Population biology of *Fusarium circinatum* Nirenberg *et* O'Donnell associated with South African *Pinus radiate* D.Don plantation trees

By

Olga Matotsi Mashandule

Submitted in partial fulfilment of the requirements for the degree

Magister Scientiae

In the Faculty of Natural & Agricultural Sciences, University of Pretoria

Pretoria

2014 December

Supervisors: Prof. E.T. Steenkamp

Prof. T.A. Coutinho

Prof. M.J. Wingfield

Declaration

I Olga Matotsi Mashandule declare that the thesis / dissertation, which I hereby submit for the degree Magister Scientiae at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

…….

……

TABLE OF CONTENTS

Acknowledgements

Thank you to my supervisors, Prof. E.T. Steenkamp, Prof. T.A. Coutinho and Prof. M.J. Wingfield, for their support, guidance, training and mentoring throughout this degree.

FABI family, thank you for your support.

Mr Zacharia Mashandule, thank you for your support and patience throughout this degree.

The Makhari family “the magnificent family” thank you for your patience, love, understanding and motivation.

My sisters, Dr. M.T. Thantsha and Dr. L.N. Moleleki, thank you for your constant encouragement and support.

Preface

The fungus *Fusarium circinatum* is the causal agent of pitch canker that was initially discovered in the south eastern United States in 1946. In South Africa this pathogen was first discovered in 1990 in a single nursery in the Mpumalanga Province where it caused significant losses due root rot and collar infections on *Pinus patula* seedlings. After this initial discovery *F. circinatum* spread to other parts of South Africa where it apparently remained limited to pine seedling nurseries. In 2006, *F. circinatum* was discovered for the first time on established plantation trees of *P. radiata* in the Western Cape of South Africa. Since then the pathogen has been detected in numerous established pine plantations in the country. Although the epidemiology of the pathogen in the pine seedling nursery is relatively well understood, not much is known regarding the emergence of pitch canker and the distribution and spread of *F. circinatum* in pine plantations. The overall aim of the research presented in this dissertation was therefore to improve current knowledge regarding the population biology of *F. circinatum* in the plantation setting.

Chapter 1 reviews the molecular basis of vegetative and sexual compatibility in Ascomyceta, which represents two of the most widely used phenotypic properties for studying the population biology of *Fusarium* populations. Vegetative compatibility enables these fungi to distinguish self from non-self, which in turn allows them to maintain their unique genetic make-up in natural populations. This system is governed by a few so-called heterokaryon incompatibility (*het*) loci which have been extensively characterized at the molecular level in *Neurospora crassa*, *Podospora anserina*, *Aspergillus nidulans* and *Cryphonectria parasitica*. Sexual compatibility allows fungi to reproduce by recognising self from non-self. This system is governed by mating type *(mat*) loci, which have been extensively characterized in *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *N. crassa*, *P. anserine*, *Cochliobolus heterstrophus* and *F. circinatum.* In this chapter, a review is also provided of the methods used to study vegetative and sexual compatibility in the laboratory and the types of population genetic information that can be gained from such studies.

In Chapter 2 the possible origin and reproductive mode of the *F. circinatum* population responsible for the outbreaks of pitch canker on established plantation trees in the Western Cape province of South Africa were considered. The first aim of this study was therefore to collect isolates of *F. circinatum* from diseased *P. radiata* seedlings and plantation trees in the

region. To determine the possible origin of these isolates, they were compared to representative isolates obtained from diseased seedlings elsewhere in the country, as well as the USA and Mexico by making use of vegetative compatibility tests and Amplified Fragment Length Polymorphism (AFLP) analysis. The final aim of this chapter was to evaluate the relative importance of the sexual cycle in the reproduction of the *F. circinatum* populations in the Western Cape. For this purpose PCR-based diagnoses of mating type and standard mating tests were employed to determine sexual compatibility and fertility. The results indicated that the outbreak in the Western Cape was due to one or more separate introductions of the disease into that area.

One of the main findings of Chapter 2 was that the mating type of the *F. circinatum* populations in the examined Western Cape plantations is skewed towards *Mat*-1. Although this may be due to the high levels of clonality observed within these populations, other factors related to the sexual process itself cannot be excluded. The research presented in Chapter 3 considered some of the factors potentially contributing to the biased inheritance of mating type in *F. circinatum*. The first aim was therefore to determine the mating types of *F. circinatum* originating from laboratory-induced sexual fruiting structures and to compare these data to what is expected under the Mendelian law of segregation. The second aim of this study was to determine whether mating type was linked to fitness attributes such as mycelial growth rate and pathogenicity. For this purpose, standard pathogenicity tests were conducted on *P. patula* seedlings and cultural growth studies were conducted. There was no association between pathogenicity and growth study, therefore, it is not known what causes this distortion in *F. circinatum*.

CHAPTER 1

Vegetative and sexual compatibility in ascomycetes – a literature study

TABLE OF CONTENTS

1 Introduction

When fungal hyphae of different individuals meet, they can fuse (anastomose) to form an individual that consists of cells containing different nuclei (Glass *et al*., 2000; Glass and Kaneko, 2003; Kaneko *et al*., 2005). A fungal isolate harbouring genetically different nuclei that coexist in a common cytoplasm is known as a heterokaryon. During the fungal life cycle, heterokaryons form part of the vegetative and sexual phases (Saupe, 2000). Sexual compatibility is controlled by genes encoded at the mating type (*mat*) loci and vegetative compatibility by those encoded at the vegetative incompatibility (*vic*) or heterokaryon incompatibility (*het*) loci (Glass and Kuldau, 1992; Glass *et al*., 2004).

In ascomycetes, several *het* loci regulate vegetative compatibility (Begueret *et al.*, 1994), while a single *mat* locus controls sexual compatibility (Leslie, 1993). The typical ascomycete *mat* locus consists of two so-called 'idiomorph' alleles that each encodes different sets of proteins. A prerequisite for meiosis and the production of recombinant progeny is that the interacting individuals harbour different idiomorphs at their *mat* loci and they should thus be of opposite mating type (Coppin *et al.*, 1997). In terms of vegetative compatibility, anastomosis and the establishment of a heterokaryon is restricted to individuals that share the same alleles at all of their *het* loci. Although such heterokaryotic cells contain nuclei of different genotypes, these nuclei may not necessarily differ at the *mat* locus (Saupe *et al.*, 2000; Wu and Glass, 2001). Also, the sexual nuclei within a heterokaryon that developed due to a sexual interaction, may not necessarily encode identical *het* alleles (Bernet, 1967; Labarere *et al*., 1974; Bourges *et al*., 1998). Therefore, the sexual recognition and vegetative recognition (also termed non-self recognition) systems operate independently from one another, although there are some exceptions (e.g. *Neurospora crassa*) where the *mat* locus determines the outcome of vegetative compatibility interactions (Leslie, 1993).

In this chapter, I review the literature pertaining to the biological and molecular aspects underlying the sexual and vegetative recognition systems of ascomycete fungi, with special reference to *F. circinatum*, the causal agent of pine pitch canker (Wingfield *et al*., 2008). I also discuss the various methods used in the laboratory to perform vegetative and sexual compatibility assays. The relevance of studying these systems in fungi, especially from a population biology point of view, is also considered.

2 Vegetative compatibility *vs* incompatibility

Hyphal fusion commences when the hyphae involved in anastomosis release diffusible signals that mediate positive autotropism or attraction of the hyphal tips (Xiang *et al.*, 2002). The mechanism of attraction (positive autotropism) is unknown (Glass *et al.*, 2004). Hyphal tip growth is arrested after physical contact. This triggers the expression of hydrolytic enzymes that break down the cell walls at the point of contact. New cell wall material (e.g. chitin) is transported to the contact point to form a new cell wall bridge between the hyphae (Glass and Kaneko, 2003; Hietala *et al.*, 2003). Next, the cytoplasms of the interacting cells fuse, thus resulting in the mixing of genetically different nuclei (Glass *et al.*, 2000). Where the two individuals are vegetatively compatible, the nuclei may migrate as in the case of *N. crassa* and *Podospora anserina* to other cells through their septal pores thus allowing the heterokaryotic cells to proliferate until almost all the cells within the mycelium are heterokaryotic (Glass *et al.*, 2000). In other cases, the heterokaryon is restricted to the fusion cells as it has been reported for species in the *Gibberella fujikuroi* complex and *Verticillium dahlia* (Glass *et al.*, 2000). If the two interacting individuals are vegetatively incompatible, a cascade of events leads to death and restriction of the fused heterokaryotic cells and sometimes the surrounding cells (Glass and Kaneko, 2003).

Previous research on the model ascomycetes *N. crassa* and *P. anserina* has demonstrated that a variety of phenotypic traits are associated with vegetative incompatibility. In *N. crassa*, it has been linked to abnormal colony morphology and growth inhibition (Saupe, 2000). Incompatible isolates of this fungus have been observed to grow 100 times slower than compatible heterokaryons. Vegetative incompatibility is also associated with reduced conidiation (Saupe, 2000). The fused cytoplasms of incompatible individuals typically become compartmentalized, granular and eventually they die off (Saupe, 2000). Septal pores that bracket the heterokaryotic cells (often the subtending cells) become plugged, thus restricting the heterokaryotic tissue (Glass *et al.*, 2000). For example, a gene “induced during incompatibility” (*idi*), *idi*-1, encodes a cell wall protein that quickly seals the septa of the cells that are adjacent to those that are dying, thus restricting cell death to the fusion cells (Dementhon *et al.*, 2003). Numerous vacuoles containing proteases and degradative enzymes

also develop in the fused cytoplasm to aid in the breakdown of the heterokaryotic and surrounding cells (Glass *et al*., 2000; Saupe *et al*., 2000).

At the nucleic acid and protein levels, incompatible heterokaryotic cells are clearly distinguishable from vegetatively compatible cells. After vegetative incompatibility has been triggered in a cell, various proteins with proteolytic and other enzymatic activities have been demonstrated to appear (Glass *et al.,* 2000). These include phenoloxidases, malate/NADH dehydrogenase, proteases (e.g. aspartyl protease and subtilisin-like serine protease), laccasses, and amino acid oxidase (Glass *et al*., 2000; Saupe *et al*., 2000; Glass and Kaneko, 2003). Vegetative incompatibility also appears to be associated with a decrease in transcription levels and total RNA synthesis (Saupe, 2000). It has further been shown to cause an overall down-regulation of gene expression and up-regulation of a limited number of key products involved in cell death and restriction of the heterokaryotic tissue (Boucherie *et al*., 1981; Bourges *et al.*, 1998).

3 Vegetative incompatibility and programmed cell death

When two interacting fungal individuals are vegetatively incompatible, features that are similar to those associated with programmed cell death (PCD) in multicellular eukaryotes are observed (Xiang and Glass, 2004). Overall, it seems that the speed at which the cells are killed depends on the number of loci with different allelic specificities between the fungal individuals (Glass *et al*., 2000; Saupe *et al*., 2000). This type of PCD is different from that observed during apoptosis (i.e. Type I PCD) in that fungi are not known to encode the required caspase with proteases to degrade proteins by digesting them at specific sites (Lam *et al.,* 2001). Also DNA-laddering, typical of apoptosis does not occur, although DNA is degraded during vegetative incompatibility (Glass and Kaneko, 2003; Marek *et al.,* 2003). Cell death caused by fungal vegetative incompatibility appears to be associated with Type II PCD (i.e. PCD due to autophagy, see below) (Pinan-Lucarre *et al.,* 2002). This is largely thought to be the case due to the increased vacuolarization after vegetative incompatibility has been triggered and the up-regulation of specific proteins (e.g. those encoded by the *idi* genes) has occurred (Klionsky and Emr, 2000).

Autophagy is a process in which cytoplasmic contents are degraded in bulk by vacuolar

enzymes to produce new pools of nutrients during vegetative incompatibility (Klionsky and Emr, 2000). It has been shown for *P. anserina,* that two *idi* genes (*idi*-6 and *idi*-7) are up regulated during certain types of vegetative incompatibility reactions. The *idi*-6 gene (also known as *pspA*) encodes a vacuolar protease that is involved in the degradation of autophagic bodies Fto prevent degradation of the newly produced nutrient pool. The *idi*-7 gene encodes an ortholog of the yeast AUT7 that has been demonstrated to be involved in the formation of autophagosomes (vesicles that take cytoplasmic material to the vacuoles) (Pinan-Lucarre *et al.*, 2002).

In addition to being induced during vegetative incompatibility, autophagy in fungi is also brought about by nutrient starvation (Klionsky and Emr, 2000). Autophagy has been shown also to be under the control of TOR (target for rapamycin) protein kinases, which are conserved from yeast to humans and appear to control growth in response to nutrient availability (Rohde *et al*., 2001). Studies have indicated that when fungal isolates are deprived of carbon and nitrogen, they show the same phenotypic effect as vegetatively incompatible reactions (Dementhon *et al*., 2003). Furthermore, Glass and Kaneko (2003) showed that the *papA* encoded aspartyl protease normally expressed during glucose starvation is also produced during vegetative incompatibility. To explain these findings, they suggested that vegetative incompatibility reactions deplete the glucose in the growth media, thus resulting in an up regulation in the expression of the *papA* gene. Dementhon *et al*. (2003) also speculated that *het* genes belong to a signaling cascade feeding into the TOR pathway or that *het* genes products affect fungal metabolism.

4 The molecular basis of vegetative incompatibility

Two genetic systems regulate vegetative incompatibility in fungi (Glass and Kaneko, 2003). The first is the so-called “allelic” system, during which vegetative incompatibility is triggered between fungal isolates that have different allelic specificities at the same *het* locus. The other is the so-called “non-allelic” system, during which vegetative incompatibility is triggered between fungal individuals that have different allelic specificities at different loci (Saupe, 2000).

Vegetative incompatibility is a well-known phenomenon of filamentous fungi, including ascomycetes, basidiomycetes and zygomycetes (Leslie, 1993; Glass and Kaneko, 2003). However, only a few *het* loci in ascomycetes have been identified and characterized at the genetic and DNA levels. Ten *het* loci and one *mat* locus in *N. crassa* have been characterized at the molecular level. Nine *het* loci in *P. anserina*, eight in *Aspergillus nidulans* and seven in *Cryphonectria parasitica* have been characterized at a molecular level (Glass *et al*., 2000). For the purposes of this review, only those *het* loci best characterized in the two model ascomycetes, *N. crassa* and *P. anserina* are considered, while the limited information on vegetative incompatibility for *Fusarium* species is also highlighted.

4.1 The *het* loci of *N. crassa*

4.1.1 *mat* locus

The *mat* locus in *N. crassa* mediates allelic vegetative incompatibility. In most other fungi, however, this locus does not seem to be involved in vegetative incompatibility (Saupe, 2000). In *N. crassa*, vegetative incompatibility is triggered when isolates that are different at the locus harbouring opposite *mat* idiomorph alleles (*mat* A-1/*mat* a-1) interact. When the interacting isolates have identical idiomorphs (*mat* A-1/A-1 or *mat* a-1/a-1) at the *mat* locus, vegetative compatibility reactions are triggered (Kronstad and Staben, 1997).

Within the *mat* idiomorphs, only specific regions appear to be associated with vegetative incompatibility. Only one of the open reading frames (ORFs) on the A idiomorph encodes a protein (MAT A-1) that has been shown to be involved in vegetative incompatibility (Glass and Kuldau, 1992). This protein apparently also determines mating type identity and plays a role in post fertilization functions (Glass and Kuldau, 1992). The ORF on the other idiomorph encodes a protein (MAT a-1) with leucine-rich repeats and a region that is homologous to High Mobility Group (HMG) transcriptional regulators (Glass and Kuldau, 1992; Saupe, 2000). Although the HMG domain appears to be required for determining mating type identity (see below; Philley and Staben, 1994), the leucine-rich repeats are involved in vegetative incompatibility. For example, four amino acid deletions at the carboxyl terminus of *mat* a-1 suppress vegetative incompatibility but not mating. Thus, despite the fact that the *mat* locus is involved in vegetative incompatibility, the biochemical mechanisms underlying this phenomenon is different from that associated with sexual recognition (Philley and Staben, 1994).

4.1.2 *het*-c

The *het-c* locus of *N. crassa* is occupied by one of three alleles (*het-c*^{OR}, *het-c*^{PA} and *het-c*^{GR}) that mediate allelic vegetative incompatibility (Saupe *et al.*, 1996; Loubradou and Turcq, 2000). A variable region of 34-48 amino acids that differs in all three alleles determines the allelic specificity (Saupe and Glass, 1997; Wu and Glass, 2001). The OR allele has a 15 base pair (bp) insertion; PA has a 30 bp insertion, while both insertions are absent in the GR allele (Saupe, 2000). Little is known about these alleles and only the *het-c*^{OR} allele (derived from the so-called Oak Ridge isolate) has been characterized. This allele encodes a non-essential polypeptide (966 amino acids) with a putative signal peptide, a coiled motif and a C-terminal glycine-rich domain. The sequence of the hydrophilic glycine-rich domain has a similar sequence to several extracellular or cell envelope proteins. A mutation in the *het-c*^{OR} affects neither fertility nor growth, but strains containing the mutant *het-c*^{OR} are compatible with strains containing either the *het-c*^{OR} or the *het-c*^{PA} allele (Saupe and Glass, 1997). These features suggest that the polypeptide encoded by *het-c*^{OR} is associated with the fungal cell wall (Saupe *et al.*, 1996) and that it plays a role mainly in vegetative incompatibility.

4.1.3 *het*-6

The *het-6* locus mediates non-allelic vegetative incompatibility that is triggered by its interaction with the *un-24* locus (situated 19 kbp distal to the *het-6* locus) (Saupe, 2000). The *het-6* locus was initially described in *N. crassa* strains that displayed hyphal growth inhibition. The locus has two alleles (*het-6*^{OR} and *het-6*^{PA}) and it is a member of a repetitive gene family, the expression of which produces a protein with no known function aside from non-self recognition (Saupe, 2000). This protein does, however, have a region (amino acid 53-242) that shares significant similarity to the proteins encoded at the *het-e* locus (amino acid 19-144) of *P. anserina* and the *tol* gene (amino acid 337-500) in *N. crassa* (Smith *et al.*, 2000).

4.1.4 *un*-24

The *un-24* locus of *N. crassa* mediates non-allelic vegetative incompatibility when it interacts with *het-6*. The allelic specificity (*un-24*^{PA} or *un-24*^{OR}) of this locus is determined by a large insertion at the C-terminal of the gene encoding the large subunit of a class I ribonucleotide reductase enzyme. The active form of this enzyme is composed of small and large subunit

dimers that play an important role in cell division. This enzyme is required for the conversion of ribonucleotide precursors to deoxyribonucleotide used in DNA synthesis and repair (Smith *et al.*, 2000). The C-terminus of *un-24* occupies the central position of the active site where the large and small subunits of the ribonucleotide reductase interact to form an active haloenzyme (i.e. the combined apoenzyme and cofactor). The vegetative incompatibility reaction between *un-24*^{PA} and *het-6*^{OR} disrupts the structure of the haloenzyme, resulting in the loss of function. Therefore, nucleotide imbalance results in cell cycle arrest and death (Saupe, 2000). It is not known which of the alleles (AP or OR) encode the small subunit of the enzyme but it can be assumed that the AP alleles in both loci (*un*-24 and *het*-6) codes for the small subunit because the OR alleles codes for the large subunit (Saupe, 2000).

4.1.5 *pin*-c

The *pin-c* (partner for incompatibility with *het-c*) locus encodes a HET domain that causes a non-allelic vegetative incompatibility reaction when it interacts with proteins encoded by the *het-c* locus. This locus is closely linked to the *het-c* locus and it is governed by three alleles that are *pin-c1*, *pin-c2* and *pin-c3*. Studies have indicated that the intensity of the incompatibility phenotype between *het-c* and *pin-c* is enhanced by the allelic interactions at the *het-c*. The *het-c* locus is, therefore, the first *het* locus known to be involved in both allelic and non-allelic interactions (Kaneko *et al*., 2005).

4.2 The *het* loci of *P. anserina*

4.2.1 *het*-s

The *het-s* locus mediates allelic vegetative incompatibility and has two alleles termed *het-s* and *het-S* (Rizet, 1952; Dalstra *et al.*, 2003). Alleles *het-s* and *het-S* are very similar, and both encode a protein that is 289 amino acids in length and they differ in only 13 amino acid residues (Balguerie *et al*., 2004; Saupe and Daskalov, 2012). Het-s and Het-S proteins consists of two domains, the N- terminal globular domain (comprising of residue 1-227 residues) termed the HeLo domain and the C-terminal domain (comprising of residue 228-289) termed the prion forming domain (PFD) (Balguerie *et al*., 2003). Differences in the functions of Het-s and Het-S are determined by the amino acid differences residing in the HeLo domain (Saupe, 2011). Studies have shown that the PFD of the Het-s protein does not require its HeLo domain to carry out its functions of infectivity and incompatibility in vivo.

On contrary, Het-S requires both the HeLo domain and the PFD to carry out its incompatibility function (Greenwald *et al*., 2010; Saupe, 2011).

The PFD of the *het-s* allele is associated with the expression of the HET-s prion and strains carrying the *het-s* allele typically display either the Het-s phenotype that harbours active HET-s prion or the Het-s* phenotype that is prion free. Like other prions, the HET-s prion also represent an infectious self-propagating amyloid polymer that is host soluble and can transform Het-s* cells to prion infected Het-s cells (Saupe, 2000). During the prion formation, the Het-s PDF undergoes transition from unstructured, to the ß-solenoid form (amyloid formation). This results in partial unfolding of the ten amino acids (residues 218-227) overlapping the HeLo domain and PFD (Saupe, 2011).

The prion form of the HET-s protein usually appears spontaneously inside the mycelium of the Het-s* strains at low frequencies. The prion free Het-s* cell undergoes a complete transformation when it comes into contact with the cytoplasm of the Het-s prion infected mycelium (Saupe, 2000). Fusions between Het-s* and Het-S hyphae result in the formation of heterokaryotic cells, while fusions between the Het-s and Het-S hyphae result in vegetative incompatibility reaction and cell death (Sarkar *et al*., 2002; Dalstra *et al*., 2005). Previous studies have shown that point mutations in *het-s* and *het-S* may also trigger incompatibility between Het-s* and Het-S hyphae (Deleu *et al*., 1993).

During the incompatibility reaction, the interaction between Het-S and Het-s activates the folding of the Het-S PFD into the ß-solenoid form (Saupe and Daskalov, 2012). This transition results in the destabilisation of the HeLo domain due to the overlap of the two domains (Saupe and Daskalov, 2012). The destabilised HeLo domain triggers the toxic and prion-inhibitory activity of the Het-S by undergoing restructuring which exposes the buried amino acids of the transmembrane (TM) helix. The activated Het-S relocate to the cell membrane where it assembles into a membrane disrupting complex, forming holes in the membrane, thus, resulting in liposomal content leakage (Mathur *et al*., 2012; Seuring *et al*., 2012).

Researchers have also discovered a protein homologue of Het-s PFD, known as NWD2 (encoded by a gene located adjacent to the het-S; see section 4.2.2.1 below). NWD2 is part of

the NWD gene family (Paoletti *et al*., 2007) with an exception of lacking a defined effector domain, instead, the N-terminal only displays a HET repeat motif (Daskalov *et al*., 2012). During the incompatibility reaction, NWD2 recognizes a ligand using its C-terminal WD-40 repeat domain and oligomerises in response to this binding. The oligomerisation results in the N-terminal extensions of NWD2 to fold into a ß-solenoid form. The ß-solenoid form of the NWD2 N-terminal activates the Het-S PFD and this triggers the HeLo toxicity (Saupe and Dasklov, 2012).

In addition to playing a role in vegetative incompatibility, the *het-s* locus is also implicated in the post fertilization steps of the sexual cycle of *P. anserina* where it causes Het-S spore killing (Dalstra *et al*., 2005). Although the genes encoded at the *het-S* locus are suppressed during the initial stages of the sexual cycle, expression of the *het-S* and *het-s* alleles may be detected as soon as young ascospores are delimited. Should the Het-S protein be expressed in a cytoplasmic background containing pre-existing Het-s prion that was formed in the maternal strain before the onset of the sexual cycle, antagonism between the two proteins result in ascospore abortion. As a result, spores carrying the *het-S* allele are killed, while the *het-s* allele is inherited in a non-Mendelian fashion (Saupe, 2011). However, overexpression of Het-s and low temperatures (18°C compared to 25°C) have been proven to favour spore killing in *P. anserina* (Saupe, 2011; Debets *et al*., 2012). In this way, *het-s* causes meiotic drive (over- or under representation of certain gametes during meiosis) as it acts as a selfish genetic element or so-called ‘segregation distorter’ (Sarkar *et al*., 2002; Dalstra *et al*., 2003; Dalstra *et al*., 2005).

4.2.2 Non-allelic interactions involving the *het*-c, *het*-d and *het*-e loci

The *het-c* locus of *P. anserina* mediates non-allelic vegetative incompatibility with the products of two unlinked genes, *het-d* and *het-e* (Loubradou and Turcq, 2000; Saupe, 2000). The *het-c* locus has four known allelic specificities (*het-c1, c2, c3* and *c4*) (Saupe *et al*., 1994) that encode a 208 amino acid glycolipid transfer protein of which the exact function is not yet clear. Mattjus *et al.* (2003) hypothesized that this protein could be involved in the regulation of the metabolic pool(s) of sphingolipid metabolites that play a role in the stress and lytic responses that are linked to cell-cell interactions. Interestingly, mutations in this locus results in abnormal ascospore formation and morphological changes (Saupe *et al*., 1995; Loubradou and Turcq, 2000). Saupe *et al.* (1994) suggested that it could be involved in the deposition of

cell wall material at the hyphal tips or on ascospore membranes. The products encoded at the *het-c* locus are, therefore, not only involved in vegetative incompatibility, but also in other cellular functions (Mattjus *et al.*, 2003).

The *het-d* locus has three specificities (*het-d1, d2* and *d3*) and has not been extensively studied in *P. anserina* (Saupe *et al*., 1994). The *het-e* locus has four alleles (*het-e1, e2, e3* and *e4*) (Saupe *et al.,* 1994) that encode a 1356 amino acid polypeptide that has a GTP-binding domain in its N-terminal (Saupe and Begueret, 1995). This domain appears to be important for vegetative incompatibility because a mutation in the GTP-binding domain results in the loss of GTP fixation and the protein becomes non-functional in the incompatibility reaction. Within the C-terminal of the HET-E protein there are WD-40 repeats that most probably mediate protein-protein interactions. These repeats are commonly found in proteins of various functions such as the β-subunit of heterotrimeric G proteins (Saupe and Begueret, 1995; Loubradou and Turcq, 2000).

