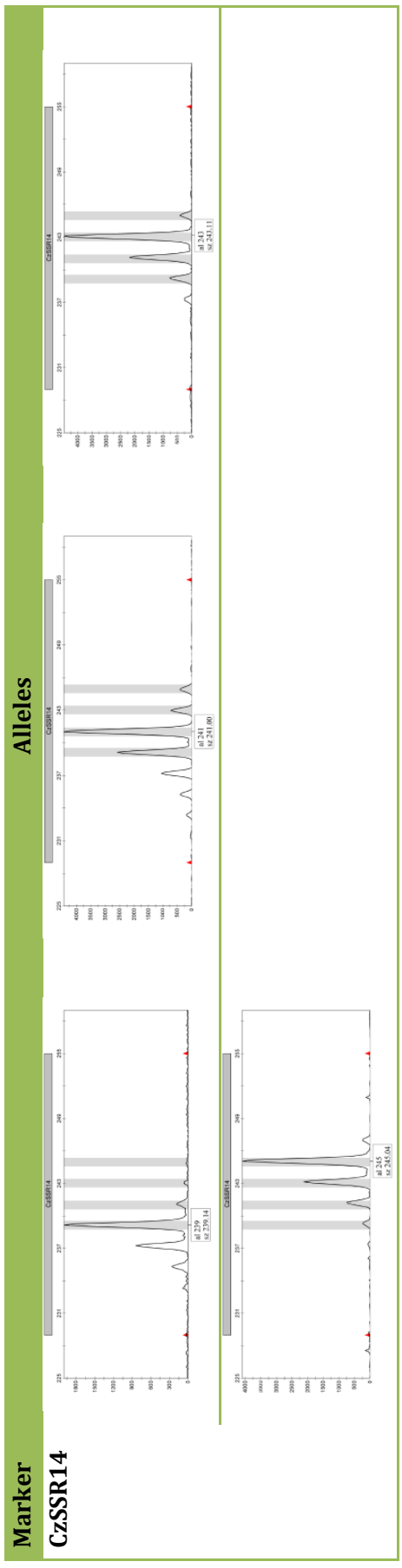


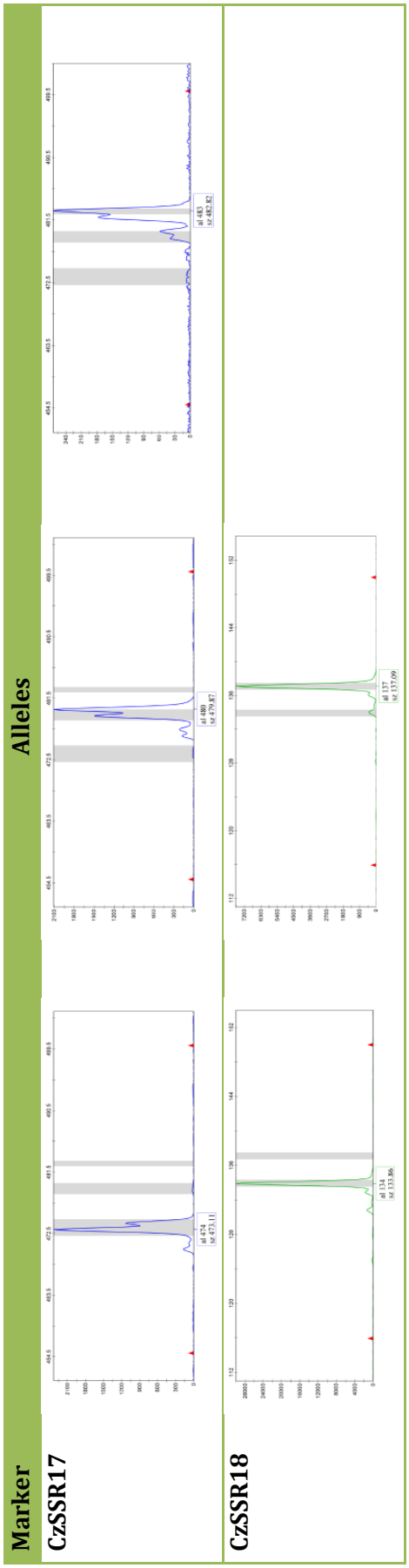












Annexure D: Microsatellite Sequences

Note: There are abbreviations for isolates that were sequenced to confirm microsatellite sequence (MK = Mkushi, OYPA = US isolate, GT = Greytown isolate, BRG = Bergville isolate (2012.BRG01), MP = Mpumalanga (2012.MP02)). Forward primer is highlighted in green, microsatellite sequence is highlighted in orange and the reverse primer is highlighted in blue.

CzSSR01

>2011_SSR02_MK TGT^15 Genbank accession: KP015832

AATTCATCGTAAGCACGACGACGGTCGACGGGCTCGAGCACGGTGACGGCGGGCCGGGACCGAAGTTATTTGCCG
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AG

>2011_SSR02_OYPA

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G

>2011_SSR02_GT

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AG

>2011_SSR02_BRG

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AG

>2011_SSR02_MP

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GGGAG

CzSSR02

>2011_SSR03_MK TGT^12 Genbank accession: KP015833

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GCGTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGT
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>2011_SSR03_OYPA

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TACTATGACGGGCAGTTGTGTAGTAGGTTGCGCGGCAGCGATGTTGCTGCTGTCATCCTCAGCCTGCTGCTGTTG
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CA

>2011_SSR03_GT

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TACTATGACGGGCAGTTGTGTAGTAGGTTGCGCGGCAGCGATGTTGCTGCTGTCATCCTCAGCCTGCTGCTGTTG
GCGTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGT
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>2011_SSR03_BRG

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>2011_SSR03_MP
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CzSSR04

>2011_SSR08_MK GTT^26 Genbank accession: KP015834
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ACGAAGATAAGAAGAATTTCAATGTGGGGCCGATGTCGAGGATGCTGGGGATGTGGATTCCCGAATAAGGAAGG
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>2011_SSR08_GT
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CzSSR05

>2011_SSR10_MK ACT^16 Genbank accession: KP015835
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>2011_SSR10_GT

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>2011_SSR10_MP
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CzSSR06

>2011_SSR13_MK TCT^15 Genbank accession: KP015836
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>2011_SSR13_BRG
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CzSSR07

>2011_SSR14_MK ATG^27 Genbank accession: KP015837
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>2011_SSR14_BRG
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CzSSR08

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