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Genetic linkage map and QTL analysis of a cross between *Fusarium subglutinans* and *Fusarium circinatum*

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by

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A thesis submitted in partial fulfillment of the degree

Philosophiae Doctor

in the

Faculty of Natural and Agricultural Sciences

Department of Genetics

Forestry and Agricultural Biotechnology Institute

University of Pretoria

South Africa

March 2012

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Declaration,

I, Lieschen De Vos, hereby declare that the thesis, which I hereby submit for the degree Philosophiae Doctor 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.

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Lieschen De Vos

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Acknowledgements

I wish to thank the following persons and institutions:

- This work was supported by the National Research Foundation (NRF), University of Pretoria, Forestry and Agricultural Biotechnology Institute (FABI), the DST/NRF Center of Excellence in Tree Health Biotechnology (CTHB), the Tree Protection Co-operative Programme (TPCP), and the Andrew Mellon Foundation.
- Profs. Brenda Wingfield, Zander Myburg and Mike Wingfield and Emma Steenkamp, as well as Drs. Albe van der Merwe, Magriet van der Nest and Mr. Quentin Santana and Simon Martin, for critical review of manuscripts, valuable advice and support.
- To Albe, for encouragement, patience and motivation when I needed it.
- Vivienne Clarence, 6th floor lab mates, and all FABI staff.
- Cathy Barnard and Mathilde Beresford: Thanks for the administrative support as well as providing an ear to listen.
- My family: it is finally finished.
- Gavin and Jarod, the two men in my life who, without, would have made for a less wonderful journey. Thank you for everything.

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Preface

The *Gibberella fujikuroi* species complex is associated with *Fusarium* species that have anamorphs in *Fusarium* section *Liseola*. This complex includes many important pathogens of agricultural crops and trees. The focus of studies in this thesis was on two species in the *G. fujikuroi* species complex, namely *Fusarium circinatum* and *Fusarium subglutinans*. Specifically, an interspecies cross between them was examined at various levels. The overall aim was to better understand the genomic architecture of the two species, as well as to consider the barriers to introgression.

Chapter 1 represents a literature review entitled "*Fusarium* genomics and underlying opportunities to understand the genetics of the pitch canker fungus". *Fusarium circinatum*, or the pitch canker fungus, and *F. subglutinans* are briefly discussed. The focus of this review was on *F. graminearum*, the current model for understanding *Fusarium* genetics. This fungus has been studied extensively with regards to genetic linkage mapping, with a genome sequence publicly available. Also, genomic sequence data for various other *Fusarium* spp. has initiated comparative studies of *Fusarium* at the genome level. Knowledge emerging from such studies are discussed in terms of their relevance in better understanding *F. circinatum*.

In **Chapter 2**, the interspecific cross between *F. circinatum* and *F. subglutinans* was investigated by producing a genetic linkage map. The aim here was to provide a useful framework for further study of the architecture of the two parental genomes, by providing amongst others, an insight into the segregation patterns of Amplified Fragment Length Polymorphism (AFLP) markers amongst the progeny. Identification of any important quantitative traits in *F. circinatum* would be facilitated with the genetic linkage map. Interspecific crosses between *F. circinatum* and *F. subglutinans* are uncommon and an aim of developing the framework linkage map was to better understand introgression barriers between these two species. Consequently, all ensuing chapters of this thesis examined these features of the genetic linkage map.

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Introgression barriers were examined by following the transmission of markers amongst the progeny of the interspecific cross in **Chapter 3**. Significant numbers of markers displaying segregation distortion (or transmission ratio distortion) in crosses that are highly divergent (as in interspecies crosses) were expected. Possible reasons for this were considered in order to clarify whether pre- or post-zygotic causes were the basis for higher than expected segregation distortion observed in the markers.

In **Chapter 4** we sought to exploit the genetic linkage map using Quantitative Trait Loci (QTLs) to examine differences in growth and morphology amongst the progeny of the interspecific cross. *Fusarium circinatum* and *F. subglutinans* are pathogens of different hosts (susceptible *Pinus* spp. and maize, respectively). Pathogenicity of the progeny was investigated on *Pinus patula* seedlings to determine whether pathogenicity was related to mycelial growth.

Genomic sequence data are available for various Fusarium spp., and using *Fusarium verticillioides* (African clade of the *G. fujikuroi* species complex), *F. circinatum* (American clade) and *Fusarium fujikuroi* (Asian clade), we were able to study representatives of the three clades of the *G. fujikuroi* species complex at the genomic level for the first time. In **Chapter 5**, we connect the physical genetic map of these three species to the genetic linkage map of the interspecific cross of *F. circinatum* and *F. subglutinans*. The primary aim here was to reveal the degree of synteny amongst these *Fusarium* spp., which are accommodated in the *G. fujikuroi* species complex. Linking the genetic map to the physical map was necessary to be able to connect data from the genetic map (*e.g.* QTLs) to actual DNA sequence.

The research chapters presented in this thesis are stand alone units (two have already been published) and some duplication between chapters was inevitable. Conclusions are drawn at the end of each chapter in the discussion sections. A synopsis of the thesis is given in the **Summary** section. A detailed summary and areas of further research that have arisen from the research done in this thesis are highlighted in **Conclusions and Future Prospects**. These will be beneficial in deciphering genomic divergence of *Fusarium* spp., particularly those in the *G. fujikuroi* species complex.

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CHAPTER 1

Fusarium genomics and underlying opportunities to understand the genetics of the pitch canker fungus

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Introduction

The field of genomics can be defined as "the scientific study of genomes, using gene mapping, nucleotide sequencing, and other techniques; the branch of molecular biology concerned with the structure, function, and evolution of genomes" (Oxford English Dictionary Online, 2010). This term was originally used in the first issue of the journal Genomics, but was coined by Dr. Thomas H. Roderick in 1987 (McKusick and Ruddle, 1987), who stated that the goal of genomics was to "promote the understanding of the structure, function, and evolution of genomes in all kingdoms of life and the application of genome sciences and technologies to challenging problems in biology and medicine".

As defined above, genomics is a broad field. Before the remarkable increase in sequenced genomes due to advances in sequencing technologies, genetic linkage mapping was the method of choice to elucidate the genomic organization as well the genetics of organisms. However, the use of genetic linkage mapping is still relevant in genomics today. As genomes of most organisms are not sequenced, elucidation of the genomic structure is still dependant on gene mapping. Also, the use of linkage maps has been invaluable in genome sequencing projects, helping to order clones during the construction of contig maps (*e.g.* Gale *et al.*, 2005). A contig map is a library of linked contigs (a group of cloned sequences that are contiguous), those that are linked represent a complete chromosomal segment. In the ensuing sections, genetic linkage mapping will be discussed focusing on research for fungi classified in the genus *Fusarium*.

Fungi residing in the genus *Fusarium* are ascomycetes. They occur worldwide, with a minimum of 80% of cultivated crops affected by at least one *Fusarium* disease (Leslie & Summerell, 2006) (*e.g.* Fusarium head blight (*Fusarium graminearum*; Trail, 2009) and the pitch canker fungus (*Fusarium circinatum*; Wingfield *et al.*, 2008). For this reason, they are of economic importance and as such have been the focus of much research.

In this review, the progress of *Fusarium* genomics will be examined. *Fusarium graminearum* (*F. graminearum* species complex), as the model for *Fusarium* genomic studies, will be described. This fungus has been studied extensively with regards to genetic linkage mapping as

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well as having a genome sequence that is publicly available (http://www.broad.mit.edu; Cuomo *et al.*, 2007). The genomes of other *Fusarium* species have also been sequenced (Table 1): *Fusarium verticillioides* (*Gibberella fujikuroi* species complex, Ma *et al.*, 2010); *Fusarium oxysporum* f. sp. *lycopersici* (*F. oxysporum* species complex, Ma *et al.*, 2010); *Fusarium solani* (*Nectria haematococca* as the sexual state; *Fusarium solani* species complex, Coleman *et al.*, 2009; Ma *et al.*, 2010) and *Fusarium virguliforme* (*F. solani* species complex, Srivastava *et al.*, 2011) are a subset of those that have been sequenced. Results from these genomes will be extrapolated to the pitch canker fungus, *Fusarium circinatum* (*G. fujikuroi* species complex, Wingfield *et al.*, 2011), for which only a working draft assembly is available at present.

The *Gibberella fujikuroi* species complex

Gibberella fujikuroi (Sawada) Wollenw. is the sexual stage associated with strains of *Fusarium* section *Liseola*. Species classified in this complex are Ascomycetes, with many being important fungal pathogens of agricultural crops globally, including commercially planted trees. Consequently, these fungi are of economic importance to the affected industries due to reductions in the yield as well as the quality of the cultivated crops. Due to this importance, the genus has received much attention, especially surrounding taxonomic issues. Species in this complex have been described using the morphological, biological and phylogenetic species concepts. The *G. fujikuroi* species complex consists of over 50 distinct phylogenetic lineages (Kvas *et al.*, 2009).

The morphological species concept is based on the similarity of morphological characters (both physical and physiological) within a species (Leslie *et al.*, 2001; Summerell *et al.*, 2003). These characters are presumed to be shared through common descent. Traditionally, the morphological species concept has been used to describe species in the *G. fujikuroi* complex (Booth, 1977; Nelson *et al.*, 1983). However, species description following this concept has been problematic, as seen with the *Fusarium subglutinans* morphological species (*F. subglutinans sensu lato*). Different species, are in fact, associated with this morphological species: *F. subglutinans sensu stricto* isolated from maize (Nirenburg & O'Donnell, 1998; O'Donnell *et al.*, 1998); *F.*

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circinatum isolated from pine species (Nirenburg & O'Donnell, 1998; Britz *et al.*, 1999); *Fusarium sacchari*, initially described as strains isolated from sugarcane but now known to have a broader host range (Nirenburg & O'Donnell, 1998; O'Donnell *et al.*, 1998); *Fusarium guttiforme* associated with disease of pineapple (Nirenberg & O'Donnell, 1998; O'Donnell *et al.*, 1998) and *Fusarium mangiferae* that causes mango malformation (Britz *et al.*, 2002a). Many *Fusarium* species have only been described based on morphology, despite these limitations. Screening of large numbers of *Fusarium* cultures still relies on the use of morphology. However, the small number of easily detectable characters between species is a serious problem.

The biological species concept defines individuals based on sexual compatibility. Individuals of the same species are reproductively compatible, *i.e.*, sexually cross-fertile with progeny being viable and fertile (Mayr, 1940; Dobzhansky, 1951; Summerell *et al.*, 2003). Within the *G. fujikuroi* species complex, *Fusarium* isolates have been formally classified into 10 mating populations or biological species that are reproductively isolated (Table 2). However, classification of *Fusarium* isolates into biological species is problematic, as more than 80% of species have no apparent sexual stage (Steenkamp *et al.*, 2002). Also, interspecific crosses have been reported in this species complex (Desjardins *et al.*, 1997, 2000; Leslie *et al.*, 2004a,b). In anticipation of changes to the International Code of Botanical Nomenclature whereby the teleomorph description would not be required for describing species, new *Fusarium* species are only being described for the anamorphic stage (Scauflaire *et al.*, 2011). The recognition of a single name nomenclature for fungi that exhibit both sexual (teleomorphic) and asexual (anamorphic) states would stabilize fungal taxonomy, with the scientific name of a species reflecting its phylogeny (Rossman & Samuels, 2005; Gräfenhan *et al.*, 2011). This "one fungus, one name" was adopted by the newly formed International Code of Nomenclature of algae, fungi and plants (Miller *et al.*, 2011).

The diagnostic phylogenetic species concept is based on the premise that a species is the smallest unit of individuals sharing ancestry and descent using diagnosable characters (Cracroft 1983). However, using this phylogenetic species approach is problematic when defining species limits (Taylor *et al.*, 2000). Consequently, the Genealogical Concordance Phylogenetic Species Recognition (GCPSR) approach is used to avoid the bias of species delimitation by utilizing the

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concordance of more than one gene genealogy (Baum *et al.*, 1995). The only prerequisite of GCPSR is that no recombination occurs in the gene being considered. The phylogenetic relationships of the *G. fujikuroi* species complex have been studied (*e.g.* O'Donnell & Cigelnik, 1997; O'Donnell *et al.*, 1998) and has proved useful in placing fungi that do not produce a sexual stage in this species complex. However, at present, species description in the *G. fujikuroi* species complex follows a polyphasic approach. A combination of the morphological, biological and phylogenetic species concepts provides a more robust method for the classification of species into the *G. fujikuroi* species complex (*e.g.* Scauflaire *et al.*, 2011).