4.2.2.1 “STAND genes”

Recent studies have shown that vegetative incompatibility genes of *het-d*, *het-e* and *het-r* loci, encode STAND (signal transduction adenosine triphosphatases with numerous domains) proteins that belongs to the HNWD gene family (Saupe *et al*., 1994; Saupe and Begueret, 1995; Loubradou and Turcq, 2000; Paoletti *et al*., 2007; Chevanne *et al*., 2009). These proteins comprises of a WD-40 repeat domain in the C-terminus, a central NACHT (nucleotide binding oligomerization domain) and an N-terminal HET domain (Leipe *et al*., 2004). STAND proteins resemble Nod-like receptors, they are thought to function as recognition receptors and are signal transducing NTPases that undergo ligand-induced oligomerisation (Saupe and Daskalov, 2012). Moreover, the STAND proteins (termed NWD2) with NACHT seem to play important roles in vegetative compatibility as they have been reported to be involved in the het-s/het-S incompatibility system (Daskalov *et al*., 2012). Chio *et al*. (2012) also reports that STAND protein of the NB-ARC-TRP type controls the *vic2* incompatinility in *Cryphonectria parasitica*.

4.2.3 Non-allelic interactions involving the *het*-r and *het*-v loci

The *het-r*/*het-v* is a non-allelic system found in *P. anserina*, with each locus carrying two alleles, *het-R* and *het-r* and *het-V* and *het-V1*, respectively (Bernet, 1967; Bourges *et al.*,

1998). This system is temperature sensitive as a strain harbouring incompatible *het-R* and *het-V* alleles will grow like the wild type at 32°C, but at 26°C it will undergo massive cell death (Dementhon *et al*., 2003). Also, when two strains harbouring incompatible alleles at the *het-r* and *het-v* loci reproduce sexually, progeny will be produced that contain both incompatible loci. The progeny of such a cross is seen to germinate normally, then upon a temperature shift, the mycelial growth stops and cell death is triggered. A strain that displays this property is termed a self-incompatible strain (SI) (Labarere *et al.*, 1974). The exact functions and identities of the genes encoded at the *het-r* and *het-v* loci remains to be determined.

4.3 Other genes/loci involved in vegetative incompatibility in *N. crassa* and *P. anserina*

4.3.1 *mod*

Mutations in the *mod* (for "modifier") genes suppress some vegetative incompatibility reactions in *P. anserina*. Five *mod* loci (*mod A, B, C, D* and *E*) have been characterized and studied in this fungus. The *mod A* locus encodes a MOD-A protein (687 amino acids) that contains a SH3 binding domain, which is involved in protein-protein interactions (Barreau *et al*., 1998). The *mod D* locus encodes an α-subunit of a heterotrimeric G-protein, while *mod E1* encodes a protein that belongs to the HSP90 family of chaparones (Loubradou *et al*., 1997). The products encoded by these loci are, therefore, highly conserved components of signal transduction pathways (Loubradou and Turcq, 2000).

Mutations at the *mod A* locus suppress the non-allelic vegetative incompatibility reactions (het-c/d, het-c/e and *het-r/v)* in *P. anserine* (Saupe, 2000). Although a mutation in the *mod A* locus suppresses the growth inhibition that is caused by the interactions of non-allelic *het* loci, it cannot inhibit cell death fully. For a mutation in the *mod A* locus to completely inhibit vegetative incompatibility, a second mutation has to occur at the *mod B* locus (Saupe, 2000). Such *mod A*-*mod B* double mutations fully suppress all non-allelic incompatibility reactions in *P. anserina*. Mutations at the *mod C* and *mod E* loci suppress the non-allelic vegetative incompatibility mediated by the *het-r* and *het-v* loci in *P. anserina.* Although the *mod* mutants suppress some vegetative incompatibility phenotypic characteristics, they also show morphological or developmental defects (Loubradou and Turcq, 2000).

4.3.2 *tol*

As mentioned earlier, the *mat* locus of *N. crassa* is implicated in vegetative incompatibility. Hyphal fusion between individuals of opposite mating type during vegetative growth typically results in growth inhibition and cell death. This process is mediated by TOL, which is encoded by the *tol* (for "tolerant) gene (Shiu and Glass, 1999). It seems that sexual development suppresses TOL activity and allows the coexistence of opposite mating type nuclei in the ascogenous hyphae. TOL mutants of opposite mating type are allowed to form viable heterokaryons, while displaying wild type growth during vegetative and sexual reproduction. Also, studies have shown that the expression of the *tol* gene is apparently activated by the formation of a heterodimer between the MAT-A1 and MAT-a1 proteins during vegetative incompatibility. This suggests that functional TOL interacts with these proteins or some other down stream products to activate cell death. The *tol* gene encodes a polyprotein made up of 1011 amino acids that has a coiled coil domain (amino acid positions 177-211) and a leucine-rich domain at amino acids 804-823. Both domains are required for TOL activity and its polypeptide shares three conserved regions with HET-6 protein in *N. crassa* and HET-E in *P. anserina* (Smith *et al*., 2000).

4.3.3 *ahc*

A mutant was isolated from *N.crassa* and shown to harbour a large deletion of 26000 bp that mapped to linkage group V of this fungus. This mutant was named *ahc* and was isolated as it escaped *het-c* mediated vegetative incompatibility and displayed slow, aconidial growth compared to wild type growth (Xiang and Glass, 2002). The *ahc* deletion spans a number of open reading frames (ORF) including the *ham* (hyphal anastomosis) and *vib-1* (*v*egetative *i*ncompatibility *b*locked) loci (Xiang and Glass, 2002; Xiang *et al*., 2002). The *ham 2* ORF encodes a highly conserved plasma membrane protein and when it was cloned into the *ahc* mutant, *het-c* mediated vegetative incompatibility remained suppressed while the *ahc* growth defects were complemented (Xiang *et al.*, 2002). The *vib-1* ORF encodes a transcriptional factor that is homologous to the *NDT80* transcriptional activator, which in *Saccharomyces cerevisiae* is responsible for completion of meiosis and sporulation (Xiang and Glass, 2004) and in *Aspergillus nidulans* it is involved in the response to carbon and nitrogen starvation (Katz *et al*., 2006). Introduction of the *vib-1* ORF into the *ahc* mutant complemented the growth defects as well as to a great extent *het-c* mediated vegetative incompatibility (Xiang

and Glass, 2002; Xiang *et al*., 2002). Later work also showed the involvement of *vib-1* in vegetative incompatibility reactions that are triggered by genetic differences at the *mat* locus (Glass and Dementhon, 2006) and at the *un-24/het-6* loci (Dementhon *et al*., 2003; Xiang and Glass, 2004). These studies demonstrated that VIB-1 is a major regulator of *N. crassa*'s responses to nitrogen and carbon starvation and is indispensable for expression of the genes involved in vegetative incompatibility and cell death (Xiang and Glass, 2002; Dementhon *et al*., 2003; Xiang and Glass, 2004).

4.4 The *het* loci of *F. circinatum* and other *Fusarium* species

Little is known regarding the genetic basis of vegetative incompatibility in *Fusarium* species. Studies have shown that *Fusarium proliferatum* carries a homologue of the *N. crassa het-c* locus (Kerenyi *et al.,* 2006). Interestingly, the *F. proliferatum het-c* is not involved in vegetative compatibility reactions, neither is it involved in the normal vegetative growth of the fungus. However, a mutation in this locus leads to a reduction in the formation of perithecia in *F. proliferatum*, which suggests that it plays a role in the formation and differentiation of the sexual structures of the fungus (Kerenyi *et al.*, 2006).

The only other information regarding the *het* loci of *Fusarium* species relates to the number of loci potentially involved in vegetative incompatibility. Wikler and Gordon (2000) have counted eight *het* loci in *Fusarium circinatum* and they also reported that each of these loci are governed by two to four alleles.

5 Vegetative incompatibility assays in the laboratory

Vegetative incompatibility is essential for maintaining the integrity and individuality in fungi (Glass *et al.*, 2000). It is an excellent mechanism for inhibiting the spread of mycoviruses and debilitated organelles within the fungal populations (Glass *et al.*, 2000). To determine whether fungal isolates are vegetatively incompatible or compatible (i.e. members of the same Vegetative Compatibility Group, VCG), one of three methods are commonly used. These include the barrage test, heterokaryon incompatibility using mutant complementation and partial-diploid analysis.

5.1 The barrage test

A barrage is defined as any form of demarcation between genetically distinct individuals when paired on a specific growth medium and may be apparent as dark lines, clearing zones, or other forms of demarcation (Micali and Smith, 2003). Formation of a barrage at the point of contact between paired fungal individuals indicates that they are vegetatively incompatible. Failure to form a barrage indicates that the paired individuals are vegetatively compatible (i.e., they belong to different VCGs). To see these barrages, compatibility assays are normally performed using growth media (e.g. media with low nitrogen levels) that do not support the production of aerial hyphae and conidia formation (Micali and Smith, 2003). The barrage test is mainly used in fungal species for which nutritional markers (see below) are difficult to obtain (Beadle and Coonredt, 1944).

The barrage test has been used to study vegetative incompatibility in a range of basidiomycetes (Worrall, 1997) and ascomycetes such as *P. anserina* (Bernet, 1965), *N. crassa* (Griffin and Rieck, 1981), *Cryphonectira parasitica* (Anagnostakis, 1977) *Ophiostoma novo-ulmi* (Milgroom and Brasier, 1997) and *Sclerotinia sclerotiorum* (Kohn *et al*., 1990). However, barrage formation is not always an indication of vegetative incompatibility as it could also result from other unknown genetic factors (Ford *et al.*, 1995). In *S. sclerotiorum*, for example, barrages may also form by chemical signaling during cell-cell contact through surface molecules (Ford *et al.*, 1995).

5.2 Heterokaryon incompatibility tests using complementation

For some fungi, strains carrying various specific, but complementary nutritional or auxotrophic markers are easy to obtain (Perkins, 1988; Micali and Smith, 2003). Separately, these auxotrophic mutant strains will not display wild type properties on a medium lacking the marker nutrient. However, when vegetatively compatible strains carrying complementary auxotrophic markers are inoculated on this medium, heterokaryon formation will allow the nutritional deficiency in each strain to be complemented and protrophic wild type-like growth of the heterokaryotic hyphae. If the strains were vegetatively incompatible, their growth remains aberrant, as a heterokaryon did not form to facilitate complementation of the markers (Micali and Smith, 2003). This approach is commonly used for fungi such as *N. crassa* and *Fusarium* species.

For *Fusarium* species, auxotrophic mutants are generated by growth on chlorate ($KCLO_3$) containing medium (Correll *et al*., 1987). Chlorate is a toxic analog of $NaNO_3$ and the fungi attempt to utilize it by using their nitrate-reducing pathway. This action results in the spontaneous emergence of mutants carrying lesions in either structural or regulatory loci of this pathway (Klittich and Leslie, 1988). In the nitrate-reducing pathway, nitrate reductase reduces NO_3 to NO_2 that is further reduced by nitrite reductase into NH_4 that is used for various functions. Nitrate reductase is also responsible for reducing ClO_3 to ClO_2.

Three types of mutants that are unable to utilize nitrate as sole nitrogen source (*nit*, NO_3-non-utilizing) are typically used for vegetative incapability tests with *Fusarium*. These are *nit1* that encodes a defective nitrate reductase gene, *nit3* that encodes a specific regulator of the nitrate and nitrite reductases, and *nitM* that encodes a molybdenum-containing co-factor that is needed for active nitrate reductase (Leslie and Summerell, 2006). This method is widely used because it can be employed to study the genetic basis of vegetative compatibility and can also be used to characterize, identify, study and map *het* loci (Leslie and Summerell, 2006).

5.3 Partial-diploid analysis method

Heterokaryon incompatibility tests using partial-diploid analysis is almost exclusively used for fungal strains that have duplication-generating chromosomal rearrangements, on which one or more *het* loci are carried (Mylyk, 1975; Perkins, 1975; Micali and Smith, 2003). Should such an appropriate strain not be available, recombinant DNA technology can be used to generate it. For example, in *N. crassa*, a cloned chromosome segment containing a known *het* locus was inserted into a genome of an isolate (Xiang and Glass, 2004). A strain with an unknown *het* gene is crossed with a strain that has a duplication generating chromosome rearrangement (Perkins, 1975). This results in the production of partially diploid homokaryotic progeny that are heterozygous for one or more specific *het* loci within the duplicated segment. As a result, these progeny are self-incompatible and characterized by slow growth and aberrant mycelium and colony properties. Partial diploids that are homozygous at a given *het* locus have a wild type appearance (Mylyk, 1975). This method was used to study the *het-e* and *het-c* loci of *N. crassa* (Perkins, 1975).

5.4 Population biology insights gained from vegetative compatibility studies

Vegetative compatibility has been used in numerous studies to understand the dynamics of fungal populations (Brasier, 1991). In the literature, the two best known examples of where vegetative compatibility was used to study fungal biology are *Ophiostoma novo-ulmi* and *Cryphonectria parasitica*. Both these fungi are destructive tree pathogens with *Ophiostoma novo-ulmi* being the casual agent of the Dutch elm disease (Brasier, 1991) that has killed elm trees across Europe, North America and central Asia (Brasier, 2000). *Cryphonectria parasitica* is the fungus that causes blight on chestnut trees and has devastated the European and North American chestnut (*Castanea dentata*) trees (Merkel, 1905). In introduced locations, these fungi have been shown to have reduced disease-causing capabilities, due to the presence of mycoviruses (Grente, 1965; Kulman, 1983; Brasier, 1988 and Griffin, 2000). As these viruses spread mainly by cell-cell contact, the diversity and number of vegetative compatibility groups within populations of these fungi will have a significant impact on viral spread and fungal survival. For example, in areas where VCG numbers were low, the mycoviruses spread faster than in areas where the number of VCGs were high (Brasier, 1986; Cortesi *et al*., 1996; Milgroom *et al.*, 1996).

For species in the genus *Fusarium*, VCG assays are routinely used to gain insights into the reproduction and spread of species and populations. For example, for *F circinatum*, a casual agent of pine pitch canker (Wingfield *et al*., 2008), VCG studies are commonly used to study the genetic diversity in specific populations by determining the number of *het* genotypes in that population (Viljoen *et al*., 1997; Cortesi and Milgroom, 1998; Wikler and Gordon, 2000; Britz *et al.*, 2005). Accordingly, it has been shown that Mexico or central America represents the likely center of origin for the pitch canker pathogen (Wikler and Gordon, 2000; Viljoen *et al*., 1997; Britz *et al.*, 2005), from where it was introduced into other regions (Wingfield *et al*., 2008). The results of VCG studies have also been used to infer the possible mode of reproduction of the fungus. Some populations are characterized by few VCGs and lack of the emergence of new VCGs, which suggest that vegetative reproduction plays a much more significant role in the biology of the fungus in that population than sexual reproduction (Wikler and Gordon, 2000; Britz *et al.*, 2005).

6 Sexual compatibility in Ascomycetes

During sexual interactions in ascomycetes, “male” strains fertilize “female” strains, where the latter produce the trichogynes, which are specialized hyphal cells at the tips of the ascogonia that eventually form the protoperithecia (Casselton, 2002). Fertilization commences when the male reproductive propagules (e.g. conidia) fuses with a trichogyne of opposite mating type. Hyphal fusion is then followed by the migration of the male nucleus (plasmo- or cytogamy) down the trichogyne into the female ascogonium, thus resulting in the coexistence of nuclei of opposite mating type in the common cytoplasm. The products of the *mat* locus are responsible for ensuring that nuclei of opposite mating types enter the ascogenous hyphae (Saupe, 2000).

The heterokaryotic cytoplasm of the zygote is mainly of maternal origin because the male gamete contributes very little cytoplasm to the heterokaryon. The nuclei then migrate into hook shaped structures called “crozier-hooks” within which karyogamy or nuclear fusion occurs to establish a diploid. Meiosis and one or more rounds of mitotic division produce mature ascospores that are eventually released from the fruiting structure (Coppin *et al.*, 1997; Kronstad and Staben, 1997).

The attraction between male and female cells and their subsequent fusion is regulated by pheromone signaling (Leberer *et al.*, 1997). These two classes of pheromones are synthesized and secreted in different pathways (Cadwell *et al*., 1995; Kronstad and Staben, 1997). The secretion of these pheromones causes the hyphae to change direction and grow towards each other (Bobrowicz *et al.*, 2002; Xiang *et al.*, 2002). Each mating type releases different hydrophobic peptide pheromones belonging to different classes in all ascomycetes. Hyphae from male isolates produce pheromones and females respond by producing trichogynes (Kronstad and Staben, 1997; Dalstra *et al*., 2003). According to Glass *et al*. (2004), it is not known whether signaling mechanisms and the hyphal fusion machinery involved in vegetative incompatibility reactions, are also used in sexual reproduction.

Based on their sexual reproductive strategies, fungal species are categorized into the three groups, which are homothallic, heterothallic and pseudohomothallic (Leslie and Summerell, 2006). True homothallic species (e.g. *Gibberella zeae* and *Aspergillus nidulans*) are able to complete their life cycles from cultures that originate from a spore (asco- or mitospore) or a

mycelial fragment that contains a single type of nucleus. They have both mating types in a single nucleus, which enables them to undergo self-fertilization although they can also outcross (Leslie and Summerell, 2006). Heterothallics, on the other hand require strains of opposite mating type that originated from distinct spores to complete their life cycle. This is due to the fact that each nucleus carries only one mating type specificity. Fungi such as *Cochliobolus heterostrophus* and *Fusarium* species in the *G. fujikuroi* complex are well known examples of heterothallic fungi (Leslie and Summerell, 2006). Like true homothallics, pseudohomothallics can also complete a life cycle from cultures that have originated from a spore, provided that the spore has two types of nuclei of opposite mating type. Vegetatively, these fungi usually grow as heterokaryons and maintain two genetically distinct loci in each cell. Pseudohomothallics pursue a heterothallic-like life cycle in which recombination between the two nuclei is tightly controlled. For example, in *Neurospora tetrasperma*, crossing over at the *mat* locus is suppressed in the bivalent strain or heterokaryon during nuclear packaging so that *mat A* and *mat a* (see below) segregates at the first meiotic division (Gallegos *et al*., 2000), while the rest of the chromosomes undergo recombination (Jacobson, 2005).

7 The molecular basis of sexual compatibility in Ascomycetes

In fungi, sexual reproduction is regulated by genes encoded at the *mat* locus. All ascomycetes have a single *mat* locus with two alternate alleles or idiomorphs (Yun *et al*., 2000). These idiomorphs each encode different proteins or sets of proteins most of which that represent transcriptional factors. In the sexual reproductive cycle, these transcription factors regulate haploid-specific genes that are required for cell-cell recognition and fusion (including cell-type-specific pheromones and receptors), as well as diploid-specific genes required for meiosis and sporulation (Shiu and Glass, 2000).

The *mat* locus is found in both homothallic and heterothallic species. The allelic mating types are termed idiomorphs due to differences in their sequences. In homothallic species both *mat 1* and *mat 2* are situated on the same chromosome, closely linked or fused (Yun *et al*., 1999). The *mat 1* and *mat 2* idiomorph organizations are different in each homothallic species. For example, studies on *Cochliobolus* species indicated unique *mat 1* and *mat 2* organizations

(Yun *et al*., 1999). However, in heterothallic species the *mat 1* and *mat 2* organizations are the same. For example, each *mat* idiomorph carries one gene that encodes for a *mat* specific DNA binding domain (HMG protein or α-domain protein) (Coppin *et al.*, 1997). Below I will briefly review the well-characterized *mat* loci of *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *N. crassa*, *P. anserina* and *C. heterstrophus*, and also point out what we currently know for *Fusarium* species.

7.1 The *mat* loci of *S. cerevisiae* and *S. pombe*

Among the ascomycetes, the mating type idiomorphs of the yeasts *S. cerevisiae* (Astell *et al*., 1981) and *S. pombe* (Kelly *et al.*, 1988) were the first to be characterized at the molecular level. In these yeasts, reproduction occurs by conjugation of cells of opposite mating type, which is then followed by meiosis and sporulation and the whole process is regulated by the products of their mating type loci (Nelson, 1996; Poggeler, 2001). Both these yeasts are heterothallic and both have the ability to become homothallic by switching their mating types. For example, in *S. cerevisiae* an α cell can become an *a* cell or *vice versa*, by moving a silent copy of the mating type locus into the active mating type locus, thereby replacing the resident idiomorph and changing the mating type of the cell (Nelson, 1996).

The mating type locus of *S. cerevisiae* is governed by the two idiomorphs *mat α* and *mat a* that are 642 bp and 747 bp in length, respectively (Astell *et al*., 1981). The *mat α* idiomorph has two ORFs that encode the proteins MATα1 and MATα2 and the *mat a* idomorph also has two ORFs encoding the MAT*a*1 and MAT*a*2 proteins. MATα1 is an α1 domain transcriptional factor that positively regulates the transcription of α-specific genes (genes required for cell-cell recognition and fusion). MATα2 is a homodomain protein that negatively regulates the transcription of *a*-specific genes, which are genes that encode the MFA1 and MFA2 pheromone precursors and STE2 α a pheromone receptor (Nelson, 1996; Poggeler, 2001).

The *mat* locus of *S. pombe* has the idiomorphs *mat1-M* and *mat1-P* that are 1104 bp and 1128 bp in length, respectively (Kelly *et al*., 1988). The *mat1-M* idiomorph encodes two proteins, which are mat1-Mm and mat1-Mc. The *mat1-P* idiomorph also encodes two proteins, which are mat1-Pc and mat1-Pm (Coppin *et al.*, 1997). mat1-Mc is an HMG protein (Grosschedl *et*

al., 1994) while mat1-Mm is an unknown class of protein (Poggeler, 2001). The mat1-Pc and mat1-Pm proteins are similar to the MATα1 and MATα2 proteins, respectively, of *S. cerevisiae* (Poggeler, 2001). mat1-Mc and mat1-Pc are required for conjugation while mat1-Pm and mat1-Mm are required for meiosis (Bolker and Kahmann, 1993).

7.2 The *mat* locus of *N. crassa*

The first filamentous ascomycete in which the *mat* locus was described is *N. crassa* (Glass *et al*., 1988). This fungus has two mating type idiomorphs, *mat* a-1 and *mat* A. The A idomorph is 5301bp in size and contains three ORFs known as *mat* A-1, *mat* A-2 and *mat* A-3. The *mat* A-1 ORF encodes a protein (MAT A-1), which has a α-box domain similar to the α1 protein of *S. cerevicieae, and* that is thought to act as a transcriptional regulator (Glass *et al.*, 1990). In addition to mediating mating type associated vegetative incompatibility reaction (see above), MAT A-1 determines mating identity and also plays a role in post-fertilization functions (Glass *et al*., 1990). The products of *mat* A-2 and A-3 are required for post-fertilization functions, and not for mating identity and mediation of heterokaryon incompatibility reaction (Glass and Staben, 1990). The MAT A-2 and A-3 proteins are also transcriptional factors, as they contain an acidic amphipathic α-helix domain and an HMG DNA-binding domain, respectively (Turgeon, 1998). The *mat* a-1 idiomorph is 3235 bp in size and has one ORF (Leslie and Summerell, 2006) that encodes MAT a-1, which is also a HMG DNA-binding protein that regulates transcription. This regulator determines mating type identity and mediates heterokaryon incompatibility reactions (see above), and is also potentially involved in ascosporogenesis (Staben and Yanofsky, 1990). To distinguish between the HMG proteins encoded at the *mat* A and *mat* a-1 idiomorphs, they were designated HMG-1 and HMG-2, respectively (Shiu and Glass, 2000).

7.3 The *mat* locus of *P. anserina*

In *P. anserina*, the *mat*+ idiomorph is 3800 bp in size and comprises of the *FPR1* gene that is homologous to the HMG-2-binding protein encoded by the *mat* a-1 gene of *N. crassa* (Shiu *et al.*, 2000). The *mat*- idiomorph is 4700 bp in size and it is comprised of the genes *FMR1*, *SMR1* and *SMR2*. These genes are homologous to *mat* A-1, *mat* A-2 and *mat* A-3 of *N. crassa* (Shiu and Glass, 2000). The N-terminal portion of *FPR1* and *FMR1* are required for mating identity and fertilization functions (Debuchy and Coppin, 1992; Debuchy *et al.*, 1993). It has

been speculated that both these genes are essential for fertilization because their products control genes that encode pheromone precursors and pheromone receptors (Debuchy, 1999). Like *mat* A-2 and *mat* A-3 of *N. crassa SMR1* and *SMR2* are responsible for post-fertilization functions (Debuchy and Coppin, 1992; Debuchy *et al.*, 1993).

7.4 The *mat* locus of *C. heterstrophus*

The *mat* idiomorphs of *C. heterostrophus* are considerably smaller than those of *N.crassa* and *P. anserina* (Coppin *et al.*, 1997). The *mat-1* idiomorph is 1297 bp and the *mat-2* idiomorph is 1171 bp in size (Coppin *et al.*, 1997). The *mat* idiomorphs of this fungus each encode a single protein that both represent transcriptional factors (Turgeon *et al*., 1993). The *mat-1* gene encodes a protein with significant homology to the α-box containing transcription factors MAT A-1 of *N. crassa* and FMR1 of *P. anserina* (Turgeon *et al*., 1993). The *mat-2* gene encodes a protein that is homolgous to the HMG-2 DNA-binding transcription factors MAT a-1 and FPR1 of *N. crassa* and *P. anserina*, respectively (Turgeon *et al*., 1993).

7.5 The *mat* locus of *F. circinatum* and other *Fusarium* species

The *mat* locus of *Fusarium* species is governed by idiomorphs *mat 1* and *mat 2* (Yun *et al.*, 2000). In all heterothalic *Fusarium* species examined to date, *mat* 1 encodes three ORFs, while *mat* 2 encodes a single ORF. The *mat 1* ORFs are MAT-1-1, MAT-1-2 and MAT-1-3, which share significant homology to the ORFs encoded at the *mat* A and *mat* – loci of *N. crassa* and *P. anserina*, respectively (Kerenyi *et al*., 2004). MAT-1-1 is the α-box domain transcription factor, MAT-1-2 contains the acidic amphipathic α-helix and MAT-1-3 has a HMG-1 DNA-binding domain. The single ORF on the *mat 2* idiomorph (MAT-2-1) encodes the transcriptional factor with the HMG-2 DNA-binding domain (Kerenyi *et al*., 2004).

An additional *mat* gene (*mat 1-2-3*) has been discovered in homothallic *Fusarium* species (Martin *et al*., 2011). However, this *mat* gene is different from the other *mat* genes in *Fusarium*, it does not have a DNA binding motif and therefore it has no effect on sexual reproduction (Kim *et al*., 2012).

8 Sexual compatibility tests in the laboratory

Induction of a teleomorph represents an important component in studies focused on understanding the biology of a fungus. In many cases, it has traditionally also been crucial for studying the taxonomy of a fungus (Summerell *et al*., 2003). Different techniques are used to test for sexual compatibility in fungi in the laboratory. These techniques can also be used to test for male and female sterility. The fact that the *mat* locus encodes proteins that are conserved among ascomycetes has also facilitated the development of DNA-based procedures for differentiating mating type.

8.1 PCR-based mating type assays

In order to determine the mating type of a heterothallic individual, tester strains of a known mating type are crossed with isolates of unknown mating type. If the crossed isolates form perithicia and viable ascospores ooze from these structures, then the mating type of the other isolate would be known (Hsieh *et al*., 1977; Klittich and Leslie, 1992; Brasier and Kirk, 2004). For the most part, this approach is time consuming because such studies invariably take many weeks or even months to complete (Klittich *et al*., 1988; Brasier and Kirk, 2004). Another disadvantage is the unavailability of tester strains for most species, as female sterility or even complete sterility makes it impossible to determine the mating type (Leslie and Summerell, 2006). Consequently, the structure of the *mat* idiomorphs and the DNA sequence information for these regions has been exploited widely for the development of more robust and reliable PCR-based methods for diagnosing the mating type of a heterothallic fungal strain. This method has been used to determine the mating type of ascomycetes such as *C. heterostrophus, Magnaporthe grisea* and *Clavicipitaceae* (Gafur *et al*., 1997; Yokoyama *et al*., 2004; Bao-Hua *et al*., 2005).

The mating type PCR has been used extensively with success to determine the mating types in different *G. fujikuroi* species complex. Kerenyi *et al*. (1999) developed primers that amplify the *mat 2* idiomorph and also standardized the mating type terminology for the *G. fujikuroi* populations. Covert *et al*. (1999) identified and sequenced the *mat 2* allele for *G. circinata* and also adapted to the standardised mating type terminology. The PCR methods by Covert *et al*. (1999) and Kerenyi *et al*. (1999) made it possible to determine the mating types of

Fusarium species without having to do crosses. Lack of amplification and the inability to amplify the *mat 1* allele were major disadvantages of these methods. These problems were then eliminated by the PCR method developed by Steenkamp *et al*. (2000). This method made it possible to amplify both *mat 1* and *mat 2* alleles successfully but only the mating types of *Fusarium* species with known sexual stage were determined. Kerenyi *et al.* (2004) took this PCR method to another level where the *mat 1* and *mat 2* alleles of *Fusarium* species with no proven sexual stage were amplified. Also, this method made it possible to determine the mating types of other related ascomycetes (*Calonectria* and *Nectria*) that have sexual stages.

8.2 Sexual crosses on media and natural substrates

In the laboratory, different media or substrates may be used for inducing the sexual reproductive cycle. These media include natural substrates such as carnation leaves, rice straw, mulberry twigs (Leslie and Summerell, 2006), or other plant material placed on water agar (Hsieh *et al.*, 1977; Dietert *et al.*, 1983; Desjardins and Beremand, 1987; Leslie and Summerell, 2006). Alternatively, media made from plant extracts such as carrot agar (Klittich *et al.*, 1988) are widely used for producing the sexual structures of *Fusarium* species (Klittich *et al.*, 1988) and species in the oomycetous genus *Phytophthora* (Brasier and Kirk, 2004). In some *Phytophthora* species such as *P. cinnamomi*, crosses are done on medium containing V-8-juice, fresh potato dextrose, cornmeal (Zentmyer *et al*., 1979) and synthetic media (Ribeiro *et al*., 1975).

For heterothallic ascomycetes, two types of strains are usually differentiated. These are female fertile or hermaphrodites and male strains (Coppin *et al*., 1997). Strains that form fruiting structures with viable ascospores are females and the ones that produce gametes that fertilize the females are male isolates. In fungi such as *Fusarium*, all isolates are considered to be able to act as males, while only certain isolates are able to act as females, hence the latter are also referred to as hermaphrodites (Nauta and Hoekstra, 1992; Leslie and Klein, 1996). To produce sexual fruiting structures in *Fusarium* species, for example, the female parents are grown on carrot agar, after which they are fertilized with spores from the male parent (Klittich *et al.*, 1988). Where the interactions are fertile, perithecia develop after about two weeks of incubation, after which they start oozing ascospores. However, isolates of opposite mating types may not always form perithecia as not all isolates are able to act as females or hermaphrodite (Leslie and Klein, 1996). In homothallic species, perithecia form

spontaneously after incubation (Leslie and Summerell, 2006).

8.3 Population biology insights gained from sexual compatibility studies

Sexual compatibility assays provide an efficient tool to study the structure and reproductive mode of a fungal population. For example, sexual reproduction is one of the modes by which mycoviruses are spread within populations of *Ophiostoma novo-ulm*i (Brasier, 1986). Sexual compatibility tests done on the *Ophiostoma novo-ulm*i population in North America indicated that all the isolates are of a single mating type. Therefore, this explained the reduction in the spread of the mycovirus because the population was multiplying clonally, therefore limiting the spread of the mycovirus (Brasier, 1986).

Sexual reproduction serves as a mode of evolution in fungal populations (Brasier, 1988). For example, *Ophiostoma novo-ulm*i has caused huge damage to elm trees across Europe, North America and central Asia (Brasier, 2000). This fungus has been spreading clonally as a single mating type until the accurance of another mating type within the same species (Brasier, 1988). Various studies have suggested that appearance of the other mating type was due to gene transfer between different species during sexual reproduction (Kile and Brasier, 1991; Brasier *et al*., 1998; Paoletti *et al*., 2006).

Sexual compatibility assays can also be used to determine the frequency of sexual and asexual reproduction in the field. A higher occurrence of female sterile isolates indicates that sexual reproduction does not occur frequently with a population because every time sexual reproduction occurs male isolates are selected against. Thus, if sexual reproduction was occurring frequently this would lead to the reduction of female sterile isolates because of negative selection (Leslie and Klein, 1996).

9 Conclusions

Vegetative and sexual compatibility are mechanisms by which nature maintains the structure and diversity of fungal populations. Sexual compatibility combines *het* genes, therefore, resulting in the formation of more diverse VCG groups. However, the absence of sexual reproduction results in clonal isolates of a fungal population.

As evolution occurs, fungal populations prefer to maintain their individuality and integrity and that is made possible by vegetative incompatibility reactions that occur in nature. This phenomenon has a vital role in inhibiting the spread of mycoviruses and debilitated organelles in the fungal population. For future research, this technique will be used to study the genetic diversity of fungal population and to trace the spread of *Fusarium circinatum* in South Africa.

10 References

1. Anagnostakis, S.L. 1977. Vegetative incompatibility in *Endothia parasitica*. Experimental Mycology 1: 306-316.
2. Astell, C.R., Ahlstrom-Jonasson, L., Smith, M., Tatchell, K., Nasmyth, K.A. and Hall, B.D. 1981. The sequence of the DNAs coding for the mating type loci of *Saccharomyces cerevisiae*. Cell 27: 15-23.
3. Balguerie, A., Dos Reis, S., Ritter, C., Chaignepain, S., Coulary-Salin, B., Forge, V., Bathany, K., Lascu, L., Schmitter, J. and Saupe, S.J. 2003. Domain organization and structure-function relationship of the HET-s prion protein of *Podospora anserina*. The EMBO Journal 22: 2071–2081.
4. Balguerie, A., Dos Reis, S., Coulary-Salin, B., Chaignepain, S., Sabou-rin, M., Schmitter, J.M. and Saupe, S.J. 2004. The sequence appended to the amyloid core region of the HET-s prion protein determine higher order aggregate organization in vivo. Journal of Cell Science 117: 2599-2610.
5. Bao-Hua, W., Guo-Dong, L., Hai-Ming, L., Yan, L. and Zong-Hua, W. 2005. Assessment of *Magnaporthe grisea* mating type by PCR. Chinese Journal of Agricultural Biotechnology 2: 1-5.
6. Barreau, C., Iskandar, M., Loubradou, G., Levallois, V. and Begueret, J. 1998. The mod-A suppressor of nonallelic heterokaryon incompatibility in *Podospora anserina* encodes a proline rich polipeptide involved in female organ formation. Genetics 149: 915-926.
7. Beadle, G.W. and Coonredt, V.L. 1944. Heterokaryosis in *Neurospora crassa*. Genetics 29: 291-308.
8. Begueret, J., Turcq, B. and Clave, C. 1994. Vegetative incompatibility in filamentous fungi*: het* genes begin to talk. Trends in Genetics Genetic 10: 441-446.
9. Bernet, J. 1965. Mode d'action des gènes de barrage et relation entre l'incompatibilite cellulaire et l'incompatibilite sexuelle chez le *Podospora anserina*. Annales des Science

Naturelles; Botinique 6: 611–768.

10. Bernet, J. 1967. Les systes d'incompatibilite chez le Podospora anserine. C. R. Academie des Science des Paris 265: 1330-1333.
11. Bobrowicz, P., Pawlak, R., Correa, A., Bell-Pedersen, D. and Ebbole, D.J. 2002. The *Neurospora crassa* pheromone precursor genes are regulated by the mating type locus and the circadian clock. Molecular Biology 45(3): 795-804.
12. Bolker, M. and Kahmann, R. 1993. Sexual pheromones and mating responses in fungi. The Plant Cell 5: 1461-1469.
13. Boucherie, H., Dupot, C.H. and Bernet, J. 1981. Polypeptide synthesis during protoplasmic incompatibility in the fungus *Podospora anserina*. Biochemimia et Biophysica Acta 653: 18-26.
14. Bourges, N., Groppi, A., Barreau, C., Clave, C. and Bequeret, J. 1998. Regulation of gene expression during the vegetative incompatibility reaction in *Podospora anserina*: characterization of the three induced genes. Genetics Society of America 150: 633- 641.
15. Brasier, C.M. 1986. The d-factor in *Ceratocystis ulmi*- its biological characteristics and implications for Dutch elm disease. Fungal Virology (ed. Buck KW). CRC Press, Boca Ralton, Florida. Pp 177-208.
16. Brasier, C.M. 1988. Rapid changes in genetic structure of epidemic populations of *Ophiostoma ulmi*. Nature 332: 538-541.
17. Brasier, C.M. 1991. *Ophiostoma novo-ulmi* sp. nov., causative agent of the current Dutch elm disease pandemics. Mycopathologia 15: 151-161.
18. Brasier, C.M., Kirk, S.A., Pipe, N.D. and Buck, K.W. 1998. Rare interspecific hybrids in natural populations of the Dutch elm disease pathogens *Ophiostoma ulmi* and *O. novo-ulmi*. Mycological Research 102: 45- 57.
19. Brasier, C.M. 2000. Interconectional spread and continuing evolution of the Dutch disease evolution. The elms: Breeding, conservation and disease management (ed. Dunn CP) Kluwer Academic Publishers, Boston. Pp 61-72.
20. Brasier, C.M. and Kirk, S. 2004. Production of gametangia by *Phytophthora ramorum* in vitro. Mycological Research 108 (7): 823-827.
21. Britz, H., Coutinho, T.A., Marasas, W.F.O and Wingfield, M.J. 2005. Diversity and differentiation of *Gibberella circinata* in South Africa. Plant Pathology 54: 46-52.
22. Cadwell, G.A., Naider, F. and Becker, J.M. 1995. Fungal lipopeptide mating pheromones: a model system for the study of protein prenylation. Microbiological

Reviews 59: 406-422.

23. Casselton, L.A. 2002. Mate recognition in fungi. Heredity 88:142-147.
24. Chevanne, D., Bastiaans, E., Debets, A., Saupe, S.J., Clavé, C. and Paoletti, M. 2009. Identification of the *het-r* vegetative incompatibility gene of *Podospora anserina* as a member of the fast evolving *HNWD* gene family. Current Genetics 55: 93-102.
25. Chio, G.H., Dawe, A.L., Churbanov, A., Smith, M.L., Milgroom, M.G. and Nuss, D.L. 2012. Molecular characterization of vegetative incompatibility genes that restricts hypovirus transmission in the chestnut blight fungus *Cryptonectria parasitica*. Genetics 190: 113-127.
26. Coppin, E., Debuchy, R., Arnaise, S. and Picard, M. 1997. Mating type and sexual development in filamentous ascomycetes. Microbiology and Molecular Biology Reviews 61: 411-428.
27. Correll, J.C., Klittich, C.J.R. and Leslie, J.F. 1987. Nitrate nonutilizing mutants of *Fusarium oxysporum* and their use of vegetative compatibility tests. Phytopathology 77:1640-1646.
28. Cortesi, P., Milgroom, M.G. and Bisiach, M. 1996. Distribution and diversity of vegetative compatibility types in subpopulations of *Cryphonectria parasitica* in Italy. Mycological Research 100: 1087-1093.
29. Cortesi, P. and Milgroom, M.G. 1998. Genetics of vegetative incompatibility in *Crphonectria parasitica*. Applied and Environmental Microbiology 64 (8): 2988-2994.
30. Covert, S.F., Briley, A., Wallace, M.M. and Mckinney, V.T. 1999. Partial MAT-2 gene structure and the influence of temperature on mating success in *Gibberella circinata*. Fungal Genetics and Biology 28: 43-54.
31. Dalstra, H.J.P., Swart, K., Debets, A.J.M., Saupe, S.J. and Hoekstra, R.F. 2003. Sexual transmission of the [Het-s] prion leads to meiotic drive in *Podospora anserina*. Genetics 100 (11): 6616-6621.
32. Dalstra, H.J.P., Zee, R., Swart, K., Hoekstra, R.F., Saupe, S.J. and Debets, A.J.M. 2005. Non-mendelian inheritance of the HET-s prion of HET-s prion domains determines the het-S spore killing system in *Podospora anserina*. Fungal Genetics and Biology 42: 836-847.
33. Daskalov, A., Paoletti, M., Ness, F. and Saupe, S.J. 2012. Genomic clustering and homology between HET-S and the NWD2 STAND protein in various fungal genomes. Plos One 7 (4): 1-16.

34. Debets, A.J.M., Dalstra, H.J.P., Slakhorst, M., Koopmanschap, B., Hoekstra, R.F. and Saupe, S.J. 2012. High natural prevalence of a fungal prion. Proceedings of the National Academy of Sciences of the United States of America 109: 1-6.
35. Debuchy, R. and Coppin, E. 1992. The mating of *Podospora anserina* functional analysis and sequence of the fertilization domains. Molecular and General Genetics 233: 113-121.
36. Debuchy, R., Arnaise, S. and Lecellier, G. 1993. The mat- allele of *Podospora anserina* contains three regulatory genes required for the development of fertilized female organs. Molecular and General Genetics 241: 667-673.
37. Debuchy, R. 1999. Internuclear recognition: A possible connection between euacomycetes and homobasidiomycetes. Fungal Genetics and Biology 27: 218-223.
38. Deleu, C., Clave, C. and Begueret, J. 1993. A single amino acid difference is sufficient to elicit vegetative incompatibility in the fungus *Podospora anserina*. Genetics 135: 45-52.
39. Dementhon, K., Paoletti, M., Pinan-Lucarre, B., Loubradou-Bourges, N., Sabourin, M., Saupe, S.J. and Clave, C. 2003. Rapamycin mimics the incompatibility reaction in the fungus *Podospora anserina*. Eukaryotic Cell 2 (2): 238-246.
40. Desjardins, A.E. and Beremand, M. 1987. A genetic system for trichothecene toxin production in *Gibberella pulicaris* (*Fusarium sambucinum*). Phytopathology 77: 678-683.
41. Dietert, M.F., VanEtten, H.D. and Matthews, P.S. 1983. *Nectria haematococca* mating population VI: Cultural parameters affecting growth, conidiation and perithicial formation. Canadian Journal of Botany 61: 1178-1184.
42. Ford, E.J., Miller, R.V., Gray, H. and Sherwood, J.E. 1995. Heterokaryon formation and vegetative compatibility in *Sclerotonia sclerotiorum.* Mycological Research 99: 241-247.
43. Gafur, A., Tanaka, C., Ouchi, S. and Tsuda, M. 1997. A PCR based method for mating determination in Cochliobolus heterostrophus. Mycoscience 38: 455-458.
44. Gallegos, A., Jacobson, D.J., Raju, N.B., Skupski, M.P. and Natvig, D.O. 2000. Suppressed recombination and a pairing anomaly on the mating type chromosome of *Neurospora tetrasperma.* Genetics 154: 623-633.
45. Glass, N.L. and Staben, C. 1990. Genetic control of mating in *Neurospora crassa*. Seminars in Cell and Developmental Biology 1: 177-184.

46. Glass, N.L., Grotelueschen, J. and Mettzenberg, R.L. 1990. *Neurospora crassa* A mating type region. Proceedings of the National Academy of Science USA 87: 4912-4916.
47. Glass, N.L. and Kuldau, G.A. 1992. Mating type and vegetative incompatibility in filamentous ascomycetes. Annual Reviews in Phytopathology 30: 201-224.
48. Glass, N.L., Jacobson, D.J. and Shiu, K.T. 2000. The genetics of hyphal fusion and vegetative incompatibility in filamentous ascomycetes fungi. Annual Reviews in Genetics 34: 165-186.
49. Glass, N.L. and Kaneko, I. 2003. Fatal attraction: Nonself recognition and heterokaryon incompatibility in filamentous fungi. Eukaryotic Cell 2 (1): 1-8.
50. Glass, N.L., Rasmussen, C., Roca, M.G. and Read, N.D. 2004. Hyphal homing and mycelial interconnectedness. Trends in Microbiology 12 (3): 135-141.
51. Glass, N. and Dementhon, K. 2006. Non-self recognition and programmed cell death in filamentous fungi. Current Opinion in Microbiology 9: 553-558.
52. Greenwald, J., Buhtz, C., Ritter, C., Kwiatkowski, W., Choe, S., Maddelein, M., Ness, F., Cescau, S., Soragni, A., Leitz, D., Saupe, S.J. and Riek, R. 2010. Molecular Cell 38 (6): 889-899.
53. Grente, J. 1965. Les formes hypovirulentes *d'Endothia parasitica* et les espoirs de lutte contre le chancre du chantaingnier. Comptes Rendus Academie Agriculture de France 51: 1033-1037.
54. Griffin, A.J.F. and Rieck, A. 1981. Perithecial distribution patterns in standard and variant strains of *Neurospora crassa*. Canadian Journal of Botany 59: 2610–2617.
55. Griffin, G.J. 2000. Blight control and restoration of the American chestnut. Journal of Forestry 98: 22-27.
56. Grosschedl, R., Giese, K. and Pagel, J. 1994. HMG domain proteins: architectural elements in the assembly of nucleoprotein structures. Trends in Genetics 10: 94-100.
57. Hietala, A.M., Korhonen, K. and Sen, R. 2003. An unknown mechanism promotes somatic incompatibility in *Cerabosidium bicorne*. Mycologia 95 (2): 239-250.
58. Hsieh, W.H., Smith, S.N. and Snyder, W.C. 1977. Mating groups in *Fusarium moniliforme*. Phytopathology 67: 1041-1043.
59. Jacobson, D.J. 2005. Blocked recombination along the mating type chromosomes of *Neurospora tetrasperma* involves both structural heterozygosity and autosomal genes. Genetics 171: 839-843.

60. Kaneko, I., Dementhon, K., Xiang, Q. and Glass, N.L. 2005. Nonallelic interactions between *het-c* and a polymorphic locus, *pin-c*, are essential for nonself recognition and programmed cell death in *Neurospora crassa*. Genetic Society of America 172: 1545-1555.
61. Katz, M.E., Gray, K.A. and Cheetham**,** B.F. 2006. The *Aspergillus nidulans xprG* (*phoG*) gene encodes a putative transcriptional activator involved in the response to nutrient limitation. Fungal Genetic Biology 43: 190-199
62. Kelly, M., Burke, J., Smith, M., Klar, A. and Beach, D. 1988. Four mating type genes control sexual differentiation in the fission yeast. The European Molecular Biology Organization Journal 7 (5): 1537-1547.
63. Kerenyi, Z., Zeller, K.A., Hornok, L. and Leslie, J.F. 1999. Molecular standardization of mating type terminology in the *Gibberella fujikuroi* species complex. Applied Applied and Environmental Microbiology 65: 4071-4076.
64. Kerenyi, Z., Moretti, A., Waalwijk, C., Olah, B. and Hornok, L. 2004. Mating type sequences in asexually reproducing *Fusarium* species. Applied and Environmental Microbiology 7(8): 4419-4423.
65. Kerenyi, Z., Olah, B., Jeney, A., Hornok, L. and Leslie, J. 2006. The homologue of *Neurospora crassa* lacks vegetative compatibility function in *Fusarium proliferatum*. Applied and Environmental Microbiology 72 (10): 6527-6532.
66. Kile, G.A. and Brasier, C.M. 1990. Inheritance and inter-relationship of fitness characters in a progeny of an aggressive x non-aggressive cross of *Ophiostoma ulmi*. Mycological Research 94: 514-522.
67. Kim, H.K., Cho, E.J., Lee, S., Lee, Y.S. and Yun, S.H. 2012. Functional analyses of individual mating type transcripts at *MAT* loci in *Fusarium graminearum and Fusarium asiaticum*. FEMS Microbiology Letters 337: 89-96.
68. Klionsky, D.J. and Emr, S.D. 2000. Autophagy as a regulated pathway of cellular degradation. Science 290: 717-1721.
69. Klittich, C.J.R., and Leslie, J.F. 1988. Nitrate reduction mutants of *Fusarium moniliforme (Gibberella fujikuroi*). Genetics 118:417-423.
70. Klittich, C.J.R., and Leslie, J.F. 1992. Identification of a second mating population within the *Fusarium moniliforme* anamorph of *Gibberella fujikuroi*. Mycological Society of America 84 (4): 541-547.

71. Kohn, L.M., Carbone, I. and Anderson, J.B. 1990. Mycelial interactions in *Sclerotonia sclerotiorum*. Experimental Mycology 14: 255-267.
72. Kronstad, J.W. and Staben, C. 1997. Mating type in filamentous fungi. Annual Reviews in Genetics 31: 245-276.
73. Kulman, E.G. 1983. Effects of hypovirulence in *Cryphonectria parasitica* and of secondary blight infections on dieback of American chestnut trees. Phytopathology 73 (7): 1030-1034.
74. Labarere, J., Begueret, J. and Bernet, J. 1974. Incompatibility in *Podospora anserina*: Comparative properties of the antagonistic cytoplasmic factors of a nonallelic system. American Society of Microbiology 120 (20): 854-860.
75. Lam, E., Kato, N. and Lawton, M. 2001. Programmed cell death, mitochondria and plant hypersensitive response. Nature 411:848-853.
76. Leberer, E., Thomas, D.Y. and Whiteway, M. 1997. Pheromone signaling and polarized morphogenesis in yeast. Current Opinion in Genetics and Development 7: 59-66.
77. Leipe, D.D., Koonin, E.V. and Aravind, L. 2004. STAND, a class of P-loop NTPases including animal and plant regulators of programmed cell death: multiple, complex domain archtectures, unusual phyletic patterns and evolution by horizontal gene transfer. Journal of Molecular Biology 343: 1-28.
78. Leslie, J.F. 1993. Fungal vegetative compatibility. Annual Reviews in Phytopathology 31: 127-151.
79. Leslie, J.F. and Klein, K.K. 1996. Female fertility and mating type effects on effective population size and evolution in filamentous fungi. Genetics 144: 557-567.
80. Leslie, J.F. and Summerell, B.A. 2006. The *Fusarium* Laboratory Manual. Ames: Blackwell.
81. Loubradou, G., Begueret, J. and Turcq, B. 1997. Mutation in an HSP90 gene affects the sexual cycle and suppresses vegetative incompatibility in the fungus *Podospora anserina*. Genetics 147: 581-588.
82. Loubradou, G. and Turcq, B. 2000. Vegetative incompatibility in filamentous fungi: a roundabout way of understanding the phenomenon. Research in Microbiology 151: 239-245.
83. Marek, S.M., Wu, J., Glass, N.L., Gilchrist, D.G. and Bostock, R.M. 2003. Nuclear DNA degradation during heterokaryon incompatibility in *Neurospora crassa*. Fungal Genetics and Biology 40: 126-137.

84. Martin, S.H., Wingfield, B.D., Wingfield, M.J. and Steenkamp, E.T. 2011. Structure and evolution of the *Fusarium* mating type locus: New insights from the *Giberella fujikuroi* complex. Fungal Genetics and Biology 48: 731-740.
85. Mathur, V., Seuring, C., Riek, R., Saupe, S.J. and Liebman, S.W. 2012. Localization of HET-S to the cell periphery, not to Het-s aggregates, is associated with Het-s-HET-S toxicity. Molecular Cell Biology 32: 139-153.
86. Mattjus, P., Turcq, B., Pike, H.M., Molotkovsky, J.G. and Brown, R.E. 2003. Glycolipid intermembrane tranfer is accelerated by HET-C2, a filamentous fungus gene product involved in the cell-cell incompatibility response. Biochemistry 42: 535-542.
87. Melkel, H.W. 1905. A deadly fungus on the American chestnut. New York Zoological Society, 10 TH Annual Report 10: 97-103.
88. Micali, C.O. and Smith, M.L. 2003. On the independence of barrage formation and heterokaryon incompatibility in *Neurospora crassa*. Fungal Genetics and Biology 38: 209-219.
89. Milgroom, M.G., Wang, K., Zhou, Y., Lipari, S.E. and Kaneko, S. 1996. Interconnectional population structure of the chestnut blight fungus, *Cryphonectria parasitica*. Mycologia 88: 179-190.
90. Milgroom, M.G. and Brasier, C.M. 1997. Potential diversity in vegetative incompatibility types of *Ophiostoma novo-ulmi* in North America. Mycologia 89: 722-726.
91. Mylyk, O.M. 1975. Heterokaryon incompatibility genes in *Neurospora crassa* detected using duplication producing chromosome rearrangements. Genetics 80: 107-124.
92. Nauta, M. and Hoekstra, R.F. 1992. Evolution of reproductive systems in filamentous ascomycetes. II. Evolution of hermaphroditism and other reproductive strategies. Heredity 68: 537-546.
93. Nelson, M.A. 1996. Mating systems in ascomycetes: a romp in the sac. Trends in Genetics 12: 69-74.
94. Paoletti, M., Buck, K.W. and Brasier, C.M. 2006. Selective acquisition of novel mating type and vegetative incompatibility genes via interspecies gene transfer in the globally invading eukaryote *Ophiostoma novo-ulmi*. Molecular Ecology 15: 249-262.
95. Paoletti, M., Saupe, S.J. and Clave, C. 2007. Genesis of a fungal non-self recognition repertoire. Plos One 2 (3): 1-10.

96. Perkins, D.D. 1975. The use of duplication generating rearrangements for studying heterokaryon incompatibility genes in *Neurospora*. Genetics 80: 87-105.

97. Perkins, D.D. 1988. Main features of vegetative incompatibility in *Neurospora*. Fungicide Genetics Newsletter 35: 44-46.

98. Philley, M.L. and Staben, C. 1994. Functional analyses of the *Neurospora crassa* MT a-1 mating type polypeptide. Genetics 137: 715-722.

99. Pinan-Lucarre, B., Paoletti, M. Dementhon, K, Coulary-Salon, B. and Clave, C. 2002. Autophagy is induced during cell death by incompatibility and is essential for differentiation in the filamentous fungus *Podospora anserina*. Molecular Microbiology 47: 321-333.

100. Poggeler, S. 2001. Mating type genes for classical strain improvement of ascomycetes. Applied Molecular Biotechnology 56: 589-601.

101. Puhalla, J.E. 1985. Classification of strains of *Fusarium oxysporum* on the basis of vegetative compatibility. Canadian Journal of Botany 63:179-183.

102. Ribeiro, O.K., Erwin, D.C. and Zentmyer, G.A. 1975. An improved synthetic medium for oospore production and germination of several *Phytophthora* species. Mycologia 67: 1012-1019.

103. Rizet, S.B. 1952. Les phenomenes de barrage chez *podospora anserina*. I. Analyse genetique des barrages entre les souches S el s. Rev. Cyto. Biol. Veg. 13: 51-92.

104. Rohde, J., Heitman, J. and Cardenas, M.E. 2001. The TOR kinases link nutrient sensing to cell growth. The Journal of Biological Chemistry 276 (13): 9583-9586.

105. Sarkar, S., Iyer, G., Wu, J. and Glass, N.L. 2002. Nonself recognition is mediated by HET-C heterocomplex formation during vegetative incompatibility. European Molecular Biology Organization Journal 21(18): 4841-4850.

106. Saupe, S., Descamps, C., Turq, B. and Begueret, J. 1994. Inactivation of the *Podospora anserina* locus *het-c*, whose product resembles a glycolipid transfer protein, drastically impairs ascospore production. Proceedings of the National Academy of Science USA 91: 5927-5931.

107. Saupe, S., Turcq, B. and Begueret, J. 1995. A gene responsible for vegetative incompatibility in the fungus *Podospora anserina* encodes a protein with a GTP-binding motif and GB homologous domain. Gene 162: 135-139.

108. Saupe, S.J., Kuldau, G.A., Smith, M.L. and Glass, N.L. 1996. The product of the het-C heterokaryon incompatibility gene of *Neurospaora crassa* has characteristics of a

glycine rich cell wall protein. Genetics 143: 1589-1600.

109. Saupe, S.J. and Glass, N.L. 1997. Allelic specificity at the *het-c* heterokaryon incompatibility locus of *Neurospora crassa* is determined by a high variable domain. Genetics 146: 12299-1309.

110. Saupe, S.J. 2000. Molecular genetics of heterokaryon incompatibility in filamentous ascomycetes. Microbiology and Molecular Biology 64 (3): 489-502.

111. Saupe, S.J., Clave, C. and Begueret, J. 2000. Vegetative incompatibility in filamentous filamentous fungi: *Podospora* and *Neurospora* provide some clues. Current Opinion in Microbiology 3: 608-612.

112. Saupe, S.J. 2011. The Het-s prion of *Podospora anserine* and its role in heterokaryon incompatibility. Seminars in Cell & Developmental Biology 22: 460-468.

113. Saupe, S.J. and Daskalov, A. 2012. The Het-S prion, an amyloid fold as a cell death activation trigger. Plos Pathogens 8: 1-3.

114. Seuring, C., Greenwald, J., Wasmwer, C., Wepf, R., Saupe, S.J., Meier. B.H. and Riek, R. 2012. The mechanism of tocicity in HET-S/HET-s prion incompatibility. Plos Biology 10 (2): 1-18.

115. Shiu, P.K.T. and Glass, N.L. 1999. Molecular characterization of *tol*, a mediator of mating type associated vegetative incompatibility in *Neurospora crassa*. Genetics Society of America 151: 545-555.

116. Shiu, P.K.T. and Glass, N.L. 2000. Cell and nuclear recognition mechanisms mediated by mating type in filamentous ascomycetes. Current Opinion in Microbiology 3: 183-188.

117. Smith, M.L., Micali, O.C., Hubbard, S.P., Mir-Rashed, N., Jacobson, D.J. and Glass, N.L. 2000. Vegetative incompatibility in the het-6 region of the *Neurospora crassa* is mediated by two linked genes. Genetic Society of America 155

118. Staben, C. and Yanofsky, C. 1990. *Neurospora crassa* a mating type region. Proceedings of the National Academy of Science USA 87: 4917-4921.

119. Steenkamp, E.T., Wingfield, B.D., Coutinho, T.A., Zeller, K.A., Wingfield, M.J., Marasas, W.F.O. and Leslie, J.F. 2000. PCR based identification of *mat-1* and *mat-2* in the *Gibberella fujikuroi* species complex. Applied and Environmental Microbiology 66 (10): 4378-4382.

120. Summerell, B.A., Salleh, B. and Leslie, J.F. 2003. A utilitarian approach to *Fusarium* identification. The America Phytopathological Society 87 (2): 117-128.

121. Turgeon, B.G. 1998. Application of mating type gene technology to problems in fungal biology. Annual Reviews in Phytopathology 36: 115-137.

122. Turgeon, B.G., Bohlmann, H., Ciuffetti, L.M., Christiansen, S.K., Yang, G., Schafer, W. and Yoder, O.C. 1993. Cloning and analyzing of the mating type genes from *Cochliobolus heterostrophus*. Molecular and General Genetics 138: 270-284.

123. Viljoen, A., Wingfield, M.J., Gordon, T.R., Marasas, W.F.O. 1997. Genotypic diversity in a South African population of pitch canker fungus *Fusarium subglutinas* f.sp. *pini*. Plant Pathology 46:590-593.

124. Wilker, K. and Gordon, T. 2000. An initial assessment of *Fusaruim circinatum* in different parts of the world. Canadian Journal of Botany 78: 709-717.

125. Wingfield, M.J., Hammerbacher, A., Ganley, R.J., Steenkamp, E.T., Gordon, T.R., Wingfield, B.D. and Coutinho, T.A. 2008. Pitch canker caused by *Fusarium circinatum*-a growing threat to pine plantations and forests worldwide. Australasian Plant Pathology 37 (4): 1-31.

126. Worrall, J. 1997. Somatic incompatibility in basidiomycetes. Mycological Society of America 89 (1): 24-36.

127. Wu, J. and Glass, N.L. 2001. Identification of specificity determinants and generation of alleles with novel specificity at the het-c heterokaryon incompatibility locus of *Neurospora crassa*. Molecular Cell Biology 21 (4): 1045-1057.

128. Xiang, Q. and Glass, L. 2002. Identification of vib-1, a locus involved in vegetative incompatibility mediated by het-c in *Neurospora crassa*. Genetics Society of America 162: 89-101.

129. Xiang, Q., Rasmussen, C. and Glass, N.L. 2002. The ham-2 locus, encoding a putative transmembrane protein, is required for hyphal fusion in *Neurospora crassa*. Genetics 160: 169-180.

130. Xiang, Q. and Glass, N.L. 2004. The control of mating type heterokaryon incompatibility by vib-1, a locus involved in het-c heterokaryon incompatibility in *Neurospora crassa*. Fungal Genetics and Biology 41: 1063-1076.

131. Yokoyama, E., Yamagishi, K. and Hara, A. 2004. Development of a PCR based mating type assay for *Clavicipitaceae*. Microbiology Letters 237: 205-212.

132. Yun, S., Berbee, M.L., Yoder, O.C and Turgeon, B.G. 1999. Evolution of the fungal self-fertile reproduction life style from Evolution of the self-sterile ancestors. Proceedings of the National Academy of Science 96: 5592-5597.

133. Yun, S. Arie, T., Kaneko, I., Yoder, O.C. and Turgeon, B.G. 2000. Molecular organization of the mating type loci in heterothallic, homothallic and sexual *Gibberella/Fusarium* species. Fungal Genetics and Biology 31: 7-20.

134. Zentmyer, G.A., Klure, L.J. and Pond, E.C. 1979. The influence of temperature and nutrition on formation of sexual structures by *Phytophthora cinnamomi*. Mycological Society of America 71 (1): 55-67.

CHAPTER 2

Emergence of novel populations of the pitch canker pathogen in the Western Cape Province of South Africa

O.M. MASHANDULE, T.A. COUTINHO, B.D. WINGFIELD, M.J. WINGFIELD and E.T. STEENKAMP*

Department of Microbiology and Plant Pathology, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria 0002

*Corresponding author email: emma.steenkamp@up.ac.za

Abstract

Fusarium circinatum, the causal agent of pine pitch canker that has caused a significant damage in many parts of the world. Pitch canker has been reported in the Western Cape Province and it poses as a threat to South African plantations. Vegetative Compatibility Group (VCG) and Amplified Fragment Length Polymorphism (AFLP) studies were used to determine the genetic diversity of the Western Cape population. However, the results of this study confirmed that a pine nursery in the Western Cape was the probable source of inoculum for the outbreaks in George and Tokai plantations due to the incompatibility between the Western Cape VCGs and the VCGs from other parts of South Africa. Therefore the outbreak in the Western Cape was the result of one or more separate introductions of the disease into that area. However, the AFLP results indicated that Western Cape isolates that were isolated from the *P. radiata* are more closely related to each other than to isolates that were obtained from other parts of South Africa.

Keywords: *Fusarium circinatum*, vegetative compatibility, sexual compatibility, AFLP

INTRODUCTION

The ascomycete fungus *Fusarium circinatum* Nirenberg and O'Donnell (1998) (teleomorph = *Gibberella circinata*) is the causal agent of pitch canker of pine (Dwinell *et al*., 1985; Storer *et al*., 1995) and Douglas fir (*Pseudotsuga menziesii*) (Gordon *et al*., 2006a). The disease affects susceptible hosts of all ages and the pathogen can infect and colonize all plant organs including seeds, exposed roots, stems, shoots, branches, cones and reproductive structures (Wingfield *et al*., 2008). Where the pitch canker pathogen affects mature or established trees, symptoms include discolouration and wilting of needles, dieback of branches from the tips to the infection site, which are typically characterized by resinous cankers (Barrows-Broaddus and Dwinell, 1983; Storer *et al*., 1995; Gordon *et al*., 2001). These cankers can develop on branches, stems, shoots and exposed roots (Barrows-Broaddus and Dwinell, 1983; Storer *et al*., 1995; Gordon *et al*., 2001). When the pitch canker fungus affects seedlings, the pathogen typically infects the roots and root collar and causes damping off, tip dieback and mortality of established seedlings (Carey and Kelly, 1994; Viljoen *et al*., 1994).

In most areas affected by pitch canker, *F. circinatum* primarily manifests itself as a problem of trees in forests or established trees in plantations (McCain *et al*., 1987; Santos and Tovar, 1991; Carlucci *et al*., 2007). Until recently, the only exceptions were South Africa (Viljoen *et al*., 1994) and Chile (Wingfield *et al*., 2002) where the pitch canker fungus was known only as a seedling pathogen in nurseries (Wingfield *et al*., 2008). However, 15 years after the initial appearance of *F. circinatum* on *P. patula* seedlings in a single nursery in the Mpumalanga Province of South Africa (Viljoen *et al*., 1994), pitch canker was discovered for the first time in an outbreak on 5 and 9 year old *Pinus radiata* trees in the Western Cape Province of South Africa (Coutinho *et al*., 2007).

In an attempt to understand the observed global distribution of *F. circinatum*, various studies have sought to clarify the population biology of this fungus (e.g., Gordon *et al*., 1996; Viljoen *et al*., 1997a; 1997b; Wikler and Gordon, 2000; Britz *et al*., 2005). Based on the high levels of genetic diversity among isolates obtained from Mexico, it is now widely believed that this region is the centre of origin of *F. circinatum*, where the fungus probably co-evolved with its pine host (Wikler and Gordon, 2000; Wingfield *et al*., 2008). From here, the pathogen was accidentally introduced to other parts of world, either directly or indirectly via other locations

(Wikler and Gordon, 2000; Wingfield *et al*., 2008). Although the pathogen may in some regions be spread mainly by wind, water splash and insects such as *Pissodes nemorensis, Conophthorus radiatae, Bradysia difformis* and *Brachydereds inacanus* (Matthews, 1962; Hurley *et al*., 2007; Romon *et al*., 2007; Wingfield *et al*., 2008), much of the observed distribution of the fungus is probably due to human activity and the movement of infected plant material, seed and soil (Wingfield *et al*., 2008). In some regions, the application of stringent quarantine measures has prevented the spread of the fungus to pitch canker-free areas. For example, in New Zealand quarantine limitations on the movement of pine, Douglas fir, soil or objects contaminated with soil have so far prevented the introduction of the disease into the country (Ormsby, 2004). In other regions, quarantine measures have been less effective. For example, in South African, *F. circinatum* has spread from a single pine seedling nursery to most commercial nurseries in the country (Wingfield *et al*., 2002; 2008; Britz *et al*., 2005).

Several questions regarding the pitch canker outbreak on established plantation trees in the Western Cape Province of South Africa remain unanswered. One of these concerns the origin of the inoculum that gave rise to this outbreak. Insight into the origin and evolution of *F. circinatum* in this region could be useful in developing effective management strategies for the pathogen. For example, knowledge regarding the possible source of inoculum for the plantation outbreaks of pitch canker is crucial for potentially preventing further spread of the fungus. Furthermore, *F. circinatum* is dimictic (Burnett, 1956; Britz *et al*., 1999), which means that in addition to asexual reproduction, this fungus is capable of reproducing sexually depending on the availability of fertile isolates of opposite mating types (Correll *et al*., 1987; Leslie, 1995; Leslie and Klein, 1996). The outcomes of these reproductive strategies are markedly different as predominantly asexual populations will be less diverse than sexual reproducing populations (Brasier, 1988; McDonald and Linde, 2002).

In this study we considered the possible origin(s) and reproductive mode(s) of the *F. circinatum* population responsible for the outbreaks of pitch canker on established plantation trees in the Western Cape province of South Africa. For this purpose we obtained a collection of *F. circinatum* isolates from diseased *P. radiata* seedlings and plantation trees in the region. To determine their possible origin(s), we compared our collection of isolates to representative isolates obtained from diseased seedlings elsewhere in the country (Viljoen *et al*., 1997a;

Britz *et al*., 2005), as well as the USA and Mexico by making use of vegetative compatibility tests (Correll *et al*., 1987; Leslie, 1993) and Amplified Fragment Length Polymorphism (AFLP) analysis (Vos *et al*., 1995). To assess the importance of the sexual cycle in the reproduction of the *F. circinatum* populations in the Western Cape, sexual compatibility and fertility tests were performed (Klittich and Leslie, 1988; Leslie and Summerell 2006). To this end, we first determined the mating type for all isolates by targeting the respective conserved α-domain (Coppin *et al*., 1997; Yoshida *et al*., 1998) and the HMG (high mobility group) domain (Arie *et al*., 1997; Coppin *et al*., 1997; Covert *et al*., 1999) of the *mat 1* and *mat 2* idiomorphs, using a diagnostic PCR approach (Steenkamp *et al*., 2000).

MATERIALS AND METHODS

F. circinatum isolates

Isolates of *F. circinatum* were obtained from the symptomatic tissue of *P. radiata* trees at the initial pitch canker outbreak site in the Tokai plantation in the Western Cape province of South Africa. In the George area of this province, isolates were obtained from *P. radiata* trees showing typical pitch canker symptoms. An additional collection of isolates was obtained from diseased *P. radiata* seedlings collected in a commercial nursery in the region, and which was previously suggested to have been the source of inoculum for the Western Cape outbreaks (Coutinho *et al*., 2007). We also included six isolates (FCC 1844, FCC 1845, FCC 1928, FCC 1985, FCC 2154 and FCC 2155) that were obtained during an outbreak in this nursery in 1996.

For all the isolations, infected plant tissue (seedlings or stems and shoots with cankers) were cut into small pieces and plated onto *Fusarium* selective media (Nelson *et al*., 1983). For the Tokai samples, isolations were also made from damage caused by the pine weevil *Pissodes nemorensis*, as well as the young insects that emerged from incubated emergence chambers. Following incubation for seven days at 25°C under a cool white fluorescent illumination, colonies resembling *Fusarium* were transferred to half-strength potato dextrose agar (PDA, Merck, South Africa). After incubation (25°C), pure cultures were prepared by inoculating single germinating conidia onto fresh PDA (Leslie and Summerell, 2006).

All isolates obtained from the Western Cape were confirmed to represent *F. circinatum* by making use of a procedure based on the specific amplification of a diagnostic portion of the intergenic spacer (IGS) region of the ribosomal RNA operon (Schweigkofler *et al.*, 2004). Genomic DNA was extracted (Möller *et al.*, 1992) from each isolate and then used as template in PCRs with the *F. circinatum*-specific primers CIRC1A and CIRC4A (Schweigkofler *et al.*, 2004). The PCR mixture contained 2.5 mM of each dNTP, 25 mM of $MgCl_2$, 10 µM of each primer, 5 U/µl of *Taq* DNA polymerase, *ca.* 50 ng/µl of template DNA and 10X PCR buffer (Roche, South Africa). The reaction conditions were a denaturation at 95°C for 30 seconds followed by 30 cycles of 95°C for 30 seconds, 62°C for 30 seconds and 72°C for 30 seconds, and a final extension at 72°C for 10 minutes. The PCR products were visualized using agarose gel electrophoresis, ethidium bromide (0.2 µg/ml) staining and a UV transilluminator (Sambrook *et al.*, 1989).

For comparative purposes, two additional sets of *F. circinatum* isolates were included (Table 2). The first set of 29 isolates represented the known vegetative compatibility groups (VCG's) originally identified (Britz *et al.*, 2005 and Viljoen *et al.*, 1997a) from nurseries elsewhere in South Africa. The second set of 42 isolates represented VCGs from California, Florida and Mexico (Wilker and Gordon, 2000). All the isolates used in this study are maintained in the *Fusarium* culture collection (FCC) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

Vegetative compatibility tests

To sort the isolates into vegetative compatibility groups, tests based on genetic complementation were used (Perkins, 1988; Micali and Smith, 2003). In this study, *nit* mutants for all the isolates were generated with medium containing 1.5 % $KCLO_3$ as described previously (Correll *et al.*, 1987). Growth in the presence of $NaNO_2$, $NaNO_3$, NH_4 or hypoxanthine as sole nitrogen sources were used to determine the phenotype of the respective *nit* mutants (Correll *et al.*, 1987). The respective *nit* mutants were then used in complementation assays to detect the formation of heterokaryons between individuals of the same VCG (Correll *et al.*, 1987). This was accomplished by pairing two mutants (for example *nit 1* and *nit 3* or *nit 1* and *nit M*) of different isolates on minimal medium containing nitrate as sole nitrogen source (Correll *et al.*, 1987). In all cases, the compatible *nit* mutants of a

specific isolate were also complemented to control for self-incompatibility (Correll *et al*., 1987).

Genetic diversity based on the VCGs for the Western Cape collection of isolates was determined by using the simple *S/N* measure proposed by Anagnostakis *et al*. (1986) where *N* is the number of isolates examined and *S* is the number of VCGs encountered. As an indication of population diversity, Shannon (*H'*) and Sheldon's (H_{EM}) indices were also determined (Sheldon, 1969). *H'* was calculated by using the equation $H' = -\Sigma\, p_i \ln p_i$, where p_i is the proportion of isolates (S) made up of the *ith* isolate. H_{EM} was calculated by using the equation ($H_{EM} = H' \ln S^{-1}$) where S is the total number of isolates in the community (Sheldon, 1969).

Sexual compatibility and fertility tests

The mating types of all isolates were determined as described previously (Steenkamp *et al*., 2000) by making use of the diagnostic PCR primers MAT 1a and MAT 1b for the *mat 1* idiomorph, and primers MAT 2c and MAT 2d for the *mat 2* idiomorph. The PCR mixture included 25 mM $MgCl_2$, 2.5 mM of each dNTPs, 10 µM of each *mat 1* or *mat 2* primer, 5 U/µl of *Taq* DNA polymerase and reaction buffer (Roche), and *ca*. 50 ng/µl of DNA template. The reaction conditions were denaturation at 94°C for 30 seconds followed by 35 cycles of 94°C for 30 seconds, 65°C for 45 seconds and 72°C for 30 seconds, and a final extension at 72°C for 10 minutes. The resulting products were analysed by electrophoresis as described above.

For the Tokai plantation and Western Cape nursery isolates, the sexual reproductive potential was evaluated by determining the extent to which isolates can act as females in crosses. For this purpose, all *mat 1* individuals were crossed with *mat 2* individuals. Because all of the Tokai isolates were *mat 1*, these were the two *mat 2* isolates (Ins 2 and Ins 3) that were routinely used as female-fertile or hermaphrodites in our laboratory. These crosses were repeated such that all the Tokai and nursery isolates were used at least once as both a female and a male. For control purposes, female fertile tester strains (MRC 7488 and MRC 6312) of opposite mating type were also crossed (Britz *et al*., 1998).

When used as females, isolates were grown on carrot agar and when used as males they were grown on complete media (Klittich and Leslie, 1988). Following incubation at 25ºC for 7 days, the spores produced on complete medium were suspended in 2.5% (v/v) Tween 60 (Merck) and used to fertilise female isolates on carrot agar (Klittich and Leslie, 1988). All crosses were incubated at 17°C under cool white and black lights (Britz *et al*., 1998; Covert *et al*, 1999; Leslie and Summerell, 2006). After the development of sexual fruiting structures (perithecia), the viability of the ascospores generated was confirmed by suspending spore masses in 1000 µl of sterile water. Ascospores were then plated onto the water agar and germination of these was examined using a light microscope.

AFLP analysis

A representative set of isolates from the Western Cape (Table 2), were subjected to AFLP analysis. The analysis also included representatives of VCGs from elsewhere in South Africa (Viljoen *et al*., 1997a; Britz *et al.*, 2005) and those from California, Florida and Mexico (Table 2). The analysis was carried out as described by De Vos *et al*. (2007), using a set of five two-base-addition *Eco*RI and *Mse*I adapter-specific selective primers (EcoRI-AA + MseI-AT, EcoRI-TC + MseI-AG, EcoRI-AA + MseI-AG, EcoRI-TC + MseI-AT, EcoRI-AA + MseI-CC). The *Eco*R1 selective primers were labelled with either the IRDyeTM 700 or IRDyeTM 800 infrared dye (LI-COR, Lincoln, USA). The amplified fragments, together with 50-70 base pair (bp) sizing standard (LI-COR), were separated and visualized with a 4200 LI-COR automated DNA sequencer (Myburg *et al*., 2001). Electronic gel images were manually scored based on the presence or absence of clear and well-resolved bands. To ensure that the AFLP profiles generated were reproducible, the AFLP procedure was repeated using DNA isolated from 20 individuals. To ensure that the scoring of the profiles was precise, any uncertainty in the scoring process was eliminated by excluding bands with low intensity, low molecular weight, and those that were closely located to each other (Bagley *et al*., 2001). The scoring process was also repeated several times.

The scored AFLP data were subjected to neighbour-joining distance analysis (Saitou and Nei, 1987) in Bionumerics (Applied Maths, Belgium) using the simple matching similarity coefficients (Kosman and Leonard, 2005), and in PHYLIP (J. Felsenstein, University of Washington; http://evolution.gs.washington.edu/phylip.html) using RESTDIST and the

restriction fragment model of Nei and Li (1979) with a restriction site length of 46 nucleotides as suggested by Felsentein (2004). Similarity matrices and tree files were visualized and/or annotated with MEGA 4 (Kumar *et al*., 1993).

RESULTS

Vegetative compatibility tests

A total of 171 isolates confirmed to represent *F. circinatum* (Figure 1) were obtained from diseased *P. radiata* seedlings and trees in the Western Cape (Table 1). For 169 of the Western Cape isolates, both *nit 1* and *nit 3* mutants were generated, but only 160 *nit M* mutants were generated. The pairing of suitable *nit*-mutants generated for the 169 Western Cape isolates of *F. circinatum* revealed that 143 of the 169 isolates were able to establish stable heterokaryons with at least one other isolate and they separated into 11 VCGs (VCO1-VCO11) (Figure 4A). None of the remaining 26 isolates was vegetatively compatible with any other isolate. This was not due to self-incompatibility between *nit*-mutants (Correll *et al*., 1987), as the pairing of compatible *nit*-mutants for a specific isolate always resulted in dense heterokaryotic growth. Each of these isolates was, therefore, considered to represent a distinct VCG (VCO12-VCO33, VCO38-VCO41) of which they were each the sole representative. Thus, of the169 *F. circinatum* isolates obtained from *P. radiata* in the Western Cape represent 26 distinct VCGs. However, none of these isolates were vegetatively compatible with any of the isolates representing VCGs from elsewhere in the country or the United States and Mexico.

A total of 9 VCGs were recovered from the 62 Tokai isolates, five (VCO1-VCO5) of which were shared by multiple isolates and three (VCO31-VCO33) that were represented by a single isolate each. 18 isolates obtained from *P. nemorensis* galleries in this location were recovered among three shared Tokai VCGs (VCO1, VCO3 and VCO4) and two single VCGs (VCO32 and VCO33). The George population of 37 isolates included 17 VCGs, seven (VCO1, VCO3, VCO5, VCO7, VCO9-VCO11) of which were shared by multiple isolates and nine (VCO12-VCO20) consisted of single isolates. The Western Cape nursery population included 19 VCGs, five (VCO2, VCO5-VCO8) of which were shared by multiple isolates and fourteen (VCO22-VCO30, VCO38-VCO41) that were represented by a single isolate each.

In terms of genetic diversity, the populations obtained from plantations trees were slightly less diverse than the population of isolates from the nursery (Table 3). The isolates from Tokai

appeared to be dominated by a single VCG (VCO1) that was associated with 74% of the isolates (Table 1). Only one of the George isolates belonged to this VCG and it was not recovered from the commercial nursery collection. Among the George isolates, two other VCGs (VCO9 and VCO11) that were not recovered from any other location appeared to be dominant and were represented by 22% and 27%, respectively, of the isolates in that location. The dominant VCGs among the nursery isolates were VCO5 and VCO6, which respectively represented 27% and 21% of the isolates. Of these, VCO6 appeared to be unique to the location, while VCO5 was the only VCG present at all three collection sites. In most cases where multiple isolates were recovered from a single tree, the isolates formed part of the same VCG (Table 1). For example, VCO3 isolates 54-1 and 54-2 both originated from the same *P. radiata* tree. However, in some instances, different VCGs were recovered from a single tree, e.g. isolates 32-1 (VCO4) and 32-2 (VCO1) originated from the same tree, as well as isolates 47-1i (VCO1) and 47-2i (VCO4) that were isolated from *P. nemorensis* galleries associated with the same tree.

Sexual compatibility and fertility tests

Application of the diagnostic PCR for scoring mating type (Steenkamp *et al*., 2000), resulted in the amplification of *ca*. 800-bp fragment from *mat 2* isolates and a *ca*. 200-bp fragment from *mat 1* isolates (Figure 2). The mating type for all of the isolates from the George and Tokai plantation trees was *mat 1* (Table 1). Among the isolates obtained from *P. radiata* seedlings, 24 were *mat 2* and 46 were *mat 1*. In most cases the isolates in a single VCG were associated with the same mating type **(**Figure 4B**)**. The only exceptions were VCO6 and VCO8, both of which only occurred in the nursery population, as well as VCO7 in which all the *mat 2* isolates originated from nursery seedlings and one *mat 1* isolate (B2H3) originated from plantation tree in the George area.

In all cases, successful crosses (*i.e*., development of perithecia) were observed approximately 14 days after mating isolates of opposite mating type (*mat 1* and *mat* 2) were placed on carrot agar. In addition to the crosses between the *F. circinatum* mating type tester strains, perithecia were observed only for crosses among the nursery isolates when isolates ks 20 and ks 45 were used as females. However, most of the perithecia from the latter cross did not exude ascospores from their ostioles. Among the crosses between the Tokai isolates and the known hermaphrodite strains, perithecia were observed only when the latter strains were used as

females. None of the isolates from Tokai were, therefore, female-fertile, but they were all able to act as males, because fertilization of the known hermaphrodite isolates resulted in the formation of perithecia from which viable ascospores oozed.

AFLP Analyses

Together with isolates representing the known VCGs in South Africa (Viljoen *et al*., 1997a; Britz *et al*., 2005), the AFLP analysis included an isolate representing each of the VCGs obtained from the Western Cape. In cases where a VCG was associated with both mating types or occurred in more than one location, a representative isolate for both mating types and from all the locations were also included. On average 31, distinct fragments were produced per AFLP primer set. Of the five primer sets used, EcoRI-AA + MseI-AG generated most (11-24) fragments per isolate, while primer set EcoRI-AA + MseI-AT generated on average the least (7-13) fragments. For the selected isolates subjected to AFLP analysis, a total of 152 distinct AFLP fragments were scored (*i.e.*, 22 for primer set EcoRI-AA + MseI-AT, 23 for EcoRI-TC + MseI-AG, 39 for EcoRI-AA + MseI-AG, 34 for EcoRI-TC + MseI-AT, and 34 for pirmer set EcoRI-AA + MseI-CC). For the isolates for which the entire AFLP procedure was repeated, identical profiles were generated.

In some cases, isolates representing distinct VCGs generated identical AFLP profiles. The representatives of VCO1 (isolate 1-1), VCO2 (isolate KS80), VCO3 (isolate 12-2i), VCO5 (isolate CBH10) and VCO28 (isolate KS53) all displayed similar AFLP profiles. This was also true for isolates KS90 (VCO5) and KS57 (VCO6), isolates 14 (VCO4) and 59-1 (VCO5), isolates KS81 (VCO29) and KS87 (VCO30), and isolates FCC5368 (VCO34) and 25-2 (VCO31). In other cases isolates representing a single VCG, but that were isolated from different locations, or that were associated with different mating types, had different AFLP profiles. This was most pronounced for the opposite mating type isolates of VCO6 (KS57 and KS7) that differed at 26 of the 152 scored AFLP fragments and VCO8 (KS25 and KS22) that differed at 70 of the 152 scored bands. In contrast, the scored AFLP fragments differed at only four or five positions for the opposite mating type isolates of VCO7 (isolates B2H3 and KS25), as well as the representatives from different locations of VCO2 (isolates KS80 and 28-2) and VCO5 (isolates KS90, CBH10 and 59-2).

All of the isolates from Tokai included in the AFLP analysis formed part of either Cluster 1 or 2 (Figure 3A). Only small proportions of the isolates examined with AFLP from the George area (four isolates) and Western Cape nursery (seven isolates) formed part of either of these clusters. However, the majority (74%) of the isolates obtained from the plantation trees in the Western Cape (Figure 3B), are represented by VCGs in Clusters 1 and 2 (Figure 3C). Similarly, at least 67% of the isolates obtained from the Western Cape nursery are represented by VCGs included in Cluster 1 and 2. Neither of the Clusters included isolates obtained from locations other than the Western Cape. The remainder of the isolates from the George area examined with AFLP analysis appeared to be related to isolates representing VCGs obtained from the Western Cape nursery (VCO7 and VCO13), or from elsewhere in South Africa (VCO9, VCO11, VCO12, VCO15, VCO18, VCO19 and VCO20). The remainder of the isolates from the Western Cape pine seedling nursery also appeared to be related to VCGs from elsewhere in South Africa (VCO39), as well as those from locations in Mexico (VCO8).

DISCUSSION

Results of this study have shown that the population of *F. circinatum* in the Western Cape is partitioned in a large number (26) of distinct VCGs. A number of common VCGs were identified among the three collections of *F. circinatum* isolates obtained from *P. radiata* trees and seedlings in the Western Cape (Figure 2A). Two VCGs were common to both the plantation tree populations, which individually also shared a VCG with the population of isolates from the Western Cape nursery. However, one VCG (VCO5) was common to all three populations. Two isolates that were collected during a 1996 disease outbreak in this Western Cape nursery (FCC 1844 and FCC 1845; Table 1) were also part of this VCG. These findings indicate that isolates of the pitch canker pathogen are being actively spread in the Western Cape region, either due to human activity in the form of the movement of infected material or because of insect vectors such as *P. nemorensis*, as suggested previously (Coutinho *et al*., 2007). The occurrence of VCGs that are shared in isolates from the Western Cape nursery and plantations in Tokai and George also supports the notion that the sources of the inoculum for the pitch canker outbreaks in the Western Cape *P. radiata* plantations probably originate from commercial pine seedling nurseries in the region also as hypothesised by Coutinho *et al* (2007).

Previous studies on populations of *F. circinatum* from primarily *P. patula* seedlings outside the Western Cape Province of South Africa indicated that the initial introduction of the pathogen into the country was probably in the form of multiple VCG genotypes (Viljoen *et al.*, 1994; Viljoen *et al.*, 1997a; Britz *et al.*, 2005). This was because 23 distinct VCGs were identified among 69 isolates of the pathogen from the Ngodwana nursery (Mpumalanga Province) (Viljoen *et al.*, 1997a). Later, after the disease spread to other nurseries in the country and Britz *et al.* (2005) reported the presence of 29 VCGs among the isolates examined. Of these, 6 VCGs were shared between the isolates originating from the initial outbreak in 1990 and the collections made during the 1990-1998 period (Britz *et al.*, 2005). However, none of the Western Cape isolates were vegetatively compatible with any of the representative isolates of VCGs known from pine nurseries in other parts of South Africa. These findings suggest that the disease in the Western Cape is probably due to a separate introduction(s) into the area. The possibility that these novel VCGs were generated due to mutations at the *vic* loci, of course, cannot be excluded (Leslie, 1993; Wikler and Gordon, 2000).

Analysis of the AFLP profiles generated in this study suggested that the majority of the isolates obtained from *P. radiata* in the Western Cape are more closely related to one another than to isolates that were obtained elsewhere in the country or the world. However, only 120 isolates of which only 29 and 42 respective isolates represented the possible diversity outside the Western Cape region and the rest of the world were included in the AFLP analyses. This precluded any conclusions regarding a possible source for the majority of the VCG and AFLP genotypes of the fungus in the Western Cape. For more exact estimations of the possible source of the genotypes observed in Western Cape plantations it would be necessary to include a much broader sample of isolates from not only South Africa, but also other parts of the world, including Mexico, as well as the application of more robust molecular markers such as those developed recently from pyrosequenced microsatellite-enriched genomic libraries (Santana *et al.*, 2009).

To study the reproductive mode the *F. circinatum* populations represented in this study, the distribution of mating type and female-fertility were considered. In both the examined plantation populations, all of the isolates belonged to a single mating type (*i.e., mat-1*). In addition, none of the isolates from plantation trees were capable of acting as females or

hermaphrodites in sexual crosses. In contrast, both mating types, as well as male-only and female-fertile strains were encountered in the commercial nursery population that was examined. Therefore, because of the presence of both male-only and female fertile strains in the examined nursery population, the pathogen is likely to have a mixed mode of reproduction in this setting, as have been reported previously for other nursery populations (Britz *et al*., 2005). However, that all of the *F. circinatum* isolates from *P. radiata* plantation trees are male-only and *mat-1* suggest the presence of a significant mutational load in the genes determining female-fertility, and that the sexual cycle would only be possible with the introduction of *mat-2* female-fertile strains. These findings, therefore, suggest that the Western Cape plantation populations of the pitch canker pathogen are reproducing mainly clonally or asexually, which is similar to the situations in California (Gordon *et al*., 2006b) and the Basque country of Spain (Iturritxa *et al*., 2011).

The diverse VCG and AFLP genotypes observed in the Western Cape populations of the pitch canker fungus may be attributed to sexual recombination and mutation. AFLP genotype and vegetative compatibility are both multilocus traits. The AFLP genotype of an isolate reflects its polymorphisms at a large number of anonymous genomic loci (Vos *et al*., 1995; Majer *et al*., 1996; Baayen *et al*., 2000), while its VCG identity reflects a specific set of 10-15 *vic* alleles (Leslie and Summerell, 2006). In the absence of sexual reproduction, the members of a VCG or those that share the same AFLP genotype are likely to be clonally related (Puhalla, 1985; Chulze *et al*., 2000; Leslie and Summerell, 2006). Sexual reproduction would generate new combinations of alleles, thus giving rise to new VCGs or AFLP genotypes, which may be the case for the *F. circinatum* populations associated with nursery seedlings in the Western Cape.

However, in the absence of sexual recombination, new VCGs or AFLP genotypes can also be generated through mutation (Deleu *et al*., 1993; Saupe *et al*., 1995; Saupe and Glass, 1997; Loubradou and Turcq, 2000). For example, Wikler and Gordon (2000) suggested that some closely related sets of *F. circinatum* isolates from the California and Florida populations potentially lost their vegetative compatibility identities through mutation, which resulted in isolates that originally belonged to the same VCG now representing distinct VCGs (Petersen and Gordon, 2005). In terms of AFLP, mutations at one or more of the anonymous loci may result in the loss or gain of specific fragments to generate a new AFLP genotype. In the

current study, this also appeared to be true for the genotypes of the *F. circinatum* in the Western Cape where, for example, certain VCGs in the plantations had identical AFLP profiles or distinct AFLP profiles were associated with the same VCG.

The findings presented in this study point towards a recent origin for *F. circinatum* in the *P. radiata* plantations in the Western Cape. This was most likely in the form of a small founder population with limited diversity (McDonald and Linde, 2002). Based on AFLP and VCG diversity, the *F. circinatum* population in the examined commercial nursery in the area is likely to be quite diverse with a presumably large effective population size (McDonald and Linde, 2002). The occurrence of sexual reproduction should, therefore, increase the overall genotypic diversity of the population and provide ample opportunity for the development of new strains with unique fitness characters. The dominant genotypes in the Western Cape plantations might thus represent isolates with specific fitness properties enabling their successful survival. Amongst other factors, these plantation isolates might be well-adapted to the climatic conditions characteristic to the specific Western Cape sites, and/or they might have the ability to overcome not only the defence mechanisms of seedlings but also those of established plantation trees. The latter is supported by the fact that one of the dominant VCGs occurring in the George and Tokai plantations (VCO5) was also isolated from diseased nursery seedlings during both the recent sampling and the sampling in 1996. Moreover, the predominance of asexual reproduction in the plantation settings will be the stable preservation of these favourable or well-adapted genotypes in the population (McDonald and Linde, 2002).

Acknowledgements

We thank the National Research Foundation (NRF), the Tree Pathology Co-operative Programme (TPCP) and the National Department of Agriculture (NDA) for financial support. Thank the companies and people involved in collections in the field etc.

References

1. Anagnostakis, S.L., Hau,B. and Kranz, J. 1986. Diversity of vegetative compatibility groups of *Cryphonectria parasitica* in Connecticut and Europe. Plant Disease 70:536-780.
2. Arie, T., Christiansen, S.K., Yoder, O.C. and Turgeon, B.G. 1997. Efficient cloning

of ascomycetes mating type genes by PCR amplification of the conserved MAT HMG box. Fungal Genetics and Biology 21: 118-130.

3. Baayen, R.P., O'Donnell, K., Bonants, P.J.M., Cigelnik, E., Kroon, L.P.N.M., Roebroeck, J.A., Waalwijk, C., 2000. Gene genealogies and AFLP analysis in the *Fusarium oxysporum* complex identify monophyletic and non-monophyletic formae speciales causing wilt and rot disease. Phytopathology 90: 891-900.
4. Bagley, M.J., Anderson, S.L. and May, B. 2001. Choice of methodology for assessing genetic impacts of environmental stressors polymorphism and reproducibility of RAPD and AFLP fingerprints. Ecotoxicology 10: 239-244.
5. Barrows-Broaddus, J and Dwinell, L.D. 1983. Histopathology of *Fusarium moniliforme* var. *subglutinans* in four species of southern pines. Phytopathology 73: 882-889.
6. Bogale, M., Steenkamp, E.T., Wingfield, M.J. and Wingfield, B.D. 2009. Diverse *Fusarium solani* isolates colonise agricultural environments in Ethiopia. European Journal of Plant Pathology 124: 369-378.
7. Brasier, C.M. 1988. Rapid changes in genetic structure of epidemic populations of *Ophiostoma ulmi*. Nature 332: 538-541.
8. Britz, H., Wingfield, M.J., Coutinho, T.A., Marasas, WFO and Leslie, J.F. 1999. Female fertility and mating type distribution in a South African population of *Fusarium subglutinans* f. sp. *pini*. Applied and Environmental Microbiology 64: 2094-2095.
9. Britz, H., Coutinho, T.A., Marasas, W.F.O and Wingfield, M.J. 2005. Diversity and differentiation of *Gibberella circinata* in South Africa. Plant Pathology 54: 46-52.
10. Burnett, J.H. 1956. The mating systems of fungi. New. Phytologist 55: 50-90.
11. Carey, W.A. and Kelly, W.D. 1994. First report of *Fusarium subglutinans* as a cause of late season mortality in longleaf pine nurseries. Plant Disease 78: 754.
12. Carlucci, A., Colatruglio, L. and Frisullo, S. 2007. First report of pitch canker caused by *Fusarium circinatum* on *Pinus halepensis* and *P. pinea* in Apulia (South Italy). Plant Disease 91 (12): 1683.
13. Chulze, S.N., Torres, A., Ramirez, M.L. and Leslie, J.F. 2000. Genetic variation in *Fusarium* section *Liseola* from non-till maize in Argentina. Applied and Environmental Microbiology 66: 5312-5315.
14. Coppin, E., Debuchy, R., Arnaise, S. and Picard, M. 1997. Mating type and sexual

development in filamentous ascomycetes. Microbiology and Molecular Biology Reviews 61: 411-428.

15. Correll, J.C., Klittich, C.J.R. and Leslie, J.F. 1987. Nitrate nonutilizing mutants of *Fusarium oxysporum* and their use of vegetative compatibility tests. Phytopathology 77: 1640-1646.
16. Coutinho, T.A., Steenkamp, E.T., Mongwaketsi, K., Wilmot, M. and Wingfield, M.J. 2007. First outbreak of pitch canker in a South African pine plantation. Australasian Plant Pathology 36: 1-6.
17. Covert, S.F., Briley, A., Wallace, M.M. and Mckinney, V.T. 1999. Partial MAT-2 gene structure and the influence of temperature on mating success in *Gibberella circinata*. Fungal Genetics and Biology 28: 43-54.
18. De Vos, L., Myburg, A.A., Wingfield, M.J., Desjardins, A.E., Gordon, T.R. and Wingfield, B.D. 2007. Complete genetic linkage maps from an interspecific cross between *Fusarium circinatum* and *Fusarium subglutinans*. Fungal Genetics and Biology 44: 701-714.
19. Deleu, C., Clave, C. and Begueret, J. 1993. A single amino acid difference is sufficient to elicit vegetative incompatibility in the fungus *Podospora anserina*. Genetics 135: 45-52.
20. Dwinell, L.D., Barrows-Broaddus, J. and Kuhlman, E.G. 1985. Pitch canker: a disease complex of southern pines. Plant Disease 69: 270-276.
21. Felsenstein J. 2004. Inferring phylogenies. Sunderland (MA): Sinauer Associates, Inc.
22. Gordon, T.R., Storer, A.J. and Okamoto, D. 1996. Population structure of the pitch canker pathogen, *Fusarium subglutinans*. f.sp. *pini*, in California. Mycological Research 100: 850-854.
23. Gordon, T.R., Storer, A.J. and Wood, D.L. 2001. The pitch canker epidemic in California. Plant Disease 85: 1128-1139.
24. Gordon, T.R., Kirkpatrick, S.C., Aegerte, B.J., Wood, D.L. and Storer, A.J. 2006a. Susceptibility of Douglas fir (*Pseudotsuga menziesii*) to pitch canker, caused by *Gibberella circinata* (anamorph = *Fusarium circinatum*). Plant Pathology 55: 231-237.
25. Gordon, T.R., Kirkpatrick, S.C., Petersen, J.C. and Friel, C.J. 2006b. Potential diversity in vegetative compatibility groupings in the California population of

Gibberella circinata. Mycological Research 110: 936-940.

26. Hurley, B.P., Govender, P., Coutinho, T.A., Wingfield, B.D., Wingfield, M.J. 2007. Fungus gnats and other Diptera in South African forestry nurseries and their possible association with the pitch canker fungus. South African Journal of Science 103: 43-46.
27. Iturritxa, E., Ganley, R.J., Wright, J., Heppe, E., Steenkamp, E.T., Gordon, T.R., and Wingfield, M.J. 2011. A genetically homogenous population of *Fusarium circinatum* causes pitch canker of *Pinus radiata* in the Basque Country, Spain. Fungal Biology 115: 288-295.
28. Klittich, C.J.R., and Leslie, J.F. 1988. Nitrate reduction mutants of *Fusarium moniliforme (Gibberella fujikuroi*). Genetics 118:417-423.
29. Kosman, E. and Leonard, J. 2005. Similarity coefficient for molecular markers in studies of genetic relationships between individuals for haploid, diploid and polyploidy species. Molecular Ecology 14: 415-424.
30. Kumar, S., Tamura, K. and Nei, M. 1993. MEGA: Molecular evolutionary genetics analysis. Pennsylvania State University: University Park, PA.
31. Leslie, J.F. 1993. Fungal vegetative compatibility. Annual Review of Phytopathology 31: 127-51.
32. Leslie, J.F. 1995. *Gibberella fujikuroi*: available populations and variable traits. Canadian Journal of Botany 73: 282-295.
33. Leslie, J.F. and Klein, K.K. 1996. Female fertility and mating type effects on effective population size and evolution in filamentous fungi. Genetics 144: 557-567.
34. Leslie, J.F. and Summerelle, B.A. 2006. The *Fusarium* laboratory manual. Ames: Blackwell.
35. Loubradou, G. and Turcq, B. 2000. Vegetative incompatibility in filamentous fungi: a roundabout way of understanding the phenomenon. Research in Microbiology 151: 239-245.
36. Majer, D., Mithen, R., Lewis, B.G., Vos, P. and Oliver, R.P. 1996. The use of AFLP fingerprinting for the detection of genetic variation in fungi. Mycological Research 100: 1107-1111.
37. Matthews, F.R. 1962. Pitch canker tip moth damage associated on slash pine seedlings. Journal of Forestry 60: 825-826.
38. McCain, A.H., Koehler, C.S. and Tjosvold, S.A. 1987. Pitch canker threatens

California pines. California Agriculture 41: 22-23.

39. McDonald, B.A. and Linde, C. 2002. Pathogen population genetics and the durability of disease resistance. Euphytica 124: 163-180.
40. Micali, C.O. and Smith, M.L. 2003. On the independence of barrage formation and heterokaryon incompatibility in *Neurospora crassa*. Fungal Genetics and Biology 38: 209-219.
41. Möller, E.M., Bahnweg, G., Sandermann, H. and Geiger, H.H. 1992. A simple and efficient protocol for isolation of high molecular weight DNA from filamentous fungi, fruit bodies and infected plant tissues. Nucleic Acid Research 2 (22): 6115-6116.
42. Myburg, A.A., Remington, D.L., O'Malley, D.M., Sederoff, R.R. and Whetten, R.W. 2001. High throughput AFLP analysis using infrared dye labelled primers and an automated DNA sequencer. Biotechniques 30 (2): 348-352, 354, 356-357.
43. Nei, M. and Li, WH. 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. Proceedings of the National Academy of Sciences of the United States of America 76: 5269-5273.
44. Nelson, P.E., Toussoun, T.A. and Marasas, W.F.O. 1983. *Fusarium* species: an illustrated manual for identification. Pennyslavania State University Press, University Park.
45. Nirenberg, H.I. and O'Donelle, K. 1998. New *Fusarium* species and combinations with the *Gibberella fujikuroi* species complex. Mycologia 90: 434-458.
46. Ormsby, M. 2004. Report on the interception of *Fusarium circinatum* (Pitch Canker) on imported seedlings of Douglas fir (*Pseudotsuga menziesii*). http://www.biosecurity.govt.NZ/imports/forest/pine-pitch-canker-interception.
47. Perkins, D.D. 1988. Main features of vegetative incompatibility in *Neurospora*. Fungicide Genetics Newsletter 35: 44-46.
48. Petersen, J.C. and Gordon, T.R. 2005. Spontaneous changes in vegetative compatibility in ascomycete *Gibberella circinata*. Phytopathology 95: 583.
49. Puhalla, J.E. 1985. Classification of strains of *Fusarium oxysporum* on the basis of vegetative compatibility. Canadian Journal of Botany 63: 179-183.
50. Romón, P. Iturrondobeitia, J.C., Gibson, K., Lindgren, B.S. and Goldarazena, A. 2007. Quantitative association of bark beetles with pitch canker fungus and effects of verbenone on their semiochemical communication in Monterey pine forests in

northern Spain. Environmental Entomology 36: 743-750.

51. Roux, J., Eisenberg, B., Kanzler, A., Coetzee, V., Kietzks, E and Wingfield, M.J. 2007. Testing of selected South African *Pinus* hybrids and families for tolerance to the pitch canker pathogen, *Fusarium circinatum*. New Forests 33: 109-123.
52. Saitou, N. and Nei, M. 1987. The neighbor joining method: A new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4: 406-425.
53. Sambrook J., Fritsch, E.F. and Maniatis, T. 1989. Molecular cloning: a laboratory manual. Cold Springs Harbour Laboratory Press, New York.
54. Santana, Q.C., Coetzee, M.P.A., Steenkamp, E.T., Mlonyeni, O.X., Hammond, G.N.A., Wingfield, M.J. and Wingfield, B.D. 2009. Microsatellite discovery by deep sequencing of enriched genomic library. Bio Techniques 46: 217-223
55. Santos, J.J.G. and Tovar, D.B. 1991. Algunos aspectos sobre el cancro resinoso de los pinos. In 'VI Simposio Nacional Sobre Parasitologia Forestal, October 1991'. P. 31. (unidad de Xcongresos del Calegio de Posgraduados montecillos: Edo, Mexico).
56. Saupe, S., Turcq, B. and Begueret, J. 1995. A gene responsible for vegetative incompatibility in the fungus *Podospora anserina* encodes a protein with a GTP-binding motif and GB homologous domain. Gene 162: 135-139.
57. Saupe, S.J. and Glass, N.L. 1997. Allelic specificity at the *het-c* heterokaryon incompatibility locus of *Neurospora crassa* is determined by a high variable domain. Genetics 146:1299-1309.
58. Schweigkofler, W., O'Donnell, K. and Garbelotto, M. 2004. Detection and quantification of *Fusarim circinatum*, the casual agent of pine pitch canker, from two California sites by using a real-time PCR approach combined with a simple spore trapping method. Applied and Environmental Microbiology 70 (6): 3512-3520.
59. Sheldon, A.L. 1969. Equitability indices: dependence on the species count. Ecology 50: 466-467.
60. Steenkamp, E.T., Wingfield, B.D., Coutinho, T.A., Zeller, K.A., Wingfield, M.J., Marasas, W.F.O. and Leslie, J.F. 2000. PCR based identification of *MAT-1* and *MAT-2* in the *Giberrela fujikuroi* Species complex. Applied and Environmental Microbiology 66 (10): 4378-4382.
61. Storer, A.J., Gordon, T.R., Wood, D.L. and Dallara, P.L. 1995. Pitch canker in California. California Department of Forestry and Fire Protection. No. 97, Sacramento.

62. Viljoen, A., Wingfield, M.J. and Marasas, W.F.O. 1994. First report of *Fusarium subglutinans f. sp. Pini* on pine in South Africa. Plant Disease 78: 309-312.
63. Viljoen, A., Wingfield, M.J., Gordon, T.R. and Marasas, W.F.O. 1997a. Genotypic diversity in a South African population of pitch canker fungus *Fusarium subglutinas* f.sp. *pini*. Plant Pathology 46: 590-593.
64. Viljoen, A., Wingfield, M.J., Gordon, T.R. and Marasas, W.F.O. 1997b. Characterisation of *Fusarium subglutinas* f.sp. *pini* causing root rot disease of *Pinus patula* seedlings in South Africa. Mycological Research 101: 437-45.
65. Vos, P., Hogers, R., Bleeker, M., Reijans, M., van de Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M. and Zabeau, M. 1995. AFLP: a new technique for DNA fingerprinting. Nucleic Acids Research 23, 4407-4414.
66. Wikler, K. and Gordon, T. 2000. An initial assessment of *Fusaruim circinatum* in different parts of the world. Canadian Journal of Botany 78: 709-717.
67. Wingfield, M.J., Jacobs, A., Coutinho, T.A., Ahumada, R. and Wingfield, B.D. 2002. First report of the pitch canker fungus *Fusarium circinatum*, on pines in Chile. Plant Pathology 51: 397.
68. Wingfield, M.J., Hammerbacher, A., Ganley, R.J., Coutinho, T.A., Steenkamp, E.T., Gordon, T.R. and Wingfield, B.D. 2008. Pitch canker caused by *Fusarium circinatum*: a growing threat to pine plantations and forests worldwide. Australasian Plant Pathology 37: 319-333.
69. Yoshida, T., Arie, T., Nomura, Y., Yoder, O.C., Turgeon, B.G. and Yamaguchi, I. 1998. Cloning mating type genes of the asexual ascomycetes Fusarium oxysporum. Proceedings of the VIIth International Congress on Plant Pathology. International Congress of Plant Pathology, Edinburgh, Scotland.

Table 1. Western Cape *F. circinatum* isolates used in this study

FCC no. [a]	Isolate no. [b]	Geographic origin [c]	Host [d]	Reference	VCG [e]	Mating type [f]
	63	Tokai, WC	*P. radiata* (T)	This study	VCO1	Mat-1
	5	Tokai, WC	*P. radiata* (T)	This study	VCO1	Mat-1
	14	Tokai, WC	*P. radiata* (T)	This study	VCO4	Mat-1
	31	Tokai, WC	*P. radiata* (T)	This study	VCO1	Mat-1
	57	Tokai, WC	*P. radiata* (T)	This study	VCO1	Mat-1
	58	Tokai, WC	*P. radiata* (T)	This study	VCO1	Mat-1
	80	Tokai, WC	*P. radiata* (T)	This study	VCO1	Mat-1
	1-1	Tokai, WC	*P. radiata* (T)	This study	VCO1	Mat-1
	1-2	Tokai, WC	*P. radiata* (T)	This study	VCO1	Mat-1
	10-1i	Tokai, WC	*P. radiata* (T)*	This study	VCO1	Mat-1
	11-2	Tokai, WC	*P. radiata* (T)	This study	VCO1	Mat-1
	12-2i	Tokai, WC	*P. radiata* (T)*	This study	VCO3	Mat-1
	17-1i	Tokai, WC	*P. radiata* (T)*	This study	VCO1	Mat-1
	19-1i	Tokai, WC	*P. radiata* (T)*	This study	VCO4	Mat-1
	2-1	Tokai, WC	*P. radiata* (T)	This study	VCO1	Mat-1
	20-1	Tokai, WC	*P. radiata* (T)	This study	VCO1	Mat-1
	20-2i	Tokai, WC	*P. radiata* (T)*	This study	VCO1	Mat-1
	25-2	Tokai, WC	*P. radiata* (T)	This study	VCO31	Mat-1
	27-2	Tokai, WC	*P. radiata* (T)	This study	VCO1	Mat-1
	28-2	Tokai, WC	*P. radiata* (T)	This study	VCO2	Mat-1
	29-1i	Tokai, WC	*P. radiata* (T)*	This study	VCO1	Mat-1
	3-2	Tokai, WC	*P. radiata* (T)	This study	VCO1	Mat-1
	30-2i	Tokai, WC	*P. radiata* (T)*	This study	VCO1	Mat-1
	32-1	Tokai, WC	*P. radiata* (T)	This study	VCO4	Mat-1
	32-2	Tokai, WC	*P. radiata* (T)	This study	VCO1	Mat-1
	33-1	Tokai, WC	*P. radiata* (T)	This study	VCO1	Mat-1
	33-2	Tokai, WC	*P. radiata* (T)	This study	VCO1	Mat-1
	34-1	Tokai, WC	*P. radiata* (T)	This study	VCO1	Mat-1
	34-2	Tokai, WC	*P. radiata* (T)	This study	VCO1	Mat-1
	37-1	Tokai, WC	*P. radiata* (T)	This study	VCO1	Mat-1
	37-2	Tokai, WC	*P. radiata* (T)	This study	VCO5	Mat-1
	38-2	Tokai, WC	*P. radiata* (T)	This study	VCO3	Mat-1
	40-2	Tokai, WC	*P. radiata* (T)	This study	VCO1	Mat-1
	46-1	Tokai, WC	*P. radiata* (T)	This study	VCO1	Mat-1
	46-2i	Tokai, WC	*P. radiata* (T)	This study	VCO32	Mat-1
	47-1i	Tokai, WC	*P. radiata* (T)*	This study	VCO1	Mat-1
	47-2i	Tokai, WC	*P. radiata* (T)*	This study	VCO4	Mat-1
	19-1	Tokai, WC	*P. radiata* (T)	This study	VCO4	Mat-1
	48-1	Tokai, WC	*P. radiata* (T)	This study	VCO1	Mat-1
	48-2	Tokai, WC	*P. radiata* (T)	This study	VCO1	Mat-1
	50-1i	Tokai, WC	*P. radiata* (T)*	This study	VCO1	Mat-1
	49-1i	Tokai, WC	*P. radiata* (T)*	This study	VCO1	Mat-1
	54-1	Tokai, WC	*P. radiata* (T)	This study	VCO3	Mat-1

	54-2	Tokai, WC	*P. radiata* (T)	This study	VCO3	Mat-1
	59-1	Tokai, WC	*P. radiata* (T)	This study	VCO5	Mat-1
	59-2	Tokai, WC	*P. radiata* (T)	This study	VCO5	Mat-1
	6-1i	Tokai, WC	*P. radiata* (T)*	This study	VCO1	Mat-1
	6-2i	Tokai, WC	*P. radiata* (T)*	This study	VCO1	Mat-1
	64-1	Tokai, WC	*P. radiata* (T)	This study	VCO1	Mat-1
	64-2	Tokai, WC	*P. radiata* (T)	This study	VCO1	Mat-1
	65-1	Tokai, WC	*P. radiata* (T)	This study	VCO1	Mat-1
	65-2	Tokai, WC	*P. radiata* (T)	This study	VCO1	Mat-1
	69-2	Tokai, WC	*P. radiata* (T)	This study	VCO1	Mat-1
	7-1i	Tokai, WC	*P. radiata* (T)*	This study	VCO33	Mat-1
	72-1	Tokai, WC	*P. radiata* (T)	This study	VCO1	Mat-1
	74-1	Tokai, WC	*P. radiata* (T)	This study	VCO1	Mat-1
	8-1i	Tokai, WC	*P. radiata* (T)*	This study	VCO1	Mat-1
	8-2i	Tokai, WC	*P. radiata* (T)*	This study	VCO1	Mat-1
	9-2i	Tokai, WC	*P. radiata* (T)*	This study	VCO1	Mat-1
	9-1i	Tokai, WC	*P. radiata* (T)*	This study	VCO1	Mat-1
FCC5051	43-2	Tokai, WC	*P. radiata* (T)	Coutinho *et al.*, 2007	VCO1	Mat-1
FCC5052	50-2i	Tokai, WC	*P. radiata* (T)	Coutinho *et al.*, 2007	VCO1	Mat-1
	Ins 2	Tokai, WC	*P. radiata* (S)	This study	VCO36	Mat-2
	Ins 3	Tokai, WC	*P. radiata* (T)	This study	VCO37	Mat-2
	ks1	Nursery, WC	*P. radiata* (T)	This study	VCO6	Mat-2
	ks2	Nursery, WC	*P. radiata* (S)	This study	VCO6	Mat-2
	ks3	Nursery, WC	*P. radiata* (S)	This study	VCO6	Mat-2
	ks4	Nursery, WC	*P. radiata* (S)	This study	VCO7	Mat-2
	ks5	Nursery, WC	*P. radiata* (S)	This study	VCO6	Mat-2
	ks6	Nursery, WC	*P. radiata* (S)	This study	VCO7	Mat-2
	ks7	Nursery, WC	*P. radiata* (S)	This study	VCO6	Mat-2
	ks8	Nursery, WC	*P. radiata* (S)	This study	VCO6	Mat-2
	ks9	Nursery, WC	*P. radiata* (S)	This study	VCO7	Mat-2
	ks10	Nursery, WC	*P. radiata* (S)	This study	VCO7	Mat-2
	ks11	Nursery, WC	*P. radiata* (S)	This study	VCO7	Mat-2
	ks12	Nursery, WC	*P. radiata* (S)	This study	VCO6	Mat-2
	ks13	Nursery, WC	*P. radiata* (S)	This study	VCO6	Mat-2
	ks14	Nursery, WC	*P. radiata* (S)	This study	VCO6	Mat-2
	ks15	Nursery, WC	*P. radiata* (S)	This study	VCO6	Mat-2
	ks16	Nursery, WC	*P. radiata* (S)	This study	VCO6	Mat-2
	ks17	Nursery, WC	*P. radiata* (S)	This study	VCO6	Mat-2
	ks18	Nursery, WC	*P. radiata* (S)	This study	VCO6	Mat-2
	ks19	Nursery, WC	*P. radiata* (S)	This study	VCO7	Mat-2
	ks20	Nursery, WC	*P. radiata* (S)	This study	VCO7	Mat-2
	ks21	Nursery, WC	*P. radiata* (S)	This study	VCO21	Mat-2
	ks22	Nursery, WC	*P. radiata* (S)	This study	VCO8	Mat-2
	ks23	Nursery, WC	*P. radiata* (S)	This study	VCO8	Mat-1
	ks24	Nursery, WC	*P. radiata* (S)	This study	VCO8	Mat-1
	ks25	Nursery, WC	*P. radiata* (S)	This study	VCO8	Mat-1

	ks26	Nursery, WC	*P. radiata* (S)	This study	VCO6	Mat-1
	ks27	Nursery, WC	*P. radiata* (S)	This study	VCO8	Mat-2
	ks28	Nursery, WC	*P. radiata* (S)	This study	VCO8	Mat-1
	ks39	Nursery, WC	*P. radiata* (S)	This study	VCO22	Mat-1
	ks40	Nursery, WC	*P. radiata* (S)	This study	VCO23	Mat-1
	ks41	Nursery, WC	*P. radiata* (S)	This study	VCO24	Mat-1
	ks43	Nursery, WC	*P. radiata* (S)	This study	VCO25	Mat-1
	ks44	Nursery, WC	*P. radiata* (S)	This study	VCO26	Mat-1
	ks47	Nursery, WC	*P. radiata* (S)	This study	VCO5	Mat-1
	ks48	Nursery, WC	*P. radiata* (S)	This study	VCO5	Mat-1
	ks49	Nursery, WC	*P. radiata* (S)	This study	VCO2	Mat-1
	ks50	Nursery, WC	*P. radiata* (S)	This study	VCO5	Mat-1
	ks51	Nursery, WC	*P. radiata* (S)	This study	VCO27	Mat-1
	ks52	Nursery, WC	*P. radiata* (S)	This study	VCO28	Mat-1
	ks53	Nursery, WC	*P. radiata* (S)	This study	VCO2	Mat-1
	ks54	Nursery, WC	*P. radiata* (S)	This study	VCO5	Mat-1
	ks55	Nursery, WC	*P. radiata* (S)	This study	VCO2	Mat-1
	ks56	Nursery, WC	*P. radiata* (S)	This study	VCO5	Mat-1
	ks57	Nursery, WC	*P. radiata* (S)	This study	VCO6	Mat-1
	ks58	Nursery, WC	*P. radiata* (S)	This study	VCO5	Mat-1
	ks59	Nursery, WC	*P. radiata* (S)	This study	VCO5	Mat-1
	ks61	Nursery, WC	*P. radiata* (S)	This study	VCO2	Mat-1
	ks62	Nursery, WC	*P. radiata* (S)	This study	VCO5	Mat-1
	ks73	Nursery, WC	*P. radiata* (S)	This study	VCO2	Mat-1
	ks76	Nursery, WC	*P. radiata* (S)	This study	VCO2	Mat-1
	ks77	Nursery, WC	*P. radiata* (S)	This study	VCO2	Mat-1
	ks78	Nursery, WC	*P. radiata* (S)	This study	VCO5	Mat-1
	ks79	Nursery, WC	*P. radiata* (S)	This study	VCO2	Mat-1
	ks80	Nursery, WC	*P. radiata* (S)	This study	VCO2	Mat-1
	ks81	Nursery, WC	*P. radiata* (S)	This study	VCO29	Mat-1
	ks82	Nursery, WC	*P. radiata* (S)	This study	VCO5	Mat-1
	ks83	Nursery, WC	*P. radiata* (S)	This study	VCO5	Mat-1
	ks84	Nursery, WC	*P. radiata* (S)	This study	VCO5	Mat-1
	ks85	Nursery, WC	*P. radiata* (S)	This study	VCO5	Mat-1
	ks86	Nursery, WC	*P. radiata* (S)	This study	VCO5	Mat-1
	ks87	Nursery, WC	*P. radiata* (S)	This study	VCO30	Mat-1
	ks88	Nursery, WC	*P. radiata* (S)	This study	VCO5	Mat-1
	ks89	Nursery, WC	*P. radiata* (S)	This study	VCO5	Mat-1
FCC1844		Nursery, WC	*P. radiata* (S)	This study	VCO5	Mat-1
FCC1845		Nursery, WC	*P. radiata* (S)	This study	VCO5	Mat-1
FCC1928		Nursery, WC	*P. radiata* (S)	This study	VCO38	Mat-2
FCC1985		Nursery, WC	*P. radiata* (S)	This study	VCO39	Mat-1
FCC2154		Nursery, WC	*P. radiata* (S)	This study	VCO40	Mat-1
FCC2155		Nursery, WC	*P. radiata* (S)	This study	VCO41	Mat-1
	ks90	Nursery, WC	*P. radiata* (S)	This study	VCO5	Mat-1
	WT (1)	George, WC	*P. radiata* (T)	This study	VCO19	Mat-1

L53A9	George, WC	*P. radiata* (T)	This study	VCO11	Mat-1
L53A8	George, WC	*P. radiata* (T)	This study	VCO9	Mat-1
CBH12	George, WC	*P. radiata* (T)	This study	VCO10	Mat-1
B1-6	George, WC	*P. radiata* (T)	This study	VCO9	Mat-1
L53A	George, WC	*P. radiata* (T)	This study	VCO11	Mat-1
CBH11	George, WC	*P. radiata* (T)	This study	VCO16	Mat-1
B1-5	George, WC	*P. radiata* (T)	This study	VCO9	Mat-1
O6	George, WC	*P. radiata* (T)	This study	VCO11	Mat-1
BG-2	George, WC	*P. radiata* (T)	This study	CCO15	Mat-1
CBH15	George, WC	*P. radiata* (T)	This study	VCO5	Mat-1
CBH19	George, WC	*P. radiata* (T)	This study	VCO17	Mat-1
B2H1	George, WC	*P. radiata* (T)	This study	VCO13	Mat-1
CBH18	George, WC	*P. radiata* (T)	This study	VCO3	Mat-1
B1-8	George, WC	*P. radiata* (T)	This study	VCO9	Mat-1
L53A7	George, WC	*P. radiata* (T)	This study	VCO11	Mat-1
L53S	George, WC	*P. radiata* (T)	This study	VCO11	Mat-1
B1-7	George, WC	*P. radiata* (T)	This study	VCO9	Mat-1
B2H6	George, WC	*P. radiata* (T)	This study	VCO14	Mat-1
O19	George, WC	*P. radiata* (T)	This study	VCO9	Mat-1
L53A5	George, WC	*P. radiata* (T)	This study	VCO9	Mat-1
L53A6	George, WC	*P. radiata* (T)	This study	VCO11	Mat-1
B1-10	George, WC	*P. radiata* (T)	This study	VCO12	Mat-1
CBH10	George, WC	*P. radiata* (T)	This study	VCO5	Mat-1
CBHA	George, WC	*P. radiata* (T)	This study	VCO5	Mat-1
B2H3	George, WC	*P. radiata* (T)	This study	VCO7	Mat-1
B2H2	George, WC	*P. radiata* (T)	This study	VCO1	Mat-1
CBHA1	George, WC	*P. radiata* (T)	This study	VCO5	Mat-1
L53H	George, WC	*P. radiata* (T)	This study	VCO11	Mat-1
L53H2	George, WC	*P. radiata* (T)	This study	VC011	Mat-1
CBH13	George, WC	*P. radiata* (T)	This study	VCO5	Mat-1
L53A4	George, WC	*P. radiata* (T)	This study	VCO11	Mat-1
B2H5	George, WC	*P. radiata* (T)	This study	VCO10	Mat-1
L53A3	George, WC	*P. radiata* (T)	This study	VCO11	Mat-1
WT1(2)	George, WC	*P. radiata* (T)	This study	VCO20	Mat-1
L53A2	George, WC	*P. radiata* (T)	This study	VCO9	Mat-1
L53A1	George, WC	*P. radiata* (T)	This study	VCO18	Mat-1

[a] FCC: *Fusarium* Culture Collection at FABI, University of Pretoria, South Africa.

[b] CMW: Culture Collection Mike Wingfield, FABI, University of Pretoria, South Africa; The isolates from Tokai were collected by E.T. Steenkamp, T.A. Coutinho and M. J. Wingfield, those from the Western Cape nursery were collected by B. Porter and the isolates from the George area were collected by J.Roux, M. Kvas and O. M. Mashandule.

[c] WC = Western Cape, RSA.

[d] Isolates obtained from mature trees or established plantation trees are indicated with (T) and those obtained from seedlings are indicated with (S). Those isolated from the galleries of *Pissodes nemorensis* are indicated with an asterisk.

[e] The VCGs of the Western Cape isolates were determined using the strategy described by Correll *et al*. (1987).

[f] Mating types were determined by PCR using the primer sets developed by Steenkamp *et al*. (2000).

Table 2. VCGs from South Africa, USA and Mexico

Isolate no.	VCG	Geographic origin	Reference
F25-9	VCG25	South Africa	Britz *et al*., 2005
F2-34	VCG29	South Africa	Britz *et al*., 2005
KK38	VCG26	South Africa	Britz *et al*., 2005
KK2	VCG27	South Africa	Britz *et al*., 2005
KK35	VCG28	South Africa	Britz *et al*., 2005
KK41	VCG24	South Africa	Britz *et al*., 2005
K014	VCG18	South Africa	Viljoen *et al*., 1997
K057	VCG8	South Africa	Viljoen *et al*., 1997
K030	VCG12	South Africa	Viljoen *et al*., 1997
K020	VCG4	South Africa	Viljoen *et al*., 1997
K203	VCG20	South Africa	Viljoen *et al*., 1997
K042	VCG3	South Africa	Viljoen *et al*., 1997
K208	VCG21	South Africa	Viljoen *et al*., 1997
K211	VCG22	South Africa	Viljoen *et al*., 1997
K165	VCG11	South Africa	Viljoen *et al*., 1997
K160	VCG17	South Africa	Viljoen *et al*., 1997
K153	VCG19	South Africa	Viljoen *et al*., 1997
K059	VCG15	South Africa	Viljoen *et al*., 1997
K049	VCG13	South Africa	Viljoen *et al*., 1997
K047	VCG9	South Africa	Viljoen *et al*., 1997
K031	VCG7	South Africa	Viljoen *et al*., 1997
K043	VCG10	South Africa	Viljoen *et al*., 1997
K004	VCG1	South Africa	Viljoen *et al*., 1997
K026	VCG14	South Africa	Viljoen *et al*., 1997
K016	VCG6	South Africa	Viljoen *et al*., 1997
K035	VCG5	South Africa	Viljoen *et al*., 1997
K037	VCG2	South Africa	Viljoen *et al*., 1997
K161	VCG16	South Africa	Viljoen *et al*., 1997
A362	C2	California	Wikler and Gordon, 2000
FSP118	C6	California	Wikler and Gordon, 2000
FSP124	C1	California	Wikler and Gordon, 2000
FSP255	C9	California	Wikler and Gordon, 2000
FSP360	C7	California	Wikler and Gordon, 2000
FSP39	C4	California	Wikler and Gordon, 2000
FSP455	C10	California	Wikler and Gordon, 2000
FSP62	C8	California	Wikler and Gordon, 2000
LA4	C5	California	Wikler and Gordon, 2000
SK2	C3	California	Wikler and Gordon, 2000
FL19	SE10	Florida	Wikler and Gordon, 2000
FL123	SE12	Florida	Wikler and Gordon, 2000
FL124	SE19	Florida	Wikler and Gordon, 2000
FL1	SE2	Florida	Wikler and Gordon, 2000
FL52	SE21	Florida	Wikler and Gordon, 2000

FL88	SE22	Florida	Wikler and Gordon, 2000
FL102	SE24	Florida	Wikler and Gordon, 2000
FL101	SE27	Florida	Wikler and Gordon, 2000
FL129	SE28	Florida	Wikler and Gordon, 2000
FL108	SE29	Florida	Wikler and Gordon, 2000
FL110	SE30	Florida	Wikler and Gordon, 2000
FL130	SE32	Florida	Wikler and Gordon, 2000
FL122	SE33	Florida	Wikler and Gordon, 2000
FL91	SE34	Florida	Wikler and Gordon, 2000
FL118	SE35	Florida	Wikler and Gordon, 2000
FL76	SE38	Florida	Wikler and Gordon, 2000
FL127	SE41	Florida	Wikler and Gordon, 2000
FL140	SE40	Florida	Wikler and Gordon, 2000
FL11	SE5	Florida	Wikler and Gordon, 2000
FL72	SE6	Florida	Wikler and Gordon, 2000
FL15	SE7	Florida	Wikler and Gordon, 2000
FL3	SE9	Florida	Wikler and Gordon, 2000
FL10	SE1	Florida	Wikler and Gordon, 2000
FL125	SE31	Florida	Wikler and Gordon, 2000
FL106	SE39	Florida	Wikler and Gordon, 2000
CMW23564	M5	Mexico	Wikler and Gordon, 2000
TEO1	M1	Mexico	Wikler and Gordon, 2000
TEO3	M2	Mexico	Wikler and Gordon, 2000
LJ	M3	Mexico	Wikler and Gordon, 2000
CMW23550	M4	Mexico	Wikler and Gordon, 2000
JAL03	M6	Mexico	Wikler and Gordon, 2000
HGO05	M7	Mexico	Wikler and Gordon, 2000

Table 3. Genetic diversity associated with the *F. circinatum* isolates obtained from the Western Cape based on VCG

***F. circinatum* population**	**No. of isolates (*N*)[a]**	**Vegetative Compatibility**			
		No. of VCGs *S*[b]	***(S/N)*[c]**	***H*[d]**	***H_{EM}*[e]**
Tokai	64	9	0.14	1.01	0.24
George	37	17	0.46	1.75	0.49
Commercial Pine Nursery	70	19	0.27	1.72	0.40
Tokai + George	101	26	0.26	1.68	0.37
Tokai + George + nursery	171	38	0.22	2.18	0.43

[a] Number of VCGs in the Western Cape

[b]Sample size at each location

[c]Number of VCGs divided by sample size

[d]Shannon diversity index (Sheldon, 1969)

[e]Sheldon index (Sheldon, 1969)

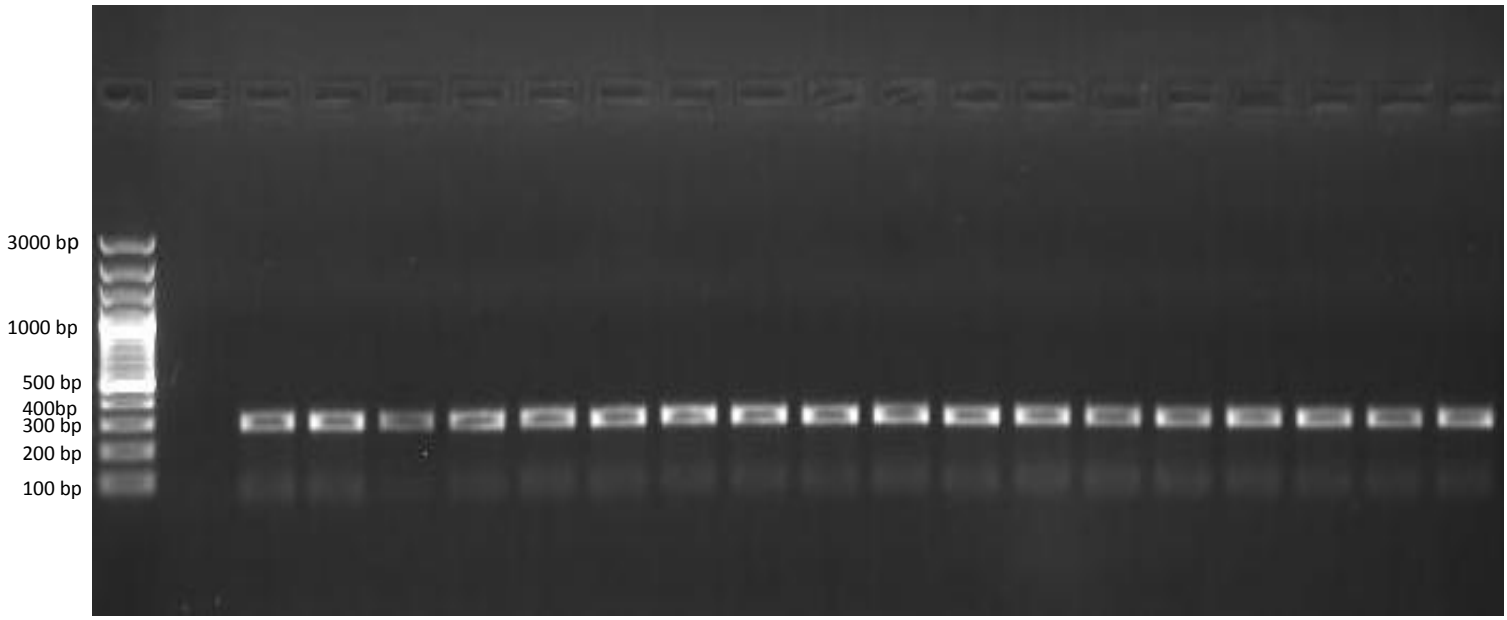

Figure 1. A 1% agarose gel showing the diagnostic 360bp DNA fragment amplified with the primers CIRC1A and CIRC4A (Schweigkofler *et al.* 2004) from *F. circinatum* isolates only.

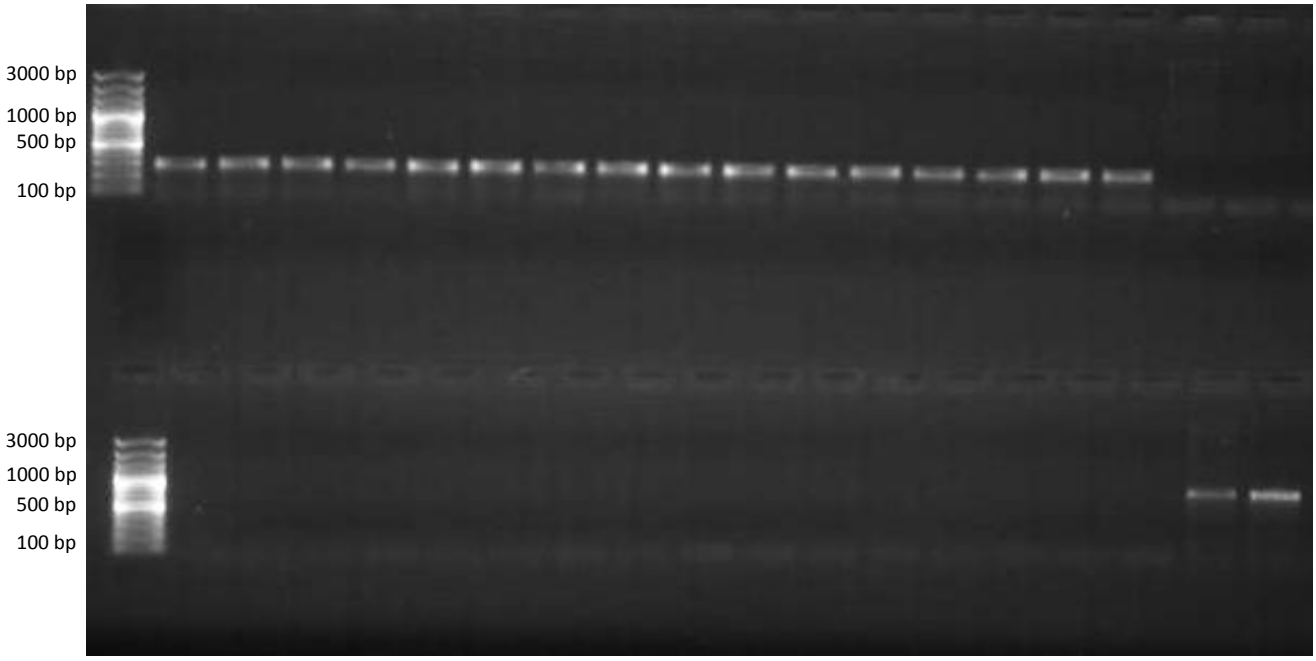

Figure 2. A 1% agarose gel indicating the PCR amplification of the *mat 2* gene (800 bp fragment) and *mat 1* gene (200bp fragment) (Steenkamp *et al*., 2000).

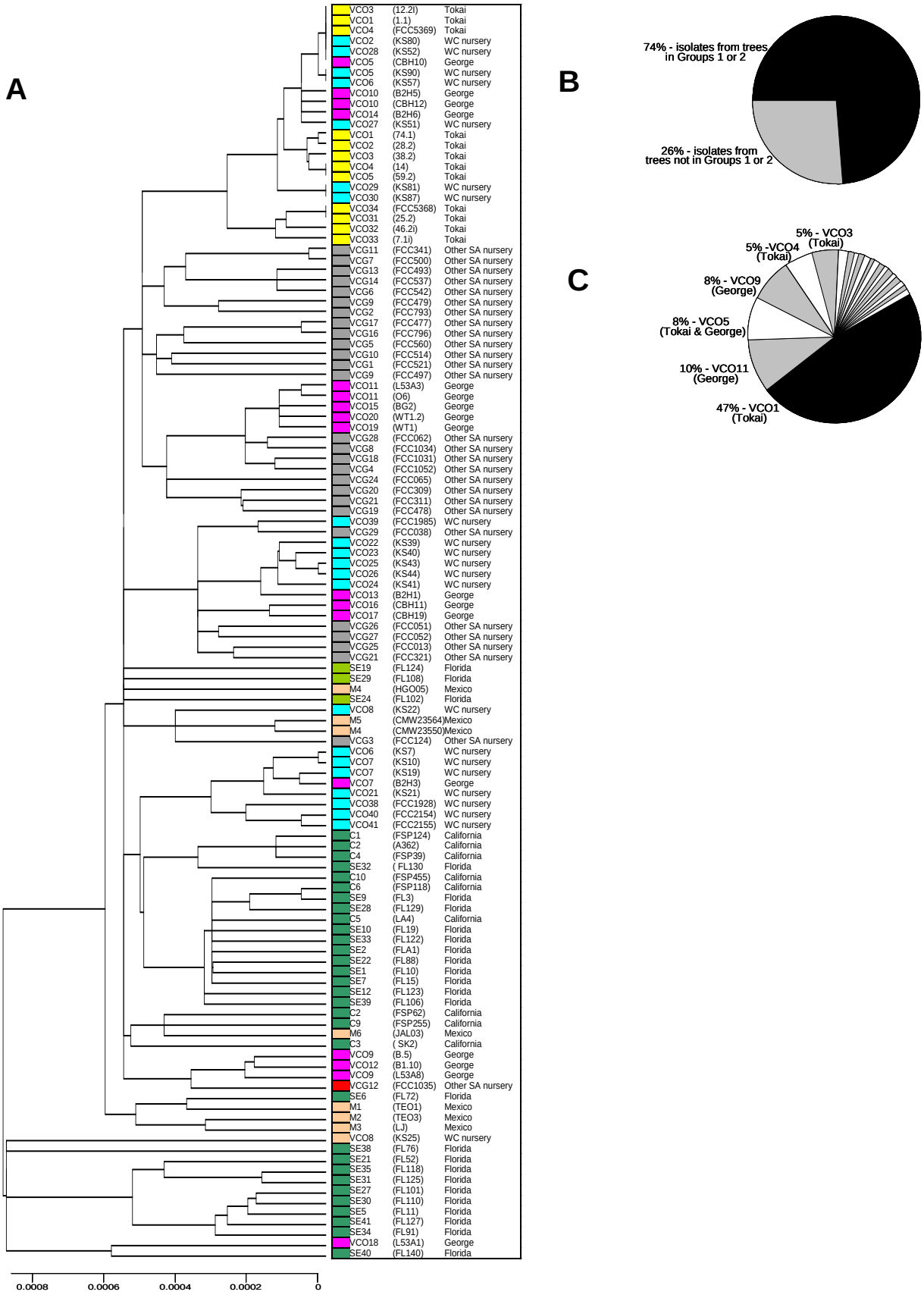

Figure 3. A: A dendogram indicating the grouping of Western Cape VCGs with known VCGs from other parts of the world. B: A pie chart indicating the grouping of isolates obtained from plantation trees in the Western Cape. C: A pie chart indicating the grouping of isolates into VCO1 to VCO5.

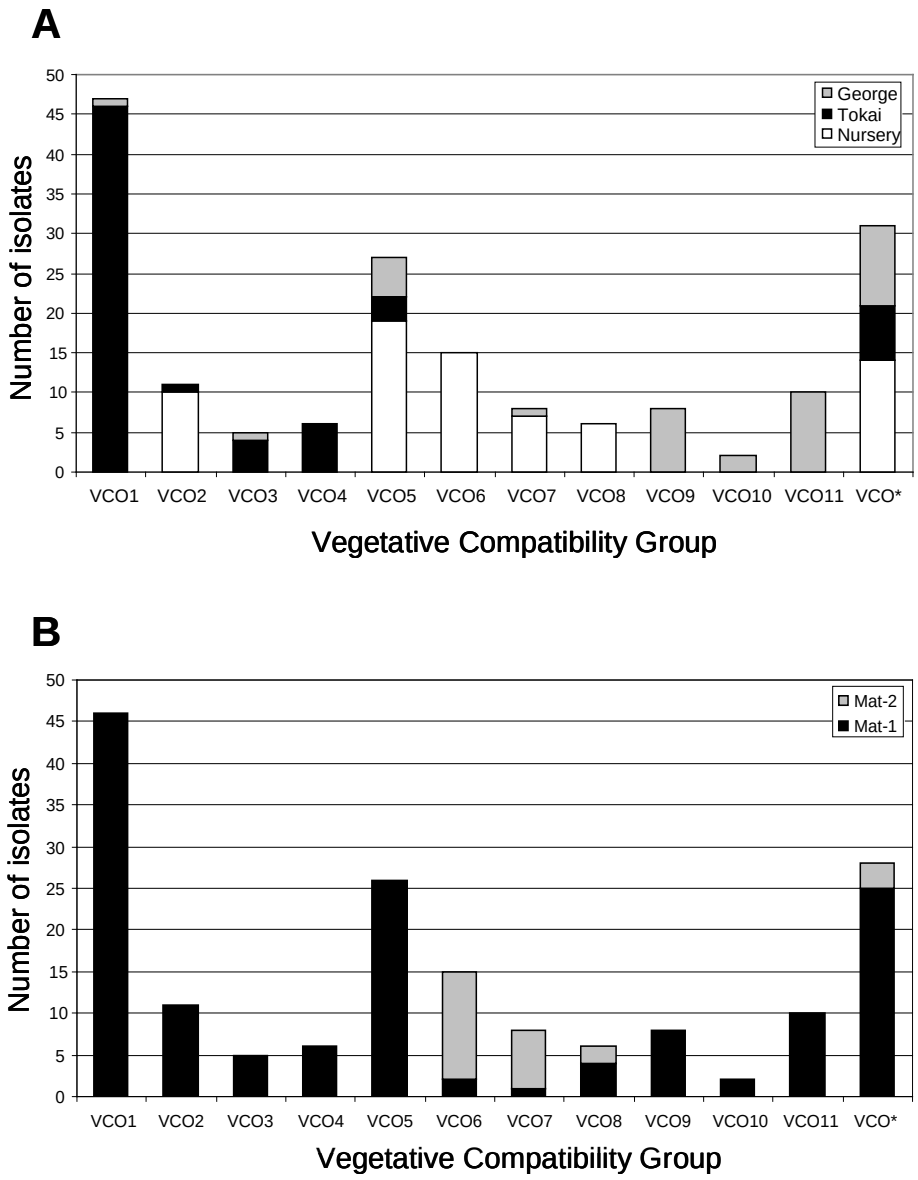

Figure 4: A: Western Cape VCG groups. B: Mating type distribution within the Western Cape isolates. * indicates VCO12-VCO33, VCO38-VCO41.

CHAPTER 3

Mating type segregation ratio distortion in *Fusarium circinatum*

O.M. MASHANDULE, T.A. COUTINHO, M.J. WINGFIELD and E.T. STEENKAMP*

Department of Microbiology and Plant Pathology, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria 0002

*Corresponding author. Email: emma.steenkamp@up.ac.za

Abstract

The pine pitch canker pathogen, *Fusarium circinatum*, is a heterothallic ascomycetous fungus that can reproduce sexually and asexually. Sexual reproduction is governed by a typical one-locus, two-allele mating type system. Therefore, mating is restricted to individuals that have opposite alleles, or idiomorphs (*mat 1* and *mat 2*) at their mating type locus (*mat*). Progeny that arise from a sexual interaction between two individuals carry either the *mat 1* or *mat 2* idiomorph. Clearly defined male/female roles are a pre-requisite in *F. circinatum* for sexual reproduction to take place and one of the isolates involved in such an interaction must thus be female-fertile (i.e. hermaphrodite). For this reason, sexual reproduction plays a crucial role in the maintenance of hermaphrodites in populations of *F. circinatum*. The aim of this study was to determine factors that contribute to mating type segregation in *F. circinatum*. The results showed that the mating type locus was severely affected by segregation distortion. This was evident in four crosses where the progeny did not segregate in the expected Medelian 1:1 ratio. No obvious association between mating type and pathogenicity or mating type and growth rate in culture was found. The cause of this distortion in *F. circinatum* remains unclear but it may be due to fitness-associated properties linked to mating type other than those considered in this study.

Keywords: Mating type, *Fusarium circinatum*

Introduction

Fusarium circinatum Nirenberg and O'Donnell (1998) (teleomorph = *Gibberella circinata*) is the causal agent of pitch canker. Pitch canker is a disease of pine (Wingfield *et al*., 2008) and Douglas fir (*Pseudotsuga menziesii*) (Gordon *et al*., 2006) that has resulted in significant economic losses in different parts of the world (Wingfield *et al*., 2008). Subsequent to its initial discovery in the south eastern United States in 1946 (Hepting and Roth, 1946), the disease has been reported from many parts of the world. These include the United States (McCain *et al*., 1987), Chile (Wingfield *et al.,* 2002), Haiti (Hepting and Roth, 1953), Italy (Carlucci *et al*., 2007), Japan (Muramoto and Dwinell, 1990), Mexico (Santos and Tovar, 1991), South Africa (Viljoen *et al*., 1994), Spain (Dwinell *et al.*, 2001; Landeras *et a*l., 2005), Korea (Lee *et al*., 2000), France (EPPO 2004), Uruguay (Alonso and Bettucci, 2009), Portugal (Braganca *et al*., 2009) and Colombia (Steenkamp *et al*., 2012).

Fusarium circinatum is a heterothallic member of the Ascomycota and has a typical one-locus, two-allele mating type system (Yun *et al.,* 2000). Sexual reproduction occurs only between individuals that have opposite alleles, or idiomorphs (mat *1* and *mat 2*) at their mating type locus (*mat*) (Coppin *et al*., 1997). However, other members of the phylum such as *Gibberella zeae* and *Aspergillus nidulans*, have both mating types in a single nucleus, and are considered as truly homothallic (Yun *et al*., 2000; Leslie and Summerell, 2006). Unlike *F. circinatum*, these ascomycetes are able to undergo self-fertilization, although they can also outcross (Leslie and Summerell, 2006). Another group, known as pseudohomothallic fungi (e.g. *Neurospora tetrasperma*), share mating characteristics with both the heterothallic and true homothallic fungi (Leslie and Summerell, 2006). They require both mating types for sexual reproduction to occur and progeny arises from a single spore (Leslie and Summerell, 2006). This is achieved by their ability to grow as heterokaryons, which gives rise to a spore that has two nuclei of opposite mating type (Gallegos *et al*., 2000; Jacobson, 2005).

The progeny produced by heterothallic species carry either the *mat 1* or *mat 2* idiomorph (Yun *et al.,* 2000). The proportions at which they occur are determined by

typical Mendelian genetics, which suggests that the two mating types will segregate among the ascospores in a 1:1 ratio (Leslie and Summerell, 2006). This is different from the situation with homothallic and pseudohomothallic fungi, where individual ascospores carry elements of both *mat* idiomorphs (Gallegos *et al*., 2000; Jacobson, 2005; Leslie and Summerell, 2006).

Clearly defined male/female roles are a pre-requisite for sexual reproduction to occur in a population of *F. circinatum* (Leslie and Summerell, 2006), which is a common trait among heterothallic Ascomycota (Coppin *et al*., 1997; Turgeon, 1998). Female strains are distinguished by their ability to form fruiting structures with viable ascospores after fertilization and male strains produce gametes that fertilize the female strains (Coppin *et al*., 1997). In *Fusarium*, all isolates are considered to be able to act as males, while only some isolates are able to act as females, hence the latter are also referred to as hermaphrodites (Nauta and Hoekstra, 1992; Leslie and Klein, 1996).

When *F. circinatum* was first found in South Africa, it occurred in a pine nursery and it remained restricted to nurseries for many years (Viljoen *et al*., 1994). In 2006, it was found to also cause pitch canker on established plantation trees in the Western Cape of South Africa (Coutinho *et al*., 2007) and the disease has subsequently appeared in plantations in many parts of the country. However, the mating type distribution of the Western Cape population (Chapter 2 of this dissertation) of the fungus is skewed towards *mat 1*. This could be due to the absence of female fertile isolates or that asexual propagation of only a few isolates with the same mating type is occurring. However, the possibility that this skewed distribution is a result of anomalies during or immediately after meiosis cannot be discounted. The aim of this study was, therefore, to investigate the apparent skewed distribution in mating type segregation by making use of laboratory crosses and to determine whether mating type is linked to fitness attributes such as mycelial growth rate and pathogenicity.

Materials and methods

Fungal isolates

A set of 30 *F. circinatum* isolates were used in this study (Table 1). Seven of these were obtained from *Pinus radiata* in the Western Cape (South Africa) during a previous study (Chapter 2 of this dissertation). These isolates were specifically selected based on the fact that they were able to form perithecia during sexual reproduction. Twenty isolates where obtained from a previous study (Viljoen *et al*., 1997; Britz *et al*., 2005), including the known mating type tester strains (MRC 6213 and MRC 7488) for this fungus (Britz *et al*., 1998).

Sexual crosses

Isolates of opposite mating type were used as males and females in crosses (Table 1) on carrot agar as described previously (Chapter 2 of this dissertation; Klittich and Leslie, 1988). After perithecium development, single-ascospore cultures were obtained by transferring an ascospore cirrhus to 100 µl sterile distilled water. The diluted ascospore suspensions were then plated onto 20% (w/v) water agar (Merck, South Africa) and incubated at room temperature for 18-24 hours to allow germination. Each single germinating spore was picked up using a sterile needle and placed on half strength potato dextrose agar (PDA) (Merck, South Africa).

In addition, two perithecia from each of the 22 crosses (Table 2) were crushed on microscope slides using cover slips. This was done in order to count the number of ascospores in each ascus.

Progeny harvested from cross “MRC 6312 X MRC 7488” were backcrossed to the parental strains to further understand segregation distortion in *F. circinatum*. These progeny were randomly chosen, therefore, F1 Mat-1 progeny (Progeny 45 and 15) were backcrossed to MRC 6213 and F1 Mat-2 progeny (Progeny 7 and 9) were backcrossed to MRC 7488.

Mating type assignment

The mating type of the progeny was determined by using the diagnostic PCR primers MAT 1a and MAT 1b for the amplification of *mat 1* idiomorph, and primers MAT 2c and MAT 2d for the amplification of *mat 2* idiomorph (Steenkamp *et al*., 2000). These PCRs were performed with genomic DNA extracted (Möller *et al*., 1992) from each progeny culture. The PCR mixture included 25 mM $MgCl_2$, 2.5 mM of each dNTPs, 10 µM of each *mat 1* or *mat 2* primer, 5 U/µl of *Taq* DNA polymerase and reaction buffer (Roche), and *ca*. 50 ng/µl of DNA template. The reaction conditions were denaturation at 94°C for 30 seconds followed by 35 cycles of 94°C for 30 seconds, 65°C for 45 seconds and 72°C for 30 seconds, and a final extension at 72°C for 10 minutes. The PCR products were visualized using agarose gel electrophoresis, ethidium bromide (0.2 µg/ml) staining and a UV transilluminator (Sambrook *et al*., 1989).

Vegetative compatibility tests

Vegetative compatibility assays as described before (Chapter 2 of this dissertation; Correll *et al*. 1987) were used to confirm that the progeny from four crosses (i.e. MRC 6217 X FCC 521, MRC 6312 X MRC 7488, Insect 3 X 39-1 and ks45 X ks15) were recombinant and not mitotic representatives of the parents used in the crosses. For this purpose, isolates were grown on complete medium (Correll *et al*., 1987) for four days at 25°C. Small pieces of agar were transferred from complete medium to Petri dishes (90 X 15 mm) containing 1.5 % chlorate medium (Correll *et al*., 1987). Each isolate was then placed on three chlorate plates and incubated at 25°C for seven days to generate as many *nit* mutants as possible. Isolates that grew as thin sectors were considered to be mutants. These mutants were screened by growing them on a minimal medium containing $NaNO_3$ (sodium nitrate) as the sole nitrogen source (Correll *et al*., 1987). Using minimal medium that was supplemented with either $NaNO_2$ (sodium nitrite), $NaNO_3$, NH_4 (ammonium) or hypoxanthine as sole nitrogen source, allowed the classification of *nit* mutants into three groups (*nit 1*, *nit 3* and *nit M*). These mutants were then used in genetic complementation studies to detect the formation of heterokaryons, as described before (Chapter 2 of this dissertation; Correll *et al*., 1987). In all cases, compatible *nit* mutants of an isolate were also complemented to rule out the possibility of self-incompatibility (Correll *et al*., 1987).

Growth study

The progeny of two crosses (i.e, MRC 6217 X FCC 521 and MRC 6213 X MRC 7488) were used to test whether isolates representing the two mating types differed in terms of colony diameter. Two replicates of each of these isolates were grown on two PDA (3.9% (w/v) PDA, Merck, South Africa) at temperatures of 10°C, 15°C, 20°C, 25°C, 30°C and 35°C. The colony diameter of the isolates was measured every second day during incubation for a period of six days. These measurements were taken using a vernier caliper with two readings taken for each isolate (vertical and horizontal across the middle of a colony diameter). An average of the two values was used as a final value.

Pathogenicity study

Progeny of two crosses, (i.e, MRC 6217 X FCC 521 and MRC 6213 X MRC 7488), were used in an inoculation study to determine if there were any differences in the pathogenicity of isolates representing the two mating types. Nine *mat 2* and 10 *mat 1* progeny isolates of the cross "MRC 6217 X FCC 521" were used. Five *mat 2* and 3 *mat 1* progeny isolates of the cross "MRC 6213 X MRC 7488" were used in this trial. For each of these isolates, inoculum was prepared by growing each of the progeny isolates in Petri dishes containing PDA medium. Thereafter, a spore suspension was collected from each Petri dish by pipetting 2ml of sterile distilled water onto the plate and retrieving this water after shaking. A haemocytometer was used to count the number of spores per millilitre.

For the pathogenicity tests, *P. radiata* seedlings were used. The apical bud of each seedling was removed using sterile shearing scissors. On the wound, 10 µl of the inoculum (50000 spore/ml) was placed using a sterile pipette. For each isolate, 10 seedlings were inoculated. For the control treatment, healthy seedlings of *P. radiata* were inoculated with sterile distilled water. The pathogenicity trial was repeated once under the same conditions. After plants were inoculated, they were watered and monitored twice a week. After eight weeks of incubation in a greenhouse at the University of Pretoria's experimental farm, a ruler was used to measure the lesion lengths on all seedlings.

To confirm that the symptoms observed where caused by *F. circinatum*, infected tissue from the stems of four seedlings (chosen randomly) representing each progeny isolate were cut into small pieces. These pieces where then plated onto half strength potato dextrose agar (PDA, Merck, South Africa) and Petri dishes where incubated for seven days at 25°C under a cool white fluorescent light. Pure cultures were obtained by inoculating single germinating conidia onto fresh PDA (Leslie and Summerell, 2006). All the re-isolated fungi where subjected to diagnostic PCR described by Schweigkofler *et al.* (2004), following a genomic DNA extraction described by Möller *et al*. (1992).

Statistical analyses

Chi-square tests were used to determine if the mating type of the progeny segregated in a Mendelian fashion. The chi-square statistic was calculated by subtracting each observed number of progeny from the corresponding expected number of progeny per cross. The difference was squared and divided by the expected number of progeny. The degree of freedom was 1 and the alpha level was 0.05.

The results of the growth and pathogenicity study were subjected to a t-test using Microsoft Excel 2010 to determine if mating type was associated with growth rate and/or pathogenicity. For the pathogenicity study, a t-test was used to compare the values (lesion length) of *mat 1* and *mat 2* progeny within each cross and the values of *mat 1* and *mat 2* progeny combined from cross 1 (MRC 6217 X FCC 521) and cross 2 (MRC 6213 X MRC 7488). According to the growth study results, maximum growth was observed at 25°C (see below), therefore a t-test was used to compared values (growth at 25°C) of *mat 1* and *mat 2* progeny within each cross and the values of *mat 1* and *mat 2* progeny combined from Cross 1 and 2.

Results

Sexual reproduction and mating type ratios

Sexual fruiting structures developed approximately 14 days after crossing isolates of opposite mating type on carrot agar. After another week of incubation, ascospores

started oozing from the perithecia. Among most of the crosses involving Western Cape isolates, mating type distribution was significantly skewed (Table 2). The mating type distribution of the progeny for cross “Insect 3 X 19-1” was 3 *mat 1* and 23 *mat 2*. For cross “Insect 3 X 39-1”, the mating type distribution for the progeny was 0 *mat 1* and 27 *mat 2*. While the mating type distribution for progeny of cross “ks 20 X ks 48” was 19 *mat 1* and 6 *mat 2*. Lastly, for cross “ks 45 X ks 15”, the mating type distribution for the progeny was 19 *mat 1* and 8 *mat 2*.

For comparative purposes, hermaphrodites obtained from elsewhere in South Africa were subjected to sexual reproduction studies on carrot agar to determine if the observed mating type segregation distortion exists among isolates from other populations of the pathogen. Among these additional 18 crosses, similar distortions in mating type distribution were also detected (Table 2). These results thus indicated that mating type segregation ratio distortion also exists among isolates from elsewhere in South Africa.

Skewness in the mating type distribution was also observed when F1 progeny was crossed back to the parental strains. When F1 progeny (15) was back -crossed to MRC 6213, 1 *mat 1* and 21 *mat 2* F2 progeny were harvested. 15 *mat 1* and 32 *mat 2* F2 progeny were harvested when F1 progeny (45) was crossed with MRC 6213. 43 *mat 1* and 0 *mat 2* progeny were harvested when F1 progeny (7) was crossed with MRC 7488. Lastly, 48 *mat 1* and 0 *mat 2* F2 progeny were harvested when F1 progeny was crossed with MRC 7488.

To determine whether the observed mating type ratios represent artefacts of the progeny isolation procedure, vegetative compatibility tests were used to examine the progeny of four crosses (MRC 6217 X FCC 521, MRC 6312 X MRC 7488, Insect 3 X 39-1 and ks45 X ks15). The *nit* mutants generated from the progeny were paired with mutants generated from parent strains in each cross and in all cases the progeny included here were vegetatively incompatible with the parental strains. The skewed mating type data were, therefore, not as a result of accidentally sampling the same individual multiple times, because all of the progeny were recombinant for vegetative compatibility. To determine whether the mating type distribution patterns might be a

consequence of some meiotic abnormalities resulting in the production of abnormal asci, four asci from the perithicia of each cross of the 22 crosses were examined. Under the microscope eight ascospores were counted in each of these asci.

Growth Study

The colony diameter of the progeny was measured every second day following incubation, which indicated that optimum growth occurred at 25°C and growth was slowest at 10°C. None of the isolates irrespective of mating type were able to grow at 35°C. Since maximum growth was recorded at 25°C, the readings (colony diameter readings) at this temperature where subjected to statistical analysis for both crosses. The p-value for cross 1 (MRC 6217 X FCC 521) and cross 2 (MRC 6213 X MRC 7488) was greater than 0.05 (Table 2), the same results were also observed when *mat 1* and *mat 2* progeny of both crosses where combined. The statistical analysis of the data thus indicated that there were no significant differences in the growth of *mat 1* and *mat 2* progeny of *F. circinatum*.

Pathogenicity study

Eight weeks post-inoculation, one of the inoculated seedlings had died, while the remaining 269 seedlings inoculated displayed the expected tip die-back symptoms. The diagnostic PCR resulted in the amplification of a *ca*. 360-bp fragment that is a diagnostic portion of the intergenic spacer (IGS) region of the ribosomal RNA operon (Schweigkofler *et al.*, 2004). All the re-isolated isolates were positive for the 360-bp fragment. The control seedlings did not show these symptoms and none of them had died.

The mean lesion length for cross 1 (MRC 6217 X FCC 521) was 2.89 cm for *mat 1* and 1.04 cm for *mat 2*. For cross 2 (MRC 6213 X MRC 7488) the mean lesion length for *mat 1* was 3.31 cm and 1.72 for *mat 2*. When all the isolates (cross 1 and cross 2) were combined, the mean lesion length for *mat 1* was 11.49 cm and *mat 2* was 16.25 cm. However, statistical analyses of the data sets showed that there were no significant difference in the pathogenicity of *mat 1* and *mat 2* progeny (Table 2) in cross 1 and cross 2 (the p-value was greater than 0.05 in both crosses). When all

isolates (cross 1 and cross 2) where combined there was no significant difference in the pathogenicity of *mat 1* and *mat 2 isolates*.

Discussion

The findings presented in this chapter show that there is a skewed distribution of mating type in the progeny of laboratory crosses of *F. circinatum*. This situation might explain the observed skewness in the distribution of mating type in the populations of *F. circinatum* in the Western Cape nursery and plantations (Chapter 2 of this Dissertation). Similar skewed mating type distribution has been observed in other fungi such as *Pleurotus osteatus* (Larraya *et al*., 2001), *F. verticilliodes* (*Kathariou* and Spieth, 1982, Seifert *et al*., 2003), *Microbotryum violaceum* (Thomas *et al*., 2003), *Silene alba* (Antonovics *et al*., 1998) and *Neurospora crassa* (Turner and Perkins, 1991).

Numerous authors have studied the phenomenon of segregation ratio distortion in various eukaryotes (Li *et al*., 2007; Axelsson *et al*., 2010). Most of these studies conclude that segregation and the processes determining it are complex and dependent on many factors (Douglas *et al*., 2003; Cai *et al*., 2011). For example, Cai *et al.* (2011), studied segregation distortion in *Lophopyrum ponticum* chromosome 7E. The mapping of chromosome 7E indicated that out of 64 markers that were analysed, 20 markers showed a normal 1:1 segregation and 44 markers showed segregation distortion. Cai *et al*. (2011), postulated that the observed segregation distortion was due to inherent genetic factors associated with the products of genes (i.e., *Sd-1* and *Sd-2*) whose products determine transmission of certain loci (Prins *et al*., 1996).

Another possible reason for the occurrence of segregation distortion is the spore killer phenomenon in Ascomycota (Nauta and Hoekstra, 1993). The spore killer gene causes segregation distortion by its ability to kill the sensitive spores that do not carry the killer gene (Nauta and Hoekstra, 1993). A cross between a sensitive strain and a killer strain results in the survival of only half of the spores that have the killer genotype (Antonovics *et al*., 1998). These killer genes are usually situated in regions where recombination is blocked in the heterozygous condition (Turner and Perkins,

1991), which is caused by the small inversions that prevent a crossing over between two loci in a cross where one is a killer locus (Kathariou and Spieth, 1982; Nauta and Hoekstra, 1993). In the current study, however, the spore killer phenomenon is unlikely to have caused the mating type distribution patterns observed, because in all of the *F. circinatum* asci examined, eight ascospores were observed.

Zamir and Tadmor (1986) indicated that segregation distortion is a result of linkage between genetic markers or factor(s). These factors are structural genes or cryptic structural genes in the parents and they could thus affect fitness or pathogenicity of the F1 progeny. However, in this study, pathogenicity and growth studies were conducted and the statistical analyses indicated that there was no significant difference in the growth rate and pathogenicity of either *mat 1* and *mat 2* progeny of *F. circinatum.* Therefore, according to the results obtained, the pathogenicity of the progeny and their fitness do not contribute to mating type segregation distortion.

As mentioned above, no difference in the pathogenicity of *mat 1* and *mat 2* isolates were detected in this study. Literature reports that other studies where able to link pathogenicity to mating type. For example, Perez-Sierra *et al*. (2007), linked pathogenicity to mating type in the population of *F. circinatum* for the first time. The study using 157 isolates of *F. circinatum* indicated that *mat 1* isolates where more virulent than *mat 2* isolates. In 2005, Nielsen *et al*. (2005) also reported a link between pathogenicity and mating type in *Cryptococcus neoformans*, where the **α** mating type was more virulent than the **a** mating type.

The Western Cape population of *F. circinatum* is characterized by relatively low genetic diverstity (Chapter 2 of this dissertation). As a result, the skewed mating type distribution of *F. circinatum* could be due to an initial introduction of only one mating type into the region followed by primarily the asexual mode of reproduction due to the general absence of female fertile isolates. Should sexual reproduction occur, however, the results of the current study suggest that most of the progeny will represent a single mating type. Although the mechanism responsible for this violation of Mendelian laws remains unclear, the results presented here suggest that anomalies during meiosis likely impact the fitness of the progeny of a specific mating type. The

results of a hybrid cross between *F. subglutinans* and *F. circinatum*, suggested that transmission ratio distortion of intact parental linkage groups to the F1 progeny during the gamete formation might be due to the presence of a distorting genetic factor in that part of a genome (De Vos *et al*., 2007). It is still unclear what determines the observed segregation distortion mating type in *F.circinatum*, but similar types of mechanisms that have been studied in intraspecific populations might be at play.

Irrespective of the exact meiotic mechanisms responsible for the segregation distortion, the results presented here suggest that only progeny carrying one of the mating types eventually survive in a population. Although ascus development was not affected, only a specific mating type survived. Ascospores representing the other mating type potentially did not germinate or the fitness of the germinated isolates could have been impaired in some way. The results presented here indicate that neither growth in culture nor pathogenicity could have given rise to the distortion. Future studies should thus attempt to evaluate other fitness properties such as the spore germination to determine the survival of ascospores.

Acknowledgements

We thank the National Research Foundation (NRF), Department of Agriculture and the Tree Pathology Co-operative Programme (TPCP) for financial support.

References

1. Alonso, R. and Bettucci, L. 2009. First report of the pitch canker fungus *Fusarium circinatum* affecting *Pinus taeda* seedlings in Uruguay. Australasian Plant Disease Notes 4: 91-92.
2. Antonovics, J., O'Keefe, K. and Hood, M.E. 1998. Theoretical population genetics of mating type linked haplo lethal alleles. International Journal of Plant Sciences 159 (2): 192-198.
3. Axelsson, E., Albrechtsen, A., Van, A.P., Li, L., Megens, H.J., Vereijken, A.L.J., Crooijmans, P.P.M.A., Groenen, M.A.M., Ellegren, H., Wlllerslev, E.

and Nielsen, R. 2010. Segregation distortion in chicken and the evolutionart consequences of female meiotic drive in birds. Heredity 105: 290-298.

4. Barrows-Broaddus, J and Dwinell, L.D. 1983. Histopathology of *Fusarium moniliforme* var. *subglutinans* in four species of southern pines. Phytopathology 73: 882-889.
5. Bragança, H., Diogo, E., Moniz, F. and Amaro, P. 2009. First report of pitch canker on pines caused by *Fusarium circinatum* in Portugal. Plant Disease 93: 1079.
6. Brasier, C.M. 1986. The d-factor in *Ceratocystis ulmi* its biological characteristics and implications for Dutch elm disease. Fungal Virology (ed. Buck KW). CRC Press, Boca Ralton, Florida. Pp 177-208.
7. Britz, H., Coutinho, T.A., Wingfield, M.J., Marasas, W.F.O., Gordon. T.R. and Leslie, J.F. 1998. *Fusarium subglutinans* f. sp. *pini* represents a distinct mating population in the *Gibberella fujikuroi* species complex. Applied and Environmental Microbiology 65 (3): 1198-1201.
8. Britz, H., Coutinho, T.A., Marasas, W.F.O and Wingfield, M.J. 2005. Diversity and differentiation of *Gibberella circinata* in South Africa. Plant Pathology 54: 46-52.
9. Cai, W., Rudolph, J.L., Harrison, S.M., Jin, L., Frantz, A.L., Harrison, D.A., Andres, D.A. 2011. An evolutionarily conserved Rit GTPase-p38 MAPK signaling pathway mediates oxidative stress resistance. Molecular Biology Cell 22(17): 3231--3241.
10. Carey, W.A. and Kelly, W.D. 1994. First report of *Fusarium subglutinans* as a cause of late season mortality in longleaf pine nurseries. Plant Disease 78: 754.
11. Carlucci, A., Colatruglio, L. and Frisullo, S. 2007. First report of pitch canker caused by *Fusarium circinatum* on *Pinus halepensis* and *P. pinea* in Apulia (South Italy). Plant Disease 91 (12): 1683.
12. Coppin, E., Debuchy, R., Arnaise, S. and Picard, M. 1997. Mating type and sexual development in filamentous ascomycetes. Microbiology and Molecular Biology Reviews 61: 411-428.

13. Correll, J.C., Klittich, C.J.R. and Leslie, J.F. 1987. Nitrate nonutilizing mutants of *Fusarium oxysporum* and their use of vegetative compatibility tests. Phytopathology 77:1640-1646.
14. Coutinho, T.A., Steenkamp, E.T., Mongwaketsi, K., Wilmot, M. and Wingfield, M.J. 2007. First outbreak of pitch canker in a South African pine plantation. Australasian Plant Pathology 36: 1-6.
15. De Vos, L., Myburg, A.A., Wingfield, M.J., Desjardins, A.E., Gordon, T.R. and Wingfield, B.D. 2007. Complete genetic maps from an interspecific cross between *Fusarium circinatum* and *Fusarium subglutinans*. Fungal Genetics and Biology 44: 701-714.
16. Douglas, R., Taylor, K. and Par, K. 2003. Common features of segregation distortion in plants and animals. Genetica 117: 27-35.
17. Dwinell, L.D., Barrows-Broaddus, J. and Kuhlman, E.G. 1985. Pitch canker: a disease complex of southern pines. Plant Disease 69: 270-276.
18. Dwinell, L.D., Fraedrich, S.W. and Adams, D. 2001. Diseases of pine caused by the pitch canker fungus. In '*Fusarium*: Paul, E. Nelson Memorial Symposium'. (Eds B.A. Summerell, J.F. Leslie, D. Backhouse, W.L. Bryden, L.W. Burgess) pp. 225-232. (The American pytopathological society: St Paul, M.N.).
19. EPPO. 2004. First report of *Gibberella circinata* in France. http://archives.eppo.org/EPPOReporting/2006/Rsf-0605.pdf.
20. Gallegos, A., Jacobson, D.J., Raju, N.B., Skupski, M.P. and Natvig, D.O. 2000. Suppressed recombination and a pairing anomaly on the mating type chromosome of *Neurospora tetrasperma*. Genetics 154: 623-633.
21. Gordon, T.R., Kirkpatrick, S.C., Aegerte, B.J., Wood, D.L. and Storer, A.J. 2006. Susceptibility of Douglas fir (*Pseudotsuga menziesii*) to pitch canker, caused by *Gibberella circinata* (anamorph = *Fusarium circinatum*). Plant Pathology 55: 231-237.
22. Gordon, T.R., Storer, A.J. and Wood, D.L. 2001. The pitch canker epidemic in California. Plant Disease 85: 1128-1139.
23. Hepting, G.H and Roth, E.R. 1946. Pitch cankers a new disease on some southern pines. Journal of Forestry 44: 742-744.

24. Hepting, G.H and Roth, E.R. 1953. Host relations and spread of the pine pitch canker disease. Phytopathology 54: 475.
25. Jacobson, D.J. 2005. Blocked recombination along the mating type chromosomes of *Neurospora tetrasperma* involves both structural heterozygosity and autosomal genes. Genetics 171: 839-843.
26. Kathariou, S. and Spieth, P.T. 1982. Spore killer polymorphism in *Fusarium moniliforme*. Genetics 102: 19-24.
27. Kerenyi, Z., Moretti, A., Waalwijk, C., Olah, B. and Hornok, L. 2004. Mating type sequences in asexually reproducing *Fusarium* species. Applied and Environmental Microbiology 7(8): 4419-4423.
28. Klittich, C.J.R., and Leslie, J.F. 1988. Nitrate reduction mutants of *Fusarium moniliforme (Gibberella fujikuroi*). Genetics 118:417-423.
29. Landeras, E., Garcia, P., Fernandez, Y. and Brana, M. 2005. Outbreak of pitch canker caused by *Fusarium circinatum* on *Pinus* spp. In northern Spain. Plant Disease 89: 1015.
30. Larraya, L.M., Perez, G., Iribarren, I., Blanco, J.A., Alfonso, M., Pisabarro, A.G. and Ramirez, L. 2001. Relationship between monokaryotic growth rate and mating type in the edible basidiomycete *Pleurotus ostreatus*. Applied and Environmental Microbiology 67 (8): 3385-3390.
31. Lee, J. K., Lee, S.H., Yang, S.I. and Lee, Y.W. 2000. First report of pitch canker disease on *Pinus rigida* in Korea. Plant Pathology Journal 16:52–54.
32. Leslie, J.F. and Klein, K.K. 1996. Female fertility and mating type effects on effective population size and evolution in filamentous fungi. Genetics 144: 557-567.
33. Leslie, J.F. and Summerelle, B.A. 2006. The *Fusarium* laboratory manual. Blackwell Publishing, USA.
34. Li, W., Zhongxu, L., Zhang, X. 2007. A novel segregation distortion in intraspecific population of Asian cotton (*Gossypium arboretum* L.) Detected bu molecular markers. Journal of Genetics 34 (7): 634-640.
35. McCain, A.H., Koehler, C.S. and Tjosvold, S.A. 1987. Pitch canker threatens California pines. California Agriculture 41: 22-23.
36. Möller, E.M., Bahnweg, G., Sandermann, H. and Geiger, H.H. 1992. A simple and efficient protocol for isolation of high molecular weight DNA from

filamentous fungi, fruit bodies and infected plant tissues. Nucleic Acid Research 2 (22): 6115-6116.

37. Muramoto, M. and Dwinell, L.D. 1990. Pitch canker of *Pinus luchuensis* in Japan. Plant Disease 74: 530.
38. Nauta, M.J. and Hoekstra, R.F. 1993. Evolutionary dynamics of spore killer. Genetics 135: 923-930.
39. Nielsen, K., Marra, R.E., Hagen, F., Boekhout, T., Mitchell, T.G., Cox, G. M. and Heitman, J. 2005. Interaction between genetic background and the mating type locus in *Cryptococcus neoformans* virulence potential. Genetics 171: 975-983.
40. Nirenberg, H.I. and O'Donelle, K. 1998. New *Fusarium* species and combinations with the *Gibberella fujikuroi* species complex. Mycologia 90: 434-458.
41. Perez-Sierra, A., Landeras, E., Leon, M., Berbegal, M., Garcia-Jimenez, J. and Armengol, J. 2007. Characterization of *Fusarium circinatum* from *Pinus* spp. In northern Spain. Mycological Research 3: 832-839.
42. Prins, R., Marais, G.F., Janse, B.H.J., Pretorius, Z.A. and Marais, A.S. 1996. A physical map of the *Thinopyrum* derived *Lr 19* translocation. Genome 39: 1013 – 1019.
43. Sambrook J., Fritsch, E.F. and Maniatis, T. 1989. Molecular cloning: a laboratory manual. Cold Springs Harbour Laboratory Press, New York.
44. Santos, J.J.G. and Tovar, D.B. 1991. Algunos aspectos sobre el cancro resinoso de los pinos. In 'VI Simposio Nacional Sobre Parasitologia Forestal, October 1991'. P. 31. (unidad de Xcongresos del Calegio de Posgraduados montecillos: Edo, Mexico).
45. Schweigkofler, W., O'Donnell, K. and Garbelotto, M. 2004. Detection and quantification of *Fusarim circinatum*, the causal agent of pine pitch canker, from two California sites by using a real-time PCR approach combined with a simple spore trapping method. Applied and Environmental Microbiology 70 (6): 3512-3520.
46. Seifert, K.A., Aoki, T., Baayen, R.P., Brayford, D., Burgess, L.W., Chulze, S., Gams. W., Geiser, D., de Gruyter, J., Leslie, J.F., Logrieco, A., Marasas, W.F.O., Nirenberg, H.I., O'Donnell, K., Rheeder, J., Samuels, G.L.,

Summerell, B.A., Thrane, U. and Waalwijk, C. 2003. The name *Fusarium moniliforme* should no longer be used. Mycological Research News 107 (6): 641-644.

47. Steenkamp, E.T., Rodas, C.A., Kvas, M. and Wingfield, M.J. 2012. *Fusarium circinatum* and pitch canker of *Pinus* in Colombia. Australasian Plant Pathology 41: 483-491.
48. Storer, A.J., Gordon, T.R., Wood, D.L. and Dallara, P.L. 1995. Pitch canker in California. California Department of Forestry and Fire Protection. No. 97, Sacramento.
49. Thomas, A., Shykoff, J., Jonot, O. and Giraud, T. 2003. Sex ratio bias in populations of the phytogenic fungus *Microbotryum violaceum* from several host species. International Journal of Plant Sciences 164 (4): 641-647.
50. Turgeon, B.G. 1998. Application of mating type gene technology to problems in fungal biology. Annual Review of Phytopathology 36: 115-137.
51. Turner, B.C. and Perkins, D.D. 1991. Meiotic drive in *Neurospora* and other fungi. The American Society of Naturalist 137 (3): 416-429.
52. Viljoen, A., Wingfield, M.J. and Marasas, W.F.O. 1994. First report of *Fusarium subglutinans f. sp. Pini* on pine in South Africa. Plant Disease 78: 309-312.
53. Wingfield, M.J., Hammerbacher, A., Ganley, R.J., Coutinho, T.A., Steenkamp, E.T., Gordon, T.R. and Wingfield, B.D. 2008. Pitch canker caused by *Fusarium circinatum*: a growing threat to pine plantations and forests worldwide. Australasian Plant Pathology 37: 319-333.
54. Wingfield, M.J., Jacobs, A., Coutinho, T.A., Ahumada, R. and Wingfield, B.D. 2002. First report of the pitch canker fungus *Fusarium circinatum*, on pines in Chile. Plant Pathology 51: 397.
55. Xu, J. and Leslie, J.F. 1996. A genetic map of *Gibberella fujikuroi* mating population A (*Fusarium moniliforme*). Genetic Society of America 143: 175-189.
56. Yun, S. Arie, T., Kaneko, I., Yoder, O.C. and Turgeon, B.G. 2000. Molecular organization of the mating type loci in heterothallic, homothallic and sexual *Gibberella/Fusarium* species. Fungal Genetics and Biology 31: 7-20.

57. Yun, S., Berbee, M.L., Yoder, O.C and Turgeon, B.G. 1999. Evolution of the fungal self-fertile reproduction life style from self-sterile ancestors. Proceedings of the National Academy of Science 96: 5592-5597.
58. Zamir, D. and Tadmor, Y. 1986. Unequal segregation of nuclear genes in plants. Botanical Gazette 147 (3): 355-358.

Table 1: *Fusarium circinatum* isolates used in this study

FCC no. [a]	Isolate no. [b]	Geographic origin [c]	Host	Mating type [d]
13		Ngodwana, MP	*P.patula*	Mat-1
65		Ngodwana, MP	*P.patula*	Mat-2
119	7488	Ngodwana, MP	*P.patula*	Mat-1
311		Ngodwana, MP	*P.patula*	Mat-1
140		Ngodwana, MP	*P.patula*	Mat-2
478	7473	Ngodwana, MP	*P.patula*	Mat-1
493	7466	Ngodwana, MP	*P.patula*	Mat-2
521	7445	Ngodwana, MP	*P.patula*	Mat-1
542	7452	Ngodwana, MP	*P.patula*	Mat-1
566	6213	Ngodwana, MP	*P.patula*	Mat-2
1031	7451	Ngodwana, MP	*P.patula*	Mat-1
1034	6217	Ngodwana, MP	*P.patula*	Mat-2
1057	7458	Ngodwana, MP	*P.patula*	Mat-1
1828		Piet Retief, MP	*P.patula*	Mat-2
1832		Piet Retief, MP	*P.patula*	Mat-2
3578		South Africa	*P.patula*	Mat-2
3580		South Africa	*P.patula*	Mat-2
3581		South Africa	*P.patula*	Mat-2
3582		South Africa	*P.patula*	Mat-2
3583		South Africa	*P.patula*	Mat-2
	Insect 3	Tokai, WC	*P.radiata*	Mat-1
	19-Jan	Tokai, WC	*P.radiata*	Mat-2
	Jan-39	Tokai, WC	*P.radiata*	Mat-2
	ks20	Nursery, WC	*P.radiata*	Mat-2
	ks48	Nursery, WC	*P.radiata*	Mat-1
	ks45	Nursery, WC	*P.radiata*	Mat-1
	ks15	Nursery, WC	*P.radiata*	Mat-2

[a] FCC: *Fusarium* Culture Collection at FABI, University of Pretoria, South Africa.

[b] CMW: Culture Collection Mike Wingfield, FABI, University of Pretoria, South Africa; The isolates from Tokai were collected by E.T. Steenkamp, T.A. Coutinho and M. J. Wingfield, those from the Western Cape nursery were collected by B. Porter and the isolates from other parts in South Africa were collected by B. Porter.

[c] WC = Western Cape, RSA.

MP = Mpumalanga, RSA.

[d] Mating types were determined by PCR using the primer sets developed by Steenkamp *et al*. (2000)

Table 2: Chi-square analysis for progeny mating type

Cross	Progeny Mat 1	Progeny Mat 2	χ^2
MRC 6213 x MRC 7488	12	38	13.52*
MRC 6217 x MRC 7488	5	107	92.8*
MRC 6217 x FCC 1031	20	5	9*
MRC 6217 x FCC 13	27	1	24.1*
MRC 6217 x FCC 478	29	0	29*
MRC 6217 x FCC 131	27	0	27*
FCC 542 x FCC 140	27	1	24.1*
FCC 119 x MRC 3580	29	0	29*
FCC 1057 x MRC 1831	24	0	24*
FCC 542 x MRC 3582	30	0	30*
FCC 542 x FCC 1828	27	2	21.56*
FCC 119 x FCC 1832	29	1	26*
FCC 542 x MRC 3578	27	0	27*
FCC 1057 x FCC 3581	29	0	29*
FCC 119 x FCC 493	27	0	27*
FCC 119 x FCC 65	29	0	29*

* Significance at 0.05 probability level

Table 3: Table indicating t-test p-values

	p-value	
	Pathogenicity study	**Growth study**
Cross 1 (6217 X 521)	0.515	0.686
Cross 2 (6213 X 7488)	0.520	0.818
Cross 1 & 2	0.600	0.771

Summary

Fusarium circinatum is a heterothalic fungus that causes pitch canker of *Pinus*. In South Africa, the disease was reported for the first time in the Western Cape Province in 2006 where it posed a significant threat to *Pinus patula* plantations. To develop effective management strategies for *F. circinatum* in the plantation setting, the overall aim of the research presented in this dissertation was to study the population biology of *F. circinatum* in selected Western Cape *P. patula* plantations. The methods used for such studies were reviewed in Chapter 1. The possible origin of the inoculum that gave rise to the Western Cape disease outbreaks were determined in Chapter 2, which also included sexual compatibility and fertility tests for determining the possible reproductive mode of the pathogen in this region. Chapter 3 investigated a possible link between sexual compatibility or mating type and fitness attributes such as mycelial growth rate and pathogenicity. The results showed that the plantation populations of the pathogen are characterized by limited genetic diversity and that the inoculum for the disease outbreaks in plantations likely originated from seedling nurseries. Based on the results of sexual compatibility and fertility assays, the plantation populations of *F. circinatum* mainly include isolates of the same mating type of which most apparently also lost the ability to act as females in sexual interactions. Although a similar skew in mating type distribution was also observed among the progeny from sexual crosses, no link could be detected between mating type and growth rate in culture or pathogenicity. Nevertheless, these data suggest, that should sexual reproduction occur, most of the resulting progeny would have the same mating type, thus potentially mimicking the mating type distribution in natural populations of the pathogen. Moreover, perithecia (sexual structures) of this pathogen have never been observed in the field. Taken together, these data point towards a primarily asexual mode of reproduction for *F. circinatum* in the Western Cape *P. patula* plantation. Whether this situation is reminiscent of a recent introduction of only a few genotypes into the respective plantations or reflects an inherent property of sexual reproduction in this fungus remains to be determined.