The evolutionary species concept defines a species as "a lineage of ancestral descendant populations which maintains its identity from other such lineages and which has its own evolutionary tendencies and historical fate" (Wiley *et al.* 1978). Thus, the morphological, biological and phylogenetic species concepts are all compatible with the evolutionary species concept, in that they are all endeavoring to define an evolutionary species. The evolutionary species concept does however not have specific recognition criteria for species recognition. This concept can be applied to all fungi and GCPSR used to diagnose individual species (Taylor *et al.* 2000).

Fusarium circinatum

Classification

The fungus, isolated from *Pinus virginiana*, was first classified as a *Fusarium* species that probably belonged to section *Liseola* (Hepting & Roth, 1946). At that time, no fruiting bodies were observed, and therefore a teleomorph genus could not be described for the pathogen. In 1949, the pitch canker fungus was identified as *F. lateritium* (Nees) emend. Snyder & Hansen f. sp. *pini* Hepting (Snyder *et al.*, 1949). In later studies the name *F. moniliforme* Sheld. var. *subglutinans* Wollenw. & Reink. was used for this pathogen isolated from pine trees affected by pitch canker (Dwinell, 1978). It was originally thought that this fungus was a secondary invader, but pathogenicity tests confirmed the development of symptoms in agreement with those caused by pitch canker. In a later study by Kuhlman *et al.* (1978) the pitch canker fungus was

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redescribed as *F. moniliforme* var. *subglutinans*. Subsequently, it was raised to the species level as *F. subglutinans* (Wollenw. & Reinking) Nelson, Toussoun & Marasas (Nelson *et al.*, 1983), although isolates not pathogenic to pine were also included in this species.

When pathogenicity tests were done on *F. subglutinans* and closely related species, only *F. subglutinans* isolates from pine were virulent on Monterey pine (*Pinus radiata*). This led to the proposal that the pitch canker fungus should be a separate *forma specialis*, namely *F. subglutinans* f. sp. *pini* (Correll *et al.*, 1991). This *forma specialis* was supported by mitochondrial DNA restriction fragment patterns that were shared by between *F. subglutinans* f. sp. *pini* isolates, but differed from non-pine *F. subglutinans* isolates (Correll *et al.*, 1992).

In 1998, using morphological characteristics, the pitch canker fungus was finally described as *F. circinatum* Nirenberg & O'Donnell, and the teleomorph was described as *Gibberella circinata* Nirenberg & O'Donnell (Nirenberg & O'Donnell, 1998). This teleomorph description was validated by Britz *et al.* (2002b). In the study by O'Donnell *et al.* (1998), *F. circinatum* was confirmed to be a phylogenetic species. *Fusarium circinatum* was also found to represent a distinct biological species (mating population H) in the *G. fujikuroi* species complex (Britz *et al.*, 1999). The changes in methods of classification for *F. circinatum* showed how the use of different species concepts as well as pathogenicity tests was necessary to ultimately arrive at a reliable species description. Use of a polyphasic approach provided a more dynamic and accurate route to species descriptions.

The pitch canker disease

Fusarium circinatum belongs to the *G. fujikuroi* species complex and was first reported on *Pinus virginiana* in the southeastern United States in 1946 (Hepting & Roth, 1946). The disease takes its name from the copious amounts of resin/pitch produced by the tree in response to infection. Pitch canker was the first disease of mature pines that was attributed to a *Fusarium* species (Snyder *et al.*, 1949). The "*circinatum*" epithet refers to the characteristic coiled hyphae that are produced by this species (Nirenberg & O'Donnell, 1998).

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The pitch canker fungus requires a wound to infect trees (Kuhlman, 1987; McCain *et al.*, 1987). In response to infection by *F. circinatum*, pine trees produces copious amounts of resin/pitch that soaks the wood behind the canker, after which the resin accumulates on and below the cankers (Hepting & Roth, 1946). Although tree death is the most important consequence of infection, pitch canker also reduces the value of the timber due to the resin staining of the wood.

The first symptoms to appear after infection are wilting and discolouration of needles (Barnard & Blakeslee, 1980). A lesion at the point of infection girdles the branch obstructing water flow. This causes the branch and needles to die from the lesion to the branch tip (Gordon *et al.*, 2001; Wikler *et al.*, 2003). The infection does not spread down the tree, limiting the appearance of visible damage (Gordon *et al.*, 2001). Repeated infections cause further dying of the crown and this can eventually lead to tree death (Blakeslee & Oak, 1979; Gordon *et al.*, 2001). In addition to branch lesions, cankers can also be found on the main stems and exposed roots (Storer *et al.*, 1997, 2002; Wikler *et al.*, 2003).

Pitch canker is regarded as one of the most serious diseases of *Pinus* spp. in the world (Wingfield *et al.*, 2002a). It has also been found on Douglas fir (*Pseudotsuga menziesii*), the only species outside the genus *Pinus*, on which it occurs (Storer *et al.*, 1994). Since the initial outbreak of pitch canker on Virginia pine (*Pinus virginiana*) in Asheville, North Carolina in 1946 (Hepting & Roth, 1946), it has also been reported from many other regions around the world. These include the USA (Hepting & Roth, 1946), Haiti (Hepting & Roth, 1953), Japan (Muramoto & Dwinell, 1990; Kobayashi & Kawabe, 1992), South Africa (Viljoen *et al.*, 1994), Spain (Dwinell *et al.*, 1998; Landeras *et al.*, 2005), Korea (Lee *et al.*, 2000), Mexico (Guerra-Santos, 1999; Britz *et al.*, 2001), Chile (Wingfield *et al.*, 2002b), France (European and Mediterranean Plant Protection Organization, 2004), Italy (Carlucci et al., 2007), Portugal (Bragança *et al.*, 2009), Uruguay (Alonso & Bettucci, 2009) and Colombia (Steenkamp *et al.*, 2012). A common thread throughout the majority of these countries was that the fungus occurs on *P. radiata* or *P. patula* trees (Viljoen *et al.*, 1994; Dwinell *et al.*, 1998; Guerra-Santos, 1999; Britz *et al.*, 2001; Wingfield *et al.*, 2002b; Landeras *et al.*, 2005; Bragança *et al.*, 2009). Based on the high levels of genetic diversity, Wikler & Gordon (2000) hypothesized that the most likely centre of origin

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for the fungus is Mexico and that the fungus entered South Africa through seed that was imported from Mexico (Wingfield *et al.,* 1999; Wikler & Gordon, 2000; Britz *et al.,* 2001).

In South Africa, the pitch canker fungus was first reported from containerized *Pinus patula* seedlings from the Sappi Ngodwana nursery where it affected over six million seedlings in a single infestation (Wikler & Gordon, 2000). The disease was characterized by post emergence root disease of seedlings leading to death (Viljoen *et al.,* 1994). *F. circinatum* spread from the initial nursery infestation to other nurseries and is now found in all pine growing regions in South Africa (Wingfield *et al.,* 2002a). This fungus has caused huge losses to nurseries involved in pine propagation, as well as problems during establishment when cuttings and seedling pines are planted in the field. Under these circumstances, *F. circinatum* is associated with basal cankers on trees up to two years old (Wingfield *et al.,* 2002a). Pitch canker remained a nursery problem for more than ten years and there was much speculation as to whether it would in fact spread to mature plantations (Viljoen *et al.,* 1994; Viljoen *et al.,* 1995; Wingfield *et al.,* 2002a). In 2005, this fear was realized when an outbreak of pitch canker was first reported on five- and nine-year-old *P. radiata* trees (Coutinho *et al.,* 2007). A pine production nursery was suspected as the source of inoculum, as it was experiencing an outbreak of pitch canker on *P. radiata* seedlings at the same time as seedlings were obtained and planted at the site that was infected.

Pinus patula and *P. radiata* are highly susceptible to pitch canker, while *Pinus elliottii* is only moderately susceptible (Hepting, 1961; Viljoen et al., 1995). *Pinus radiata* is one of the most economically important conifer species in the world (Balocchi et al., 1999; Hodge, 1999). It is the primary plantation species in Chile, New Zealand and Australia (Balocchi et al., 1999; Hodge, 1999; Wingfield et al., 2002a). Monoculture with such a susceptible species has raised concerns that pitch canker could eradicate entire plantations and their associated industries in these countries. In South Africa, pine plantations constitute 52.5% of the land area under plantation in the forestry industry (Anonymous, 2009). Of this, *P. patula* makes up the majority of *Pinus* spp. planted (Roux et al., 2007). Other important species planted in this country include *P. elliottii* and *P. taeda* and to a lesser extent, *P. radiata*. In this respect it is important to note that *F. circinatum* was first isolated from mature *P. radiata* trees in South Africa (Coutinho et

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al., 2007) and has since also been found on *P. greggii* (Southern provenance) trees (http://www/fabinet.up.ac.za/tpcp/pitch_canker_mature_trees).

To understand the spread of the disease, several studies have focused on identifying resistant *Pinus* species (Kuhlman *et al.*, 1982b; Gordon *et al.*, 1998a, b; Storer *et al.*, 1999, 2002; Hodge & Dvorak, 2000; Schmale & Gordon, 2003; Kayihan *et al.*, 2005; Dvorak *et al.*, 2009; Woo *et al.*, 2010) as well as resistant hybrid pines (Roux *et al.*, 2007; Kim *et al.*, 2008). Studies are underway to investigate the defence mechanisms employed by resistant trees (Bonello *et al.*, 2001; Morse *et al.*, 2004; Kayihan *et al.*, 2005). The ability of this fungus to overcome resistance will be related to the genetic diversity of the pathogen and its ability to undergo sexual recombination. Only eight genotypes of the fungus are present in California (Correll *et al.*, 1992; Gordon *et al.*, 1996), which is a very small genetic basis in comparison to the genetic diversity that has been reported from South Africa (Viljoen *et al.*, 1997a, b; Britz *et al.*, 2005). Host resistance could thus be more readily overcome in South Africa than in other parts of the world, which have lower genetic diversity. Due to the heterothallic mating system of *F. circinatum* (Steenkamp *et al.*, 2000), the presence of both mating types may result in outcrossing between genotypes, which may increase the genetic diversity of populations. In contrast to the situation in South Africa, *F. circinatum* populations in other parts of the world generally have lower levels of genetic diversity, and in some cases, only one mating type is present (Iturritxa *et al.*, 2011). The absence of both mating types in a population will therefore result in a lower evolutionary potential of *F. circinatum* populations, leading to more durable resistance in the host trees if there is already resistance in the local tree population.

Genetic linkage mapping

Classical linkage mapping is used to determine the order (arrangement) of genes or markers on a chromosome. Genes that are found on homologous chromosomes usually belong to the same linkage group and can be studied by following their inheritance patterns (Russell, 2010). Genes are linked when they exist on the same linkage group or unlinked when they exist on different linkage groups (Hedrick, 2000). The linkage of genes is disrupted when crossing over

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(recombination) occurs during meiosis. The probability of recombination between genes increases directly proportionally to the distance between them. Linked genes that are close together tend to be preferentially transmitted together to the next generation. Therefore, by performing crossing experiments, linked and unlinked genes can be studied in the offspring. Linkage was first demonstrated by Thomas H. Morgan on *Drosophila melanogaster* (Morgan, 1910).

Classical linkage mapping was concerned with gene (trait) order and the genetic distance between them. However, the physical nature of these genes could not be studied until the advent of modern linkage mapping (Botstein *et al.,* 1980). Polymorphic DNA markers provided the basis of this technology and using these markers, desirable genes could be located if they showed linkage to the markers. Consequently, the gene of interest could be cloned and sequenced in an approach called map-based cloning (*e.g.* Sharma *et al.,* 2005). Therefore, genetic markers have provided the means to connect phenotypes to genes on the genetic maps of organisms.

Different polymorphic DNA markers have been employed to characterize linkage in the genomes of organisms. These include among others, RAPDs (Random Amplified Polymorphic DNA; Williams *et al.,* 1990), RFLPs (Restriction Fragment Length Polymorphisms; Botstein *et al.,* 1980), AFLPs (Amplified Fragment Length Polymorphisms; Vos *et al.,* 1995) and SSRs (Simple Sequence Repeats or microsatellites; Litt & Luty, 1989). These markers are anonymous in nature; they are detectable because of variation in sequence, yet the function, if any, of the sequence is unknown. The choice of marker(s) to be used is individual and related to the organism to be mapped. These marker classes have the potential to provide a large number of DNA markers that can be used to increase the density of genetic maps.

The polymorphic markers can be grouped as either dominant or co-dominant. Dominant markers analyze many loci at the same time (*e.g.* AFLPs and RAPDs), whilst co-dominant markers analyze only one locus at a time (*e.g.* RFLPs and SSRs). Dominant markers are high-throughput, allowing the analysis of many loci at once without requiring the need for prior information about their sequence. Co-dominant markers are more informative than dominant

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markers as different allelic variations at the specific locus can be distinguished. Prior knowledge of the sequence is required which is costly.

The use of linkage maps has been important in genome sequencing projects (*e.g.* Gale *et al.*, 2005). Genetic markers have provided a set of reference points along chromosomes to help order clones during the construction of contig maps. Approximately 110 fungal genome sequencing projects have been completed and ~ 60 more species have been targeted for sequencing (Cuomo & Birren, 2010), with this number increasing every year. Not all species will be sequenced, thus genetic linkage mapping will play an important role in the identification of genes involved in pathogenicity and other phenotypic characters. Sequencing of related organisms still promises to be the most accurate method to identify genes of interest (*e.g.* Martin *et al.*, 2011). The physical location of a marker in the genome of an organism can be used to identify linked open reading frames, or genes, that may be related to the phenotypic character associated with the marker (*e.g.* Christians *et al.*, 2011). Furthermore, the availability of a genetic linkage map enables the identification of QTLs (Quantitative Trait Loci), which could aid in programs focusing on marker-assisted selection (*e.g.* Larraya *et al.*, 2002).

Genetic linkage mapping in fungi

With the advent of polymorphic DNA marker technologies, genetic linkage maps have been generated for a wide range of organisms. These include insects (Tan *et al.*, 2001), vascular plants (Remington *et al.*, 1999) and fish (Liu *et al.*, 2003). Fungal genomes have also been mapped using molecular markers. These include ascomycetes such as *Cochliobolus heterostrophus* (Tzeng *et al.*, 1992) and *Magnaporthe grisea* (Romao & Hamer, 1992), basidiomycetes like *Phanerochaete chrysosporium* (Rhaeder *et al.*, 1989) and *Agaricus bisporus* (Kerrigan *et al.*, 1993), as well as the yeast *Cryptococcus neoformans* var. *neoformans* (Forche *et al.*, 2000).

Genetic linkage maps are constructed for an organism for a variety of reasons. Commonplace aims are to examine the genetics of these organisms as well as their genome organization (Marra

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et al., 2004). These maps are also used as a framework for further genomic study, particularly with regard to identifying possible pathogenicity and virulence factors (Cumagun *et al.*, 2004), and even in identifying economically important traits designed for breeding of cultivated mushrooms (Kerrigan *et al*., 1993).

Amongst a vast array of applications, genetic maps aid in the identification of desirable genes (*e.g.* Kang *et al.*, 2011), identifying QTLs linked to desirable traits (*e.g.* Cumagun *et al.*, 2004) and even to show synteny between related species (*e.g.* Kaló *et al.*, 2004). Mapping can be performed for any organism and, with the advent of DNA polymorphic markers, a greater number of markers can be generated leading to saturated maps. Genetic linkage mapping thus provides a robust tool to study the genetic architecture of genomes.

Genetic linkage mapping in *Fusarium*

Genetic linkage mapping in *Fusarium* spp. dates back to 1983 when the first rudimentary map of *Fusarium verticillioides* showing three linkage groups containing nine markers was generated (Puhalla & Spieth, 1983; Table 3). Auxotrophic, colour mutants and mating type phenotypes were mapped and were found to segregate as if they were each under the control of a single nuclear gene locus. In a later study, this was improved to 12 markers on four linkage groups (Puhalla & Spieth, 1985; Table 3). This included a vegetative incompatibility gene (*vic-1*). Vegetative incompatibility was found to be heterogenic and under nuclear gene control. These two studies demonstrated that *F. verticillioides* (and possibly other *Fusarium* spp.) could be used experimentally to construct genetic linkage maps.

A more robust map was generated for *F. verticillioides* using primarily RFLP markers (Xu & Leslie, 1996; Table 3). Thirteen linkage groups were identified and linked to the 12 chromosomes previously identified for *F. verticillioides* (Xu *et al.*, 1995). Comparisons were also drawn to the limited *F. verticillioides* linkage map (Puhalla & Spieth, 1985). Interestingly, the Xu & Leslie (1996) map showed that chromosome 12 seems to be a dispensable chromosome. The map generated for *F. verticillioides* was not saturated, *i.e.* contained several

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significant gaps, making it difficult to locate biologically important genes. Three genetic markers (*FUM1*, affecting fumonisin biosynthesis, *SK* spore killer and the mating type idiomorphs) also had no close flanking markers. For this reason an expanded map for this fungus was generated (Jurgenson *et al.*, 2002b; Table 3).

To saturate the existing genetic linkage map of Xu & Leslie (1996), 486 AFLP markers were added (Jurgenson *et al.*, 2002b). AFLP markers were identified that mapped closer to the three genetic markers. The absence of chromosome 12 in three progeny from the previous genetic map (Xu & Leslie, 1996) was again demonstrated. The authors anticipated that this genetic linkage map would aid in identifying genes of interest, help order clones in the construction of contigs as well as support investigations into the genomic architecture of this fungus and related *Fusarium* spp. in the *G. fujikuroi* species complex.

Because *F. oxysporum* f. sp. *lycopersici* is asexual, the process of parasexual crossing was used to generate fusion products construct a mitotic linkage map of this fungus (Teunissen *et al.*, 2003) (Table 3). Using a modified AFLP technique that incorporated a *Foxy*-specific primer with the standard AFLP adaptor primers, 102 elements were found to be polymorphic between the two parents. *Foxy* is a transposable element that is a member of an active family of short interspersed nuclear elements (Mes *et al.*, 2000). Using this approach, 23 linkage groups were identified. The high number of linkage groups, in comparison to chromosomes, is due to the low amount of coverage of the transposable elements on the map, as well as the low population size (32 fusion products). The linkage map revealed that *Foxy*-AFLP markers were clustered, indicating that the transposable elements did not transpose very far from their initial insertion sites. Due to their high level of polymorphisms, *Foxy*-AFLPs were also shown to be informative in this clonally propagating species.

Another *Fusarium* for which a genetic linkage map has been produced is *F. graminearum* (Jurgenson *et al.*, 2002a; Table 3). Using the genetic map, nine linkage groups were identified and segregation distortion (markers showing significant departure from the expected Mendelian segregation ratio) was detected in five of these linkage groups. Half of the progeny did not display any crossovers in four of the linkage groups, which indicates that recombination

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suppression is present. Also, two chromosomal structural rearrangements were found in the form of heterozygous inversions (Jurgenson *et al.*, 2002a; Bowden *et al.*, 2008). The authors postulated that their map could be used for QTL studies, to order genomic libraries during genome sequencing and to examine the genomic organization of *F. graminearum* (Jurgenson *et al.*, 2002a).

Cumagun *et al* (2004) used the genetic map of *F. graminearum* (Jurgenson *et al*., 2002a) to identify QTLs responsible for pathogenicity and aggressiveness on wheat. Pathogenicity was linked to loci affecting pigmentation, perithecium production and the level of mycotoxin production. Additionally, two QTLs for aggressiveness were linked to the *Tri5* gene, which encodes the enzyme trichodiene synthase in *F. graminearum*. This is the first enzyme involved in the pathway for trichothecene (a mycotoxin that is a protein synthesis inhibitor in eukaryotes) (Proctor *et al*., 1995; Brown *et al.*, 2001). It includes the *Tri13* gene product that determines whether the mycotoxins DON or NIV are produced (Lee *et al.*, 2002). The specific genes involved in pathogenicity and aggressiveness were not identified, although this information would have provided some insight into interactions between the pathogen and the host.

In genetic linkage mapping, distance is given as centiMorgans (cM). One cM is defined as the distance that separates two genes between which there is a 1% chance of recombination (Rieger *et al.*, 1991). The physical distance (in base pairs) of 1 cM is dependent on the rate of recombination per physical distance and often covers a vast area that could include a number of genes and open reading frames (Kang *et al.*, 2011). Thus, linking of a QTL to a specific gene is complex as a QTL could span hundreds of open reading frames.

The genetic linkage map of *F. graminearum* (Jurgenson *et al.*, 2002a), was also used to aid in the location of *Tri15*, a gene involved in trichothecene production (Alexander *et al.*, 2004). This gene was mapped to *F. graminearum* linkage group II, separated from the main trichothecene gene cluster. Consequently, with the aid of the existing linkage map, *Tri15* was found to represent a fourth distinct trichothecene locus (Alexander *et al.*, 2004). Employment of the genetic linkage map was also used to investigate the relationship between polymorphic markers and the trichothecene gene cluster, although no linkage was determined (Desjardins *et al.*, 2004).

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Genetic linkage maps can be used to answer important questions about any organism, as indicated above. As will be shown in the next section, these maps can play an integral role in further genomic studies. With the aid of genetic maps, various *Fusarium* genomic sequences have revitalized research on this economically important genus of fungi.

The genome sequence of *Fusarium graminearum*

The first draft genome sequence of *F. graminearum* was released to the public in 2003 (www.broadinstitute.org). The genome is 36.1 Mb in size and a high level of genome coverage (~10X Sanger sequencing) was obtained (Cuomo *et al.*, 2007). However, the major ribosomal repeats and telomeres were not included in the assembly, resulting in a genome size prediction closer to 38 Mb (Goswami & Kistler, 2004).

An intraspecific cross for *F. graminearum* was used to generate a genetic map to validate and anchor the draft genome assembly (Gale *et al.*, 2005; Table 3). As with the Jurgenson *et al.* (2002a) genetic map, nine linkage groups were obtained. These nine linkage groups aligned with 22 scaffolds, leaving only five unanchored scaffolds (Gale *et al.*, 2005). These 22 scaffolds anchored 99.83% of the draft genome assembly and could be assembled into four chromosomes. A large-insert genomic DNA library (bacterial artificial chromosomes; BACs) was used to generate a BAC-based physical map of *F. graminearum* to augment the genomic resources for this fungus (Chang *et al.*, 2007). Two BAC libraries were developed with a genomic coverage of 11.9X. From these libraries, a physical map was generated consisting of 26 contigs that span 39.2 Mb. This physical map was integrated with the genomic sequence and the genetic linkage map of Gale *et al.* (2005). The BAC-based map is highly consistent with the genetic map and genomic sequence. Also, four clones were identified that span gaps in the genomic sequence.

As expected, the genomic sequence of *F. graminearum* has provided significant insight into this fungus. There are 13 718 genes on four chromosomes that encode predicted proteins (Wong *et al.*, 2011). Of these, 2001 are orphan genes, *i.e.* they show no similarity to any other sequenced genes from other organisms, and 5812 show homology to proteins that have no known function

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(Güldener *et al.*, 2006). Repetitive and duplicated DNA did not occur frequently (Goswami & Kistler, 2004; Gale *et al.*, 2005; Cuomo *et al.*, 2007). The fact that *F. graminearum* is homothallic and sexual recombination is rare (Bowden & Leslie, 1992, Walker *et al.*, 2001) may explain the small number of repetitive sequences, as the opportunity to acquire new repeats is limited by the low amount of sexual recombination. In a study examining simple repetitive sequences (SSRs) found in completed fungal genomes, the total number of SSRs in fungal species was not proportional to their genome sizes (Karaoglu *et al.*, 2004). *Neurospora crassa*, which has a genome size comparable to *F. graminearum*, has five times more SSRs. Interestingly, for *F. graminearum*, there was a lower frequency of CG/GC repeats than expected in all the fungal genomes compared, even though the four other fungal genomes investigated also had very fewer CG/GC repeats than expected. There is no explanation for the frequency variation of certain repeats in different fungal genomes (Karaoglu *et al.*, 2004). Generally, genomes that displayed low GC content were more likely to be dominated by A/T-rich microsatellites (Lim *et al.*, 2004).

The presence of infrequent repetitive DNA could also be due to repeat-induced point mutations (RIPs; Selker *et al.*, 1987). This is a genome wide defence system whereby duplicated sequences are identified and C:G to T:A transition mutations are introduced during meiosis (Watters *et al.*, 1999). The proposed mechanism of RIP involves the methylation of cytosine followed by deamination (Freitag *et al.*, 2002). It has been suggested that RIP decreases the homology of duplicated sequences, thereby reducing the incidence of crossovers during meiosis that could lead to chromosomal rearrangements (Cambareri *et al.*, 1991). This genomic defence system, which maintains genomic structure, has previously been identified in *F. graminearum* (Cuomo *et al.*, 2007), even though sexual recombination is limited.

A second genome sequence in *Fusarium* was generated for an additional strain of *F. graminearum* (Cuomo *et al.*, 2007). This strain was one of the parents used by Jurgenson *et al.* (2002a) to compile a genetic linkage map. This second genome sequence had a ~ 0.4X coverage (Cuomo *et al.*, 2007). When the two *F. graminearum* sequences were compared, 10 495 SNPs (single-nucleotide polymorphisms) were identified and mapped to the four chromosomes. Regions that displayed high SNP density were located on the telomeric regions of the

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chromosomes, as well as in the interstitial regions of three of the chromosomes. Ancestral chromosome fusion was cited as a possible reason for high SNP density in interstitial regions of chromosomes. Regions displaying high SNP density also showed the highest recombination rate and lower G + C content, which led to the hypothesis that interstitial regions represent ancestral telomeres (Cuomo *et al.*, 2007). This could explain the low number of chromosomes found in *F. graminearum*, in comparison to members of the *G. fujikuroi* complex, of which six are known to have 12 chromosomes (Xu *et al.*, 1995).

The 2001 orphan genes identified in the *F. graminearum* sequence are concentrated in the regions of high SNP density, in other words in the telomeric regions (Cuomo *et al.*, 2007). In *Magnaporthe oryzae*, genes that are involved in niche specificity are overrepresented in the subtelomeric regions (Farman, 2007). It has been hypothesized that the orphan genes, with their limited phylogenetic distribution, are major pathogenic genes that are specific to *F. graminearum* infection. Similarly, *F. graminearum* genes that are specifically expressed during plant infection are overrepresented in regions of high SNP density (Cuomo *et al.*, 2007). As more genomic data becomes available, more information regarding these elusive genes will be obtained.

A third assembly of *F. graminearum* was generated for the same strain as the original genome sequence. The genome size was found to be 36.45 Mb with ~10X coverage. This sequence was used to independently verify the genetic and physical mapping of Gale *et al.* (2005; Lee *et al.*, 2008). The Jurgenson *et al.* (2002a) map was aligned to the third *F. graminearum* genome assembly, which verified that four chromosomes were present. Using the genome sequence, the original nine linkage groups (Jurgenson *et al.*, 2002a) were reduced to six. These six linkage groups aligned with nine supercontigs, anchoring 99.2% of the assembly. Several gaps were also identified, suggesting incomplete genome assembly in the Gale *et al.* (2005) study.

The genetic linkage maps of *F. graminearum* (Jurgenson *et al.*, 2002a; Gale *et al.*, 2005) proved beneficial in the assembly of the *F. graminearum* genomic sequence (Gale *et al.*, 2005; Lee *et al.*, 2008) by anchoring and validating the draft genome assembly as well as confirmation of the presence of four chromosomes in *F. graminearum* (Jurgenson *et al.*, 2002a; Gale *et al.*, 2005; Lee *et al.*, 2008). Additionally, clones that spanned gaps in the genome sequence were useful to

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assemble scaffolds. Sequencing the genome of *F. graminearum* provided an estimation of genome size and yielded insights into the genomic structure of the fungus. This was primarily evident in the presence of the ancestral chromosomal fusion and the identification of a genomic defence system, namely RIPs.

The process of generating genetic linkage maps and the genome sequences of *Fusarium* spp. has enabled the generation of comparative studies between these species. These studies have started to answer many questions about the genetic architecture of these species as well as questions relating to pathogenicity.

Fusarium comparative genome studies

The staggering number of whole genomic sequence data available for fungi has enabled comparative genomic analysis between fungi that up to a decade ago was impossible (*e.g.* Ma *et al.*, 2010). In *Fusarium*, these studies have highlighted, amongst others, the genomic location of pathogenicity-related genes and how the process of loss or gain of these regions allowed related isolates to evolve in different ecological niches (Ma *et al.*, 2010).

In a genomic comparative study among *F. graminearum*, *F. verticillioides* and *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*; 15 chromosomes), the *Fol* genome was 44% larger than that of *F. verticillioides*, and 65% larger than *F. graminearum* (Ma *et al.*, 2010). The three *Fusarium* species carry a core genome that has a high level of synteny. The larger genome of *Fol*, comprising more than 25% of the total, is due to additional unique sequences that predominate in four extra chromosomes, the *Fol* lineage-specific (*Fol* LS) regions. The *Fol* LS regions contain genes related to pathogenicity including effector genes (avirulence [*avr*] genes encode for effectors that are key pathogenicity components for virulence; De Wit *et al.*, 2009). The horizontal transfer of LS chromosomes between strains of *F. oxysporum* converted a non-pathogenic strain into a pathogenic strain. The transfer of an entire group of pathogenicity genes could increase the severity of a given disease rapidly, and affect disease management (Ma *et al.*, 2010).

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When extending this *Fusarium* comparative study to *Fusarium solani* (17 chromosomes; Coleman *et al.*, 2009), the conserved core genome was also found in *F. solani* (Ma *et al.*, 2010). The *Fol* LS regions were absent in the *F. solani* genome. In addition, *F. solani* had three additional supernumerary chromosomes, or LS chromosomes, that comprised more repeat sequences, more unique and duplicated genes, as well as having a lower G + C content to the other *F. solani* chromosomes (Coleman *et al.*, 2009). The function of these *F. solani* LS chromosomes was postulated to enable isolates to occupy different environmental niches due to the presence of host-specific genes.

Synteny studies showed that there is on average a 90% sequence identity between *Fol* and *F. verticillioides* (Ma *et al.*, 2010). *Fusarium verticillioides* has analogous chromosomes in *Fol*, except for one major translocation event of a chromosome and a small number of local rearrangements. Similarly, *F. verticillioides* and *F. graminearum* displayed 80% sequence identity. The previously identified regions that displayed a high SNP density in *F. graminearum* (Cuomo *et al.*, 2007) were shown to correspond to the telomeric regions of *F. verticillioides* (Ma *et al.*, 2010). Interestingly, these highly polymorphic regions have continued to evolve as subtelomeres, even though chromosomal fusion has occurred. It has been postulated that epigenetic modification persists in denoting these fused sites as subtelomeres.

The *F. verticillioides* strain (7600) that was sequenced has no sequence data or genetic markers for the twelfth chromosome (Ma *et al.*, 2010). From the genetic linkage map of this *Fusarium*, it was found that this chromosome is possibly a dispensable chromosome, which in some progeny may be duplicated, or partially or totally deleted (Xu & Leslie, 1996; Jurgenson *et al.*, 2002b). Strain 7600 of *F. verticillioides* that was sequenced does not have chromosome 12 (J.F. Leslie, personal communication). It has been shown that some fungal dispensable chromosomes confer advantages to the fungus by way of pathogenicity (Funnell & VanEtten, 2002) and the ability to colonize different environmental niches, as with the supernumerary chromosomes discussed above.

Interestingly, the supernumerary chromosomes and lineage-specific regions associated with pathogenicity, all contain higher than usual numbers of transposable elements (Rep & Kistler,

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2010). It will be interesting to learn whether these transposons play a role in the mobility of these regions, or whether this is merely coincidence.

Interspecific crosses in the *G. fujikuroi* species complex

Fusarium subglutinans was first reported on maize in Nebraska where it caused kernel mould disease (Sheldon, 1904). The fungus causes disease on members of the Gramineae and is an ubiquitous pathogen of maize in all maize growing countries (Booth, 1971), where it causes stalk, root and ear rot disease as well as seedling blight (Burgess *et al.*, 1981). This causes yield loss, reduction in grain quality, as well as the maize being rendered toxigenic due to the presence of mycotoxins produced by the fungus (Booth, 1971).

Both *F. circinatum* (O'Donnell *et al.*, 1998) and *F. subglutinans* (Nelson *et al.*, 1983) reside in the *G. fujikuroi* species complex. They represent different biological species (Table 2): *F. circinatum* belongs to mating population H (Britz *et al.*, 1999) and *F. subglutinans* to mating population E (Nelson *et al.*, 1983), and are phylogenetically distinct species (Nirenberg & O'Donnell, 1998; O'Donnell *et al.*, 1998).

In a study by Desjardins *et al.* (2000), *Fusarium* isolates from maize and the closely related wild teosinte (*Zea* spp.) in Mexico and Central America, were examined. Based on morphology, isolates were identified as *F. subglutinans*. However, using mating experiments, one isolate from teosinte was moderately fertile when crossed to an isolate of *F. circinatum*. It was hypothesized that the isolates from Mexico and Central America represent a new mating population in the *G. fujikuroi* species complex, even though one isolate was fertile with an isolate from mating population H. However, by utilizing phylogenetic analyses and sexual compatibility tests, these *F. subglutinans* isolates where shown to belong to mating population E (Steenkamp *et al.*, 2001). The isolate from teosinte that was moderately fertile with an isolate of *F. circinatum* thus represents an example of an interspecific cross in the *G. fujikuroi* species complex.

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Despite the use of the biological species concept in species delineation in the *G. fujikuroi* species complex, hybridization of other closely related species has also been reported (Desjardins *et al.*, 1997, 2000; Leslie *et al.*, 2004a, b). In *Fusarium fujikuroi* (mating population C) and *Fusarium proliferatum* (mating population D), a few isolates are sexually compatible (Desjardins *et al.*, 1997; Leslie *et al.*, 2004b), and a naturally occurring hybrid has been found (Leslie *et al.*, 2004a). These authors suggested that the standard tester strains for these two biological species may be represented by distinct species, but that a hybrid swarm might exist between these two species. It could also be possible that the two species are in the final stages of speciation and that some individuals in each species could still overcome crossing barriers.

The interspecific cross between *F. circinatum* and *F. subglutinans* (Desjardins *et al.*, 2000) provides an unique opportunity to study genetic differentiation between these two different species, using genetic linkage mapping. Due to the wide nature of such a cross, a large number of polymorphic markers could be generated with ease. A genetic linkage map would, in addition, estimate genome length and might help determine why this interspecific cross is possible. With QTL mapping, any quantitative trait of interest could be mapped on existing genetic linkage maps.

Inconsistencies are encountered when dealing with an interspecific cross versus an intraspecific cross (*e.g.* Rieseberg & Linder, 1999; Burke & Arnold, 2001). One of these discrepancies is the higher than expected transmission ratio distortion (TRD) or segregation distortion that could be indicative of isolating mechanisms that have arisen due to speciation (Bradshaw & Stettler, 1994). In an interspecific cross of *F. graminearum*, segregation distortion was found in the genetic linkage map of Jurgenson *et al.* (2002a). TRD has been attributed to linkage between molecular markers and factor(s) that function in the pre- and postzygotic stages of reproduction (Zamir & Tadmor, 1986), especially when these distorted markers cluster nonrandomly amongst the linkage groups (Schwarz-Sommer *et al.*, 2003; Myburg *et al.*, 2004; Hall & Willis, 2005). Genes that display non-Mendelian inheritance are enhancing their own transmission to the next generation. The process of preferential inheritance of alleles occurs during meiosis (prezygotic) or afterwards (postzygotic) and can be explained by three mechanisms: Chromosome loss,

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greater genomic divergence between the two parental species, such as found in interspecific crosses and linkage to a lethal gene (Bradshaw & Stettler, 1994).

Comparative genomic studies between different *Fusarium* species should deliver the key understandings of a range of molecular mechanisms for this fungus. At present, the genome of *F. circinatum* has been sequenced to 10X coverage (Wingfield *et al.*, 2012), although the sequence is not yet publicly available. The same pitch canker isolate that was used to generate the interspecific cross has been sequenced. A comparison of a subset of sequenced polymorphic (AFLP) markers from the genetic linkage map of the interspecific cross to the available *Fusarium* genomic, would aid in synteny studies of these *Fusarium* species, and add *F. circinatum* and *F. subglutinans* to the comparative genomics era.

Conclusion

In this literature review, I have used *F. graminearum* as the model *Fusarium* on which genomic work had been done. Comparative studies between related organisms promise to advance our understanding of host-pathogen interactions. Members of the Eumycota possess specific suites of genes that enable them to occupy their specific niche (postulated to be represented by the orphan genes). Identification of these orphan genes would be a first step in the elucidation of the molecular mechanisms behind fungal virulence and pathogenesis in *F. circinatum*. This would enhance our understanding of *Fusarium* biology, and possibly reveal new approaches to control economically important diseases these fungi cause on crops and trees.

Members of the *G. fujikuroi* species complex are of economic interest globally due to the impact they have on agricultural crops and commercially planted trees. Two of these, *F. circinatum* and *F. subglutinans*, are the subject of chapters of this thesis, specifically an interspecific cross between these two species (Desjardins *et al.*, 2000). Morphologically, *F. circinatum* and *F. subglutinans* fall into the *F. subglutinans sensu lato* species. Using the biological and phylogenetic species concepts, these were found to represent two separate species (Nelson *et al.*, 1983; Nirenburg & O'Donnell, 1998; O'Donnell *et al.*, 1988; Britz *et al.*, 1999). Taken together,

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this illustrates how close these two species are phylogenetically (O'Donnell *et al.*, 1998; Steenkamp *et al.*, 1999, 2000). It also highlights problems that arise from identifying *Fusarium* species using the biological species concept. Interspecific hybridization has been well documented in other fungi (*e.g.* Lind *et al.*, 2005), as well as within the *G. fujikuroi* species complex (Desjardins *et al.*, 1997; Leslie *et al.*, 2004b). The interspecific cross between *F. circinatum* and *F. subglutinans* has provided an unique opportunity to study genetic differentiation by way of genetic linkage mapping using polymorphic AFLP markers. This has provided the basis for the remaining studies of this thesis. The genetic linkage map of the interspecific cross will be used as a platform for further genomic studies, in particular, QTL identification of traits, as well as using the genetic linkage map to anchor the *F. circinatum* genomic sequence.

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Table 1. *Fusarium* genomes that have been sequenced.

Organism	Strain	Biological property	Genome size (Mb)
Fusarium circinatum	Fsp34[1]	Pine pitch canker disease	43.97
Fusarium culmorum	[2]	Seedling blight and other diseases of cereals	unknown
Fusarium fujikuroi	IMI58289[2]	*Bakanae* disease in rice seedlings	42.96
Fusarium graminearum	PH-1& GZ3639[3]	Fusarium head blight of small grains	36.45
Fusarium langsethiae	[2]	Kernel pathogen of oats, wheat and barley	unknown
Fusarium mangiferae	[4]	Causes mango malformation	unknown
Fusarium oxysporum f. sp. *lycopersici*	4287[5]	Fusarium vascular wilt of tomato	61.36
F. oxysporum	Fo47[6]	Biological control of wilt diseases	49.66
F. oxysporum	FOSC 3-a[6]	Necrotic diseases in immunocompetent humans	47.91
F. oxysporum f. sp. *pisi*	HDV247[6]	Wilt disease of peas	55.19
F. oxysporum f. sp. *lycopersici*	MN25[6]	Fusarium vascular wilt of tomato	48.64
F. oxysporum f. sp. *radicis-lycopersici*	CL57[6]	Tomato crown rot	49.36
F. oxysporum f. sp. *vasinfectum*	[6]	Wilt disease of cotton	52.91
F. oxysporum f. sp. *cubense*	IIS[6]	Fusarium wilt of banana	46.55
F. oxysporum f. sp. *melonis*	[6]	Wilt disease of melon	54.03

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Table 1. *(continued)*

Organism	Strain	Disease	Genome size (Mb)
F. oxysporum f. sp. *conglutinans*	PHW808[6]	Cabbage yellow disease, *Arabidopsis* wilt disease	53.58
F. oxysporum f. sp. *raphani*	PHW815[6]	Radish wilt disease, *Arabidopsis* wilt disease	53.5
Fusarium pseudograminearum	[2,7]	Fusarium crown rot of wheat and barley	unknown
Fusarium solani	77-13-4[8]	Soilborne pathogen (broad plant host range)	54.43
Fusarium verticillioides	7600[5]	Fusarium ear rot of maize	41.78
Fusarium virguliforme	Mont-1[9]	Soybean sudden death syndrome	50.90

[1]Wingfield *et al.*,2011
[2]http://www.fgsc.net/Fusarium/2011FusWkshp_Program.htm
[3]Cuomo *et al.*, 2007
[4]Personal communication by Dr. Stanley Freeman
[5]Ma *et al.*, 2007
[6]*Fusarium* Comparative Sequencing Project, Broad Institute of Harvard and MIT (http://0-www.broadinstitute.org.innopac.up.ac.za/)
[7]Gardiner *et al.*, 2011
[8]Coleman *et al.*, 2009
[9]Srivastava *et al.*, 2011

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Table 2. Mating populations of the *Gibberella fujikuroi* species complex and their corresponding anamorphs and teleomorphs, first description of the teleomorph and host plants.

Mating population	Anamorph	Teleomorph	Teleomorph description	Host originally described on
A	*F. verticillioides*	*G. moniliformis*	Hsieh *et al.* (1977)	Maize, rice
B	*F. sacchari*	*G. sacchari*	Leslie *et al.* (2005)	Sugarcane, sorghum
C	*F. fujikuroi*	*G. fujikuroi*	Hsieh *et al.* (1977)	Rice
D	*F. proliferatum*	*G. intermedia*	Kuhlman (1982a)	Rice, maize, sorghum
E	*F. subglutinans*	*G. subglutinans*	Nelson *et al.* (1983)	Maize
F	*F. thapsinum*	*G. thapsina*	Klittich & Leslie (1992)	Sorghum, maize
G	*F. nygamai*	*G. nygamai*	Klaasen & Nelson (1996)	Soil, soil debris, millet, sorghum
H	*F. circinatum*	*G. circinata*	Britz *et al.* (1999)	Pine
I	*F. konzum*	*G. konza*	Zeller *et al.* (2003)	Prairie grass
J	*F. xylarioides*	*G. xylarioides*	Lepoint *et al.* (2005)	Coffee

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Table 3. Genetic linkage maps of *Fusarium* spp. Information given was provided by the relevant reference, unless stated otherwise.

Organism	Number of linkage groups	Number of polymorphic markers mapped	Map length (cM)	Physical distance per unit of recombination (kB/cM)	Average distance between markers (cM/interval)
Fusarium verticillioides[1]	3	9	N/A	N/A	N/A
Fusarium verticllioides[2]	4	12	N/A	N/A	N/A
Fusarium verticllioides[3]	13	143	1452	32	10
Fusarium verticllioides[4]	12	486 + 150[a]	2188	21	3.4
Fusarium graminearum[5]	9	1048	1286	28[b]	2.8
Fusarium oxysporum f. sp. *lycopersici*[6]	23	83	N/A	N/A	N/A
Fusarium graminearum[7]	9	235	1234	29	5.3[c]

[1]Puhalla & Spieth, 1983
[2]Puhalla & Spieth, 1985
[3]Xu & Leslie, 1996
[4]Jurgenson *et al.*, 2002b
[5]Jurgenson *et al.*, 2002a
[6]Teunissen *et al.* 2003
[7]Gale *et al.* 2005
[a]150 polymorphic markers are from the map of Xu & Leslie, 1996
[b]Genome size of 36.45 Mb from Cuomo *et al.* (2007) was used to calculate the physical distance per unit of recombination
[c]Calculated from Gale *et al.*(2005).

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CHAPTER 2

Complete genetic linkage maps from an interspecific cross between *Fusarium circinatum* and *Fusarium subglutinans*[1,2]

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[1]De Vos, L., Myburg, A.A., Wingfield, M.J., Desjardins, A.E., Gordon, T.R., 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.

[2]The text from this publication is reproduced in full. It has however, been reformatted to conform with the layout of the thesis.

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Abstract

The *Gibberella fujikuroi* complex includes many plant pathogens of agricultural crops and trees, all of which have anamorphs assigned to the genus *Fusarium*. In this study, an interspecific hybrid cross between *G. circinata* and *G. subglutinans* was used to compile a genetic linkage map. A framework map was constructed using a total of 578 AFLP markers together with the mating type (*MAT-1* and *MAT-2*) genes and the histone (H3) gene. Twelve major linkage groups were identified (n=12). Fifty percent of the markers showed significant deviation from the expected 1:1 transmission ratio in a haploid F_1 cross (P<0.05). The transmission of the markers on the linkage map was biased towards alleles of the *G. subglutinans* parent, with an estimated 60% of the genome of F_1 individuals contributed by this parent. This map will serve as a powerful tool to study the genetic architecture of interspecific differentiation and pathogenicity in the two parental genomes.

Introduction

Gibberella fujikuroi (Sawada) Wollenw. is the species complex associated with species that have anamorphs in *Fusarium* section *Liseola*. These include many important fungal pathogens of agricultural crops and trees. The *Fusarium* species associated with this complex include at least eleven different biological species (mating population A–K), which are reproductively isolated (Nirenberg & O'Donnell, 1998; Samuels *et al.*, 2001; Zeller *et al.*, 2003; Phan *et al.*, 2004; Lepoint *et al.*, 2005).

Traditionally, the morphological species concept has been used to describe species in the *G. fujikuroi* complex, but differentiation between species following this approach has generally been unsatisfactory. One example is *F. subglutinans sensu lato* within which three mating populations (B, E and H) have been identified, based on patterns of inter-isolate fertility. Recognition of these mating populations as distinct species is supported by multigene phylogenies (Nirenburg & O'Donnell, 1998; O'Donnell *et al.*, 1998). *Fusarium subglutinans*

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sensu stricto is used for strains isolated from maize and belongs to mating population E (Nirenburg & O'Donnell, 1998; O'Donnell *et al.*, 1998). *Fusarium circinatum* is the name applied to isolates from pine that cause pitch canker, which are associated with mating population H of the *G. fujikuori* complex (Nirenburg & O'Donnell, 1998; Britz *et al.*, 1999). The biological species concept has been used extensively to characterise other species in the *Gibberella fujikuroi* complex. However, this approach has limited value because the majority of species have no apparent sexual stage (Steenkamp *et al.*, 2002).

Fusarium circinatum (mating population H), also known as the pitch canker fungus, is a pathogen of many pine species, and is especially damaging to *Pinus patula* and *Pinus radiata* (McCain *et al.*, 1987; Viljoen & Wingfield, 1994). This fungus was first discovered in the United States in 1946 (Hepting & Roth, 1946) and has since spread to many other parts of the world including South Africa. In the latter country it causes root rot and damping off of *P. patula* and other susceptible pine species in seedling nurseries (Viljoen & Wingfield, 1994).

Fusarium subglutinans (mating population E) is a common pathogen of domesticated maize (*Zea mays* spp. *mays*). Desjardins *et al.* (2000) studied isolates of *Fusarium* from maize and the closely related wild teosinte (*Zea* spp.) in Mexico and Central America, in an attempt to characterize these isolates and determine an appropriate mating population for them. Strains identified based on morphology as *F. subglutinans* from maize and teosinte, were infertile when crossed with tester strains from mating populations B, E and H. However, one strain of *F. subglutinans* isolated from teosinte was moderately fertile in a cross with an isolate of *F. circinatum* (mating population H). Desjardins *et al.* (2000) suggested that all *F. subglutinans* strains treated in this study represent a fourth distinct mating population associated with *F. subglutinans.* This was due to the almost complete infertility with the standard tester strains. Steenkamp *et al.* (2001) applied phylogenetic analyses and sexual compatibility tests to show that *F. subglutinans* isolates from teosinte belong to mating population E. They are phylogenetically more closely related to each other than to other mating populations within the *G. fujikuroi* species complex (O'Donnell *et al.*, 1998; Steenkamp *et al.*, 1999, 2000), indicating they share a greater ancestry. Hybridization of closely related fungal species, such as mating population E and H forming the basis of this study, has been documented in other fungi (Lind et

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al., 2005) as well as within the *Liseola* section of *Fusarium* (Desjardins *et al.*, 1997; Leslie *et al.*, 2004b).

Fusarium circinatum and *F. subglutinans* threaten forestry and maize production in South Africa. Both these industries are considered integral parts of South Africa's economy with maize being considered to be the staple diet of South Africans with approximately 25% of South Africa's total arable land use being planted to maize. Therefore, both commercial forestry and maize are of economic importance in South Africa and maintaining these sectors is of important to the economy of the country.

The cross between *F. circinatum* and *F. subglutinans* made by Desjardins *et al.* (2000) provided us with a unique opportunity to study genetic differentiation using genetic linkage mapping. Genetic linkage maps have also been used to study other fungi including *F. verticillioides* (Xu & Leslie, 1996; Jurgenson *et al.*, 2002b) and *F. graminearum* (Jurgenson *et al.*, 2002a; Gale *et al.*, 2005). In general, AFLP markers are preferred when generating linkage maps. The objective of our study was to use AFLP markers (Vos *et al.*, 1995) to generate a genetic linkage map for an interspecific cross between *G. circinata* and *G. subglutinans*. This map should provide a useful framework for further study of the architecture of the two parental genomes and elucidation of the genetic determinants of pathogenicity to pine.

Materials & Methods

Fungal isolates

Isolates used for genetic linkage analysis were F_1 progeny from the cross between *G. circinata* (maternal parent; *MAT-1*) and *G. subglutinans* (paternal parent; *MAT-2*) from the study of Desjardins *et al.* (2000). The parents of this cross were isolates MRC7828 (*G. subglutinans*) and MRC7870 (*G. circinata*) (Table 1). Ninety-four F_1 isolates were randomly selected from 226 viable ascospore progeny obtained from 14 perithecia for use in this study.

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DNA isolation

Isolates were grown on half strength PDA (potato dextrose agar; 20% potato dextrose agar and 5% agar) for 7 days at 25°C in the dark. Mycelium was harvested and 300 µl extraction buffer (200 mM Tris [pH 8], 250 mM NaCl, 25 mM EDTA [pH 8], 0.5% w/v SDS) was added (Raeder & Broda, 1985). This mixture was homogenised at 4 m/s for 20 s using the Fastprep FP120 (QBIOgene, Farmingdale, NY, USA) system. Following homogenisation, the tissue was frozen in liquid nitrogen and then thawed in boiling water for 5 minutes. Phenol-chloroform (1:1) extractions were performed (10 600xg for 5 minutes) until all cell debris had been removed. Thereafter, 0.1 volumes of 3 M sodium acetate (pH 8) and two volumes of cold absolute ethanol were added and the Eppendorf tubes were inverted five times. After centrifugation at 10 600xg for 5 min, 1 ml 70% ethanol was added to the pellet and allowed to stand for 5 min at room temperature (Sambrook *et al.*, 1989). The precipitated DNA was centrifuged for a further 5 min at 2700xg and dried under vacuum. The DNA was resuspended in 500 µl low TE (10 mM Tris [pH 8], 0.1 mM EDTA).

AFLP analysis

AFLP analysis was performed essentially following the protocol of Vos *et al.* (1995). Restriction digestion of the genomic DNA was performed using *Eco*RI and *Mse*I (Zeller *et al.*, 2000). These restriction fragments were ligated to the corresponding enzyme-specific oligonucleotide adapters (Vos *et al.*, 1995). Preselective amplifications were performed with zero-base-addition *Eco*RI and *Mse*I adapter-specific primers using the following PCR conditions: 1 cycle of 30 s at 72°C, 25 cycles of 30 s at 94°C, 30 s at 56°C and 1 min at 72°C with an increase of 1 s per cycle and a final elongation step of 2 min at 72°C. Final selective amplifications used *Eco*RI and *Mse*I primers (Table 2) with two-base-additions. The *Eco*RI primer was labelled with the infrared dyes, IRDye™ 700 or IRDye™ 800 (LI-COR, Lincoln, NE). PCR conditions were as follows: 13 cycles of 10 s at 94°C, 30 s at 65°C with a decrease of 0.7°C per cycle and 1 min at 72°C followed by 23 cycles of 10 s at 94°C, 30 s at 56°C and 1 min at 72°C with an increase of 1 s per cycle and a final elongation step of 1 min at 72°C.

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AFLP fragment analysis was performed on a model 4200 LI-COR® automated DNA sequencer as described by Myburg *et al.* (2001). Electrophoresis run parameters were set to the following: 1500 V, 35 mA, 35 W, 45°C, motor speed 3 and signal filter 3. Electrophoresis prerun time was set to 30 min and the run time to 4 h.

Digital gel images obtained from the LI-COR system were analysed using the Saga^{MX} AFLP® Analysis Software package (LI-COR) according to the manufacturers' instructions. Only markers that were polymorphic for the two hybrid parent strains were scored with a '0' indicating absence, '1' indicating presence of bands and 'X' indicating missing data.

Additional marker analysis

PCR identification of the mating types of all the isolates was performed as described by Steenkamp *et al.* (2000). It was not possible to perform the multiplex PCR as described, but superior results were achieved when the *MAT-1* or *MAT-2* primer sets were used separately. An annealing temperature of 65°C was used for the *MAT-2* PCR and 67°C for *MAT-1*.

Because the F_1 isolates were hybrids of *F. circinatum* and *F. subglutinans*, PCR-RFLP analysis of the histone H3 gene was used to distinguish between parental alleles (Steenkamp *et al.*, 1999). This PCR-RFLP technique successfully determined the parental origin of the histone H3 alleles segregating in the F_1 isolates. The results of all these amplifications were scored as a '0' for band absent and '1' for band present.

Framework linkage map construction

Chi-square analysis was performed on all markers to test for departure from the expected Mendelian segregation ratio (1:1) using a significance threshold of $\alpha = 0.05$. All markers, including those showing transmission ratio distortion, were included in framework map construction in order to optimise map coverage.

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Based on the origin of each marker, the data were separated into two parental data sets. Linkage analysis was performed on the separate and joint data sets to obtain separate parental framework linkage maps and a F_1 framework linkage map. A maternal map of the *F. circinatum* parent and a paternal map of the *F. subglutinans* parent were generated, representing the two linkage phases of the F_1 map. The parental (linkage phase) maps were constructed separately to increase the confidence of marker ordering in the F_1 map. Markers having greater than 10% missing data were dropped from the data sets before framework map construction in MAPMAKER Macintosh V2.0 (Lander *et al.,* 1987). Data were regarded as F_2 backcross configuration to accurately analyse segregation in the haploid F_1 genomes (Xu & Leslie, 1996). The Kosambi mapping function was used. The haploid chromosome number of *F. subglutinans* is known (n = 12) (Xu *et al.*, 1995). This information was used when distributing markers into linkage groups by evaluating the LOD linkage thresholds from 6 to 14, in incremental steps of 1.0, by using the 'Group' command (Myburg *et al.*, 2003). The parental marker sets was separated into at least 12 linkage groups at LOD thresholds of 9 and 10.

To select framework markers, markers in each linkage group were subjected to the 'First Order' command of MAPMAKER to attain a starting order. Using the 'Drop Marker' command, internal markers that expanded the map by more than 11 cM were dropped. After a marker had been dropped, the 'First Order' step was repeated. Using the 'Ripple' function, the support of the remaining markers was evaluated. Markers that did not have a LOD interval support of at least 1.5 were removed from the map. The 'First Order' step was again repeated after each marker was dropped. Finally the terminal markers were evaluated with the 'TwoPoint/LOD Table' command. Terminal markers that showed stronger pairwise linkage to internal markers than to adjacent markers were dropped (Myburg *et al.*, 2003).

In order to combine the parental maps and construct a single integrated map (referred to herein as the "F_1 map"), marker presence/absence data from the maternal data set was recoded to indicate that band absent ('0') represented *F. circinatum* markers. The two data sets (the recoded maternal data set and the paternal data set) were then combined into one data set and map construction was performed using the MAPMAKER program as described above. Markers were distributed into linkage groups using the 'Group' command by evaluating the LOD linkage

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thresholds from 6 to 14, in incremental steps of 1.0. The mapping set was separated into 12 major linkage groups at a LOD threshold of 9. Higher thresholds were used for the 'Drop Marker' command and 'Ripple' functions, with markers expanding the map length by 10 cM and not having a LOD interval support of at least 3.0 being dropped respectively.

Data from the F_1 map were subjected to the Graphical GenoTyping program (GGT) (Van Berloo, 1999). This was used to inspect the distribution of crossovers for each chromosome. This program was also used to determine if any nonrecombinant or duplicate progeny was found to show that this interspecific cross was the product of a heterothallic event.

Bin mapping of accessory markers

AFLP markers that did not meet framework marker criteria were mapped to the framework map as accessory markers using the bin mapping function of the MapPop V1.0 program. This program places accessory markers into map intervals contained in a previously constructed high confidence framework map (Vision *et al.*, 2000). Only markers with $P > 0.95$ were placed into framework intervals. MapPop does not allow for the placement of accessory markers outside the terminal framework markers of each linkage group. Thus, AFLP markers not placed with MapPop were assessed for linkage to terminal markers using the 'Two Point/LOD' function of MAPMAKER (Myburg *et al.*, 2003). Markers showing any linkage to the terminal markers at LOD 3.0 were placed as terminal accessory markers.

Estimated genome coverage and length

The total genome length (L) of the F_1 map was estimated using the Hulbert estimate (Hulbert *et al.*, 1988) as modified in method 3 of Chakravarti *et al.* (1991), giving $L = n(n-1)d/k$. Here n is the total number of markers, d is the map distance which corresponds to the LOD threshold at which linkage was determined (Z) and k is the number of markers linked at the LOD threshold of Z or greater. The linkage threshold of LOD 9.0 (Z) was used to estimate genome length as the mapping set was separated into 12 linkage groups at this threshold.

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Theoretical map coverage was calculated using the formula $c = 1 - e^{-2dn/L}$, where c is the proportion of the genome within *d* cM of a framework marker, *n* is the number of framework markers in the map and *L* is the estimated genome length (Lange & Boehnke, 1982).

Results

DNA isolation and AFLP analysis

DNA was successfully isolated from the 94 selected F_1 individuals as well as from the parents of the interspecific cross between *F. circinatum* and *F. subglutinans*. AFLP analyses were performed on these individuals (Table 2). Five hundred and seventy-eight polymorphic AFLP markers were identified with an average of 45 polymorphisms per primer combination. In total, 56% of AFLP fragments were polymorphic. One marker was dropped for linkage analysis as it had more than 10% missing data. Missing data was defined as bands that could not be scored with confidence due to local gel irregularities, weak amplification, etc. This represented only 1% of the final data set.

Five hundred and eighty two markers (578 AFLP markers and four other genetic markers) were generated. The parent-specific alleles of the *MAT* and H3 marker loci were considered as four different markers for mapping purposes. Of the 582 markers, 50% deviated significantly from the expected 1:1 ratio for a haploid F_1 cross ($\alpha = 0.05$, Table 3). One hundred and seventy-nine markers (31%) differed at the 1% level of significance and 97 (17%) at the 0.1% level of significance (results not shown). Only 12 of the markers that showed transmission ratio distortion at $\alpha = 0.05$ were skewed towards the *F. circinatum* parent with the remainder being skewed towards the *F. subglutinans* parent. The number of markers generated from each parent (296 from *F. subglutinans* and 286 from *F. circinatum*) did not differ significantly ($P = 0.68$).

Framework linkage map construction

All markers, including those showing transmission ratio distortion, were evaluated during framework map construction using the criteria described before. Using MAPMAKER, mapping

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sets were separated into at least 12 major linkage groups at LOD thresholds of 9 and 10. Subsequent analyses were performed on the linkage groups obtained at the LOD threshold of 9.0, as at this threshold more markers were incorporated into the 12 major linkage groups.

Twelve linkage groups emerged for the two parental framework maps as well as the F_1 map (Figure 1). This corresponds to the haploid chromosome number reported for *F. subglutinans* (Xu *et al.*, 1995). Only 252 markers (43%) met our criteria for framework markers in the F_1 map (Table 3). Less stringent framework marker criteria were subsequently used for construction of the parental (linkage phase) framework maps to ensure that all markers placed in the F_1 map were also present in the parental maps. The 252 markers in the F_1 map corresponded to 104 markers in the *F. subglutinans* parental map and 148 markers in the *F. circinatum* parental map.

Linkage groups in the F_1 map ranged in size from ~141 cM (Linkage Group 10) to ~358 cM (Linkage Group 1). The total observed length of the map was 2 774 cM and the average distance between markers was 12 cM. Significantly (P = 0.041) more markers from the *F. circinatum* parent were incorporated into the F_1 map than from the *F. subglutinans* parent (Table 3).

The *MAT* idiomorphs mapped to Linkage Group 3 (Figure 1). Linkage Group 3, therefore, corresponds to chromosome 6 as previously reported for *F. verticillioides* (Xu & Leslie, 1996). The histone H3 gene mapped to linkage group 11 (Figure 1).

Based on output from the Graphical GenoTyping (GGT) program, the estimated proportion of the genome of the F_1 progeny that was descended from *F. subglutinans* was 59.8% and from *F. circinatum* 39.7%, with 0.5% of the genome being unknown due to missing data that was scored as 'X'.

Using the GGT program, the number of progeny lacking any crossovers on each linkage group was determined (Table 4). Of the 12 linkage groups, Linkage Group 5 ($P < 0.05$) and 6 ($P < 0.001$) showed significant deviation from the expected 1:1 origin of markers. Both of these linkage groups had a substantial number of markers from *F. circinatum* (Table 4). This was also reflected in the fact that a significantly greater number of markers were placed in the F_1 map

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from *F. circinatum.* F_1 progeny that received intact linkage groups tended to inherit these from the *F. subglutinans* parent, which was significant for Linkage Group 7 and 11 ($P < 0.05$), 1, 4, 8 and 12 ($P < 0.01$) and 10 ($P < 0.001$). No duplicate progeny was found, supporting the view that the interspecific cross forming the basis of this study was the product of a heterothallic event.

Bin mapping of accessory markers

Using MapPop and MAPMAKER, 82% of the remaining markers were placed in the intervals between framework map markers as well as outside the terminal markers (Table 3). Approximately 10% of markers were not mapped to the framework maps. This is most likely due to scoring error or the markers being too distant from terminal markers ($\theta > 0.45$) to include them in the final map.

Estimated genome coverage and length

Estimation of genome length using the method of Hulbert (Hulbert *et al.*, 1988; Chakravarti *et al.*, 1991) showed that the Hulbert estimate was 16% lower than the observed map length for the F_1 map (Table 3). Using the Hulbert estimate of genome length, an estimated 99% of loci in the F_1 hybrid map were within 20 cM of a framework marker and an estimated 89% of loci were within 10 cM of a framework marker (Table 3).

Discussion

The F_1 progeny analysed in this study were the product of an interspecific cross between *F. circinatum* and *F. subglutinans*. Because these fungi are haploid, analysis of segregation patterns in F_1 progeny is similar to that of a backcross population in a diploid organism. No prior cloning or sequence data were required for the AFLP analyses and a large number of markers (average 45 markers produced per primer combination) were generated. Twelve linkage groups were found for the framework maps, which is consistent with the haploid chromosome

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number of *F. subglutinans* (Xu *et al.*, 1995). The haploid chromosome number for *F. circinatum* is not known.

In this study, 582 polymorphic markers were generated and of these 252 were used to compile an F_1 framework linkage map. Two separate framework maps were also generated for the parental strains of this interspecific cross in order to evaluate the stability of marker ordering and map distances in the parental (linkage phase) maps and the F_1 map. The ordering of markers in two different (mutually exclusive) sets allowed us to independently evaluate the possible local effects of specific marker combinations and their associated errors. As could be expected, the parental maps were generally shorter than the F_1 map due to lower map coverage. This is shown clearly in Linkage Group 1H, which has no markers originating from *F. circinatum* at the top end of the linkage group. The parental maps were also less inflated due to the presence of fewer markers. It has been shown previously that the addition of markers expands the length of linkage groups (Jurgenson *et al.*, 2002b), which could be due to possible scoring error (Hackett & Broadfoot, 2003). A lower threshold was used in constructing the parental framework maps so that all markers present in the F_1 map could also be placed in the parental maps. When the same threshold was used for the parental and the F_1 maps, several markers could not be placed in the parental maps, especially in cases where map intervals became very large due to low map coverage.

The addition of the parental maps in this study has allowed us to compare the parental maps and the F_1 map. Even though a reasonable comparison could be drawn between them, significant differences do exist. Increasing the number of markers (as is the case with the F_1 map) provided better map coverage at linkage group terminals as well as increased statistical rigour to the framework linkage map.

Genetic maps have been published for other *Fusarium* species (Xu & Leslie, 1996; Jurgenson *et al.*, 2002a, b; Gale *et al.*, 2005). The genetic map of *F. verticillioides* had a total length of 1452 cM and a physical distance per unit of recombination of ~32 kb/cM (Xu & Leslie, 1996). Addition of AFLP markers to the existing RFLP map increased the map length to 2188 cM and the physical distance per unit of recombination decreased to ~21 kb/cM (Jurgenson *et al.*,

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2002b). The genetic map of *F. graminearum* had a map length of 1286 cM (Jurgenson *et al.*, 2002a). However, phylogenetic evidence has subsequently shown that this map was based on an interspecific cross between *F. graminearum* and *F. asiaticum* (O'Donnell *et al.*, 2004). A genetic map of *F. graminearum* had a map length of 1234 cM (Gale *et al.*, 2005). The F_1 map in this study had a map length of 2774 cM and the physical distance per unit of recombination was ~20 kb/cM. Thus, the F_1 map produced in this study is consistent with previous published maps for *Fusarium* spp.

The observed map length for *F. subglutinans* in this study was only 5.6% larger than the *F. circinatum* map. This is despite the fact that there were 42.3% more framework markers included in the *F. circinatum* map. Conversely, approximately 60% of the F_1 genome was descended from the *F. subglutinans* parent. This is explained by the average spacing between framework markers being greater for *F. subglutinans* leading to a larger observed map length.

The two parental species, *F. circinatum* and *F. subglutinans*, shared 44% AFLP identity. Leslie *et al.* (2001) noted that, within the *Liseola* section of *Fusarium*, strains that share >65% band identity represent the same biological species. In contrast, those of different species usually share no more than 40% band identity, and often significantly less. Although the two parental strains used in this study represent discrete taxa, our results showed a higher level of band identity (44%) than isolates studied in other mapping studies using AFLP analysis for intraspecific crosses in *Fusarium* (Jurgenson *et al.*, 2002a). Furthermore, in the map of an interspecific cross between *F. graminearum* and *F. asiaticum*, although not in the *Liseola* section of *Fusarium*, 50% band identity was observed between the two isolates used to construct the genetic map (Jurgenson *et al.*, 2002a). In an interspecies cross between *G. fujikuroi* (mating population C) and *G. intermedia* (mating population D) band identity was approximately 50% (Desjardins *et al.*, 1997; Leslie *et al.*, 2004b). Although in separate mating populations, the authors hypothesized that these two species might be consolidated into a single species. Thus, genetic similarity as determined from the percentage band identity using AFLPs appears to be consistent with relationships inferred from phylogenetic analyses based on differences in DNA sequences. The two parental strains in this study are different species, but are more closely

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related than other members of the mating populations in the *Liseola* section of *Fusarium*, based on AFLP similarity.

Mendel's postulate of segregation dictates that during the formation of gametes, the paired unit factors segregate randomly so that each gamete receives one or the other with equal likelihood (Klug & Cummings, 1994). Zamir & Tadmor (1986) attributed transmission ratio distortion to linkage between markers and genetic factor(s) that affect the fitness of gametes leading to unbalanced transmission of parental alleles to the next generation. In the present study, there was genome-wide selection for alleles of the *F. subglutinans* parent, with an estimated 59.8% of F_1 progeny genomes being received from the *F. subglutinans* parent. The F_1 progeny also showed a tendency to inherit intact parental linkage groups originating from the *F. subglutinans* parent (Table 4). Of the 292 markers that exhibited transmission ratio distortion, 96% were skewed towards the *F. subglutinans* parent. This interspecies cross therefore showed a clear bias towards the transmission of *F. subglutinans* alleles. We were not able to determine what proportion of the distorted loci exhibited epistatic interactions. However, 15% of the distorted markers exhibited a segregation ratio of approximately 3:1 ($P < 0.05$). In haploid organisms this is an indication of epistasis with two independent loci being involved in producing the phenotypic trait.

Marker loci exhibiting transmission ratio distortion suggest the presence of a distorting genetic factor in that region of the genome. However, it has also been shown that the greater the genetic divergence between the parental lines, the higher the levels of transmission ratio distortion (Paterson *et al.*, 1991; Grandillo & Tanksley, 1996). In the present study, approximately 50% of the markers showed transmission ratio distortion. A more extreme case of transmission ratio distortion was reported in an interspecific cross between the domesticated tomato (*Lycopersicon esculentum*) and a wild relative (*L. pennellii*), which showed 80% skewed segregation (DeVicente & Tanksley, 1993). Transmission ratio distortion ranging from 11 to 66% has also been reported in basidiomycetous fungi (*e.g.* Larraya *et al.*, 2000; Lind *et al.*, 2005) as well as in other ascomycetes (*e.g.* Xu & Leslie, 1996; Leslie *et al.*, 2004b; Gale *et al.*, 2005). The authors of these studies have hypothesized that the distortion could be attributed to several factors such as bias in the collection of spores used for the mapping population (Larraya *et al.*, 2000; Lind *et*

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al., 2005), error in scoring (Xu & Leslie, 1996), differential viability of certain ascospores (Xu & Leslie, 1996), or to structural rearrangements of chromosomes that may have caused distorted segregation patterns (Gale *et al.*, 2005).

The *F. subglutinans* x *F. circinatum* cross showed a clear preferential inheritance of alleles as well as complete chromosomes from the *F. subglutinans* parent rather than from the *F. circinatum* genome. This suggests a general fitness benefit for F_1 progeny that inherited *F. subglutinans* alleles. The reasons for this unidirectional distortion are not clear at present. It is possible that ascospores with *F. subglutinans* alleles were generally more viable on the specific crossing medium that was used, or that there were fewer negative interactions of *F. subglutinans* alleles with the hybrid genetic background. In this case, fitness selection would be due to the additive effects of individual genetic factors as mentioned previously. It is also possible that selection occurred in some cases against recombinant gametes because of co-evolved gene complexes that were broken up by recombination (Jurgenson *et al.*, 2002a), but this would not explain the unidirectional bias observed.

Despite the use of the biological species concept in species delineation, interspecific crosses in *Fusarium* section *Liseola* have been reported previously. *Fusarium fujikuroi* (mating population C) and *F. proliferatum* (mating population D) are defined as being different biological species, yet a few isolates have been shown to be sexually compatible (Desjardins *et al.*, 1997; Leslie *et al.*, 2004b), indicating the limitations of the biological species concept in the *Liseola* section of *Fusarium*. A naturally occurring hybrid has also been identified (Leslie *et al.*, 2004a). One hypothesis to explain the presence of a naturally occurring hybrid is that of a hybrid swarm, possibly being geographically separated or occurring on a specific host. The standard tester strains are represented by distinct species, but a hybrid swarm might naturally exist between *F. fujikuroi* and *F. proliferatum* (Leslie *et al.*, 2004b). The authors hypothesized that these two species might be consolidated into a single species. It may also be possible that the two species are in the final stages of speciation with some individuals in each species still being able to overcome crossing barriers. In the present study, isolates representing mating populations E and H of *G. fujikuroi* are phylogenetically closely related (O'Donnell & Cigelnik, 1997; O'Donnell *et al.*, 1998), but are less similar to each other than mating populations C and D are to each other

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(Steenkamp *et al.*, 2001). It is also important to highlight the fact that progeny used in this study were obtained from a laboratory cross rather from a natural cross and that only one isolate of *F. subglutinans* has been found to cross to one isolate of *F. circinatum*. The absence of host-specific factors in the laboratory cross may have helped to overcome natural crossing barriers between these two species.

The framework map generated in this study will be used to identify QTLs for important quantitative traits such as pathogenicity in *F. circinatum*, which is an economically important pine tree pathogen. In a previous study (Friel *et al.*, 2002), F_1 progeny of this same cross were found to be avirulent on pine trees. However, a backcross population involving a single F_1 individual crossed to the *F. circinatum* parental strain exhibited a wide range of virulence. We have constructed a similar backcross (unpublished results). This experimental population would be useful to identify QTLs associated with pathogenicity in the *F. circinatum* and *F. subglutinans* genomes and will aid us in gaining a better understanding of the genetic basis of *F. circinatum* virulence on *Pinus* species. It is reasonable to expect that there would be significant synteny between the genomes of *F. subglutinans* and *F. circinatum*, and that of *F. verticillioides*, which is completely sequenced and publicly available (http://www.broad.mit.edu). These resources should facilitate further investigation into the genetic determinants of pathogenicity of the pitch canker fungus.

The framework linkage map generated in this study will provide a means for studying genetic architecture of crossing barriers between *F. subglutinans* and *F. circinatum*. This will be possible with a more in depth analysis of the genome-wide pattern of segregation distortion observed in this mapping study. As mentioned, the genomic sequence of *F. verticillioides* is available and this genome is comparable to that of *F. subglutinans* in that there are also 12 chromosomes and the genome size is similar (Xu & Leslie, 1995). By sequencing selected AFLP framework markers generated in this study and finding a matching sequence in the *F. verticillioides* genome, we should be able to link the 12 linkage groups of this study to the *F. verticillioides* chromosomes. Putative hybrid fitness loci and pathogenesis-related QTLs could be fine-mapped and possibly identified by studying the corresponding genomic regions in the *F. verticillioides* genome.

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Acknowledgements

We thank the University of Pretoria, National Research Foundation (NRF), members of the Tree Pathology Cooperative Program (TPCP), the Mellon Foundation and the THRIP initiative of the Department of Trade and Industry (DTI) in South Africa for financial assistance.

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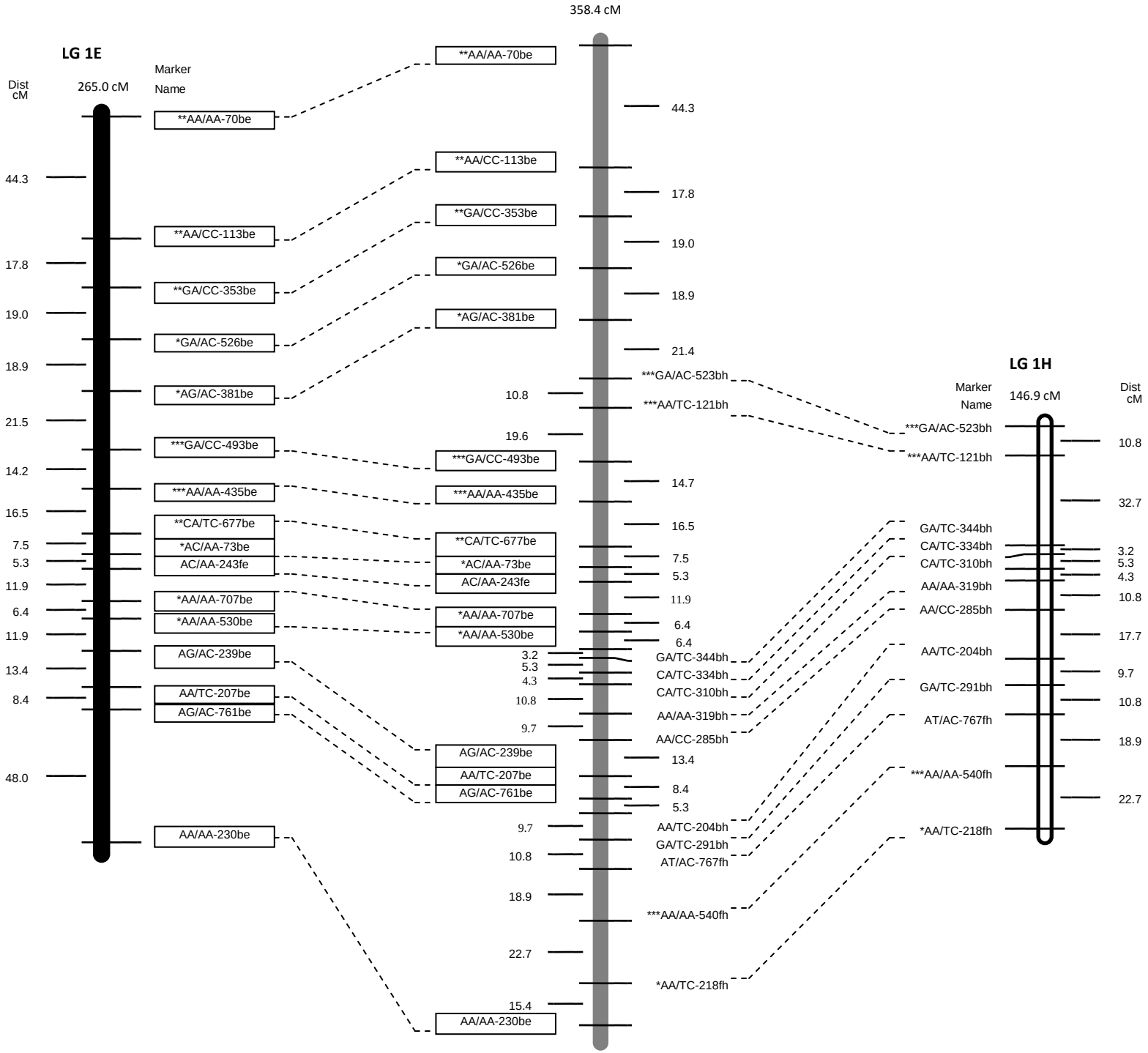

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Figure 1. Integrated framework maps of the cross between *F. subglutinans* and *F. circinatum*. Linkage group numbers followed by an 'E' indicate the *F. subglutinans* parental framework map and a 'H' the *F. circinatum* parental framework map. Bars shaded in black designate the *F. subglutinans* linkage map, those that are not shaded the *F. circinatum* linkage map and those that are shaded in grey the integrated F_1 map of *F. subglutinans* and *F. circinatum*. Distances are given in centiMorgan (cM) Kosambi and the total map length of each linkage group is given at the top of each linkage group. Marker names consist of the *Mse*I selective nucleotides followed by the *Eco*RI selective nucleotides and the molecular size (bp), followed by a b (bright) or f (faint) indicating the quality of the fragment, and an 'e' and 'h' indicating markers originating from either *F. subglutinans* and *F. circinatum*, respectively. Marker names that are blocked originated from the *F. subglutinans* parent and unblocked from the *F. circinatum* parent. The dotted lines indicate those markers shared between maps. Markers exhibiting transmission ratio distortion are indicated with an asterisk (*$P < 0.05$, **$P < 0.01$, ***$P <$ 001).

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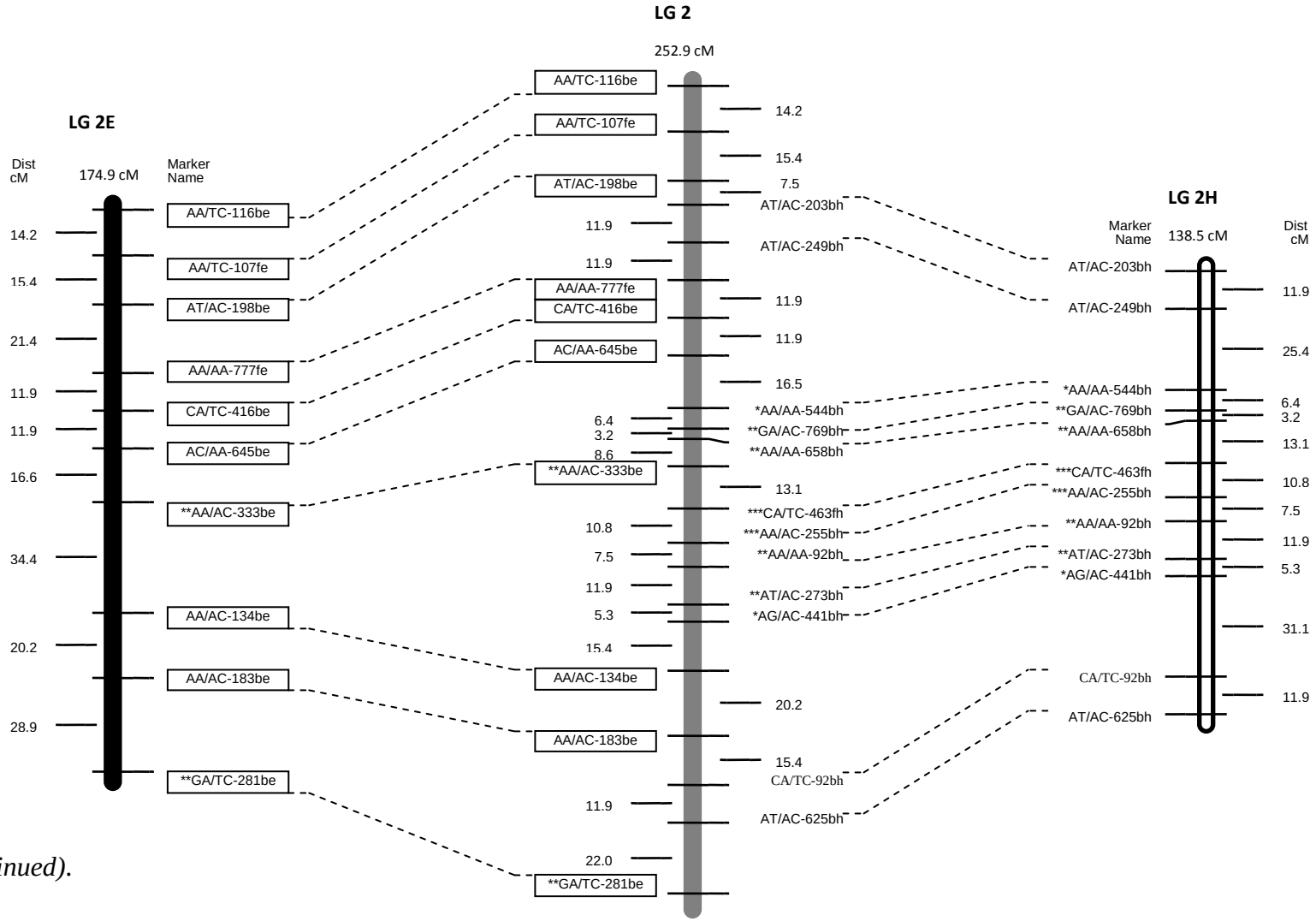

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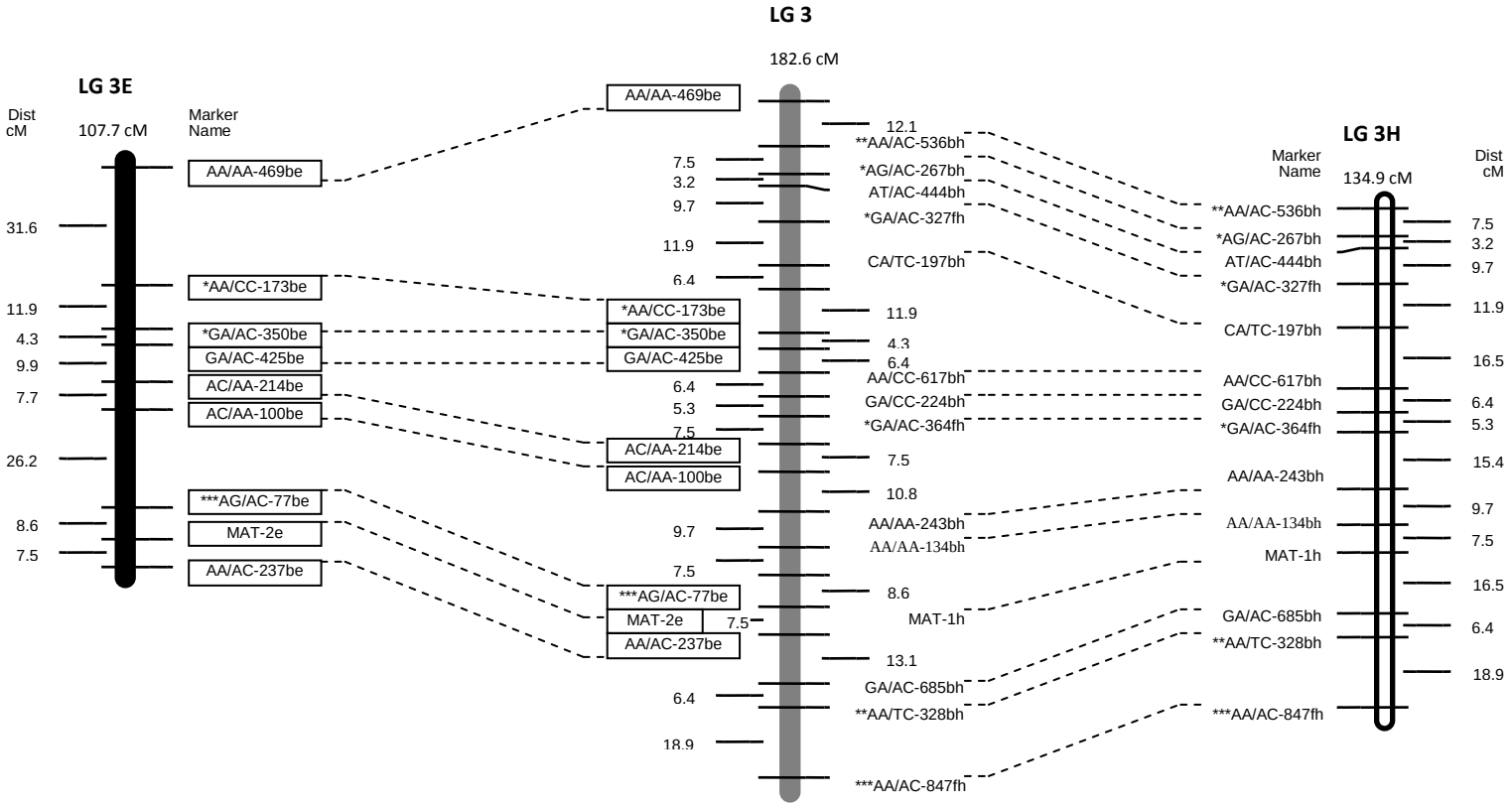

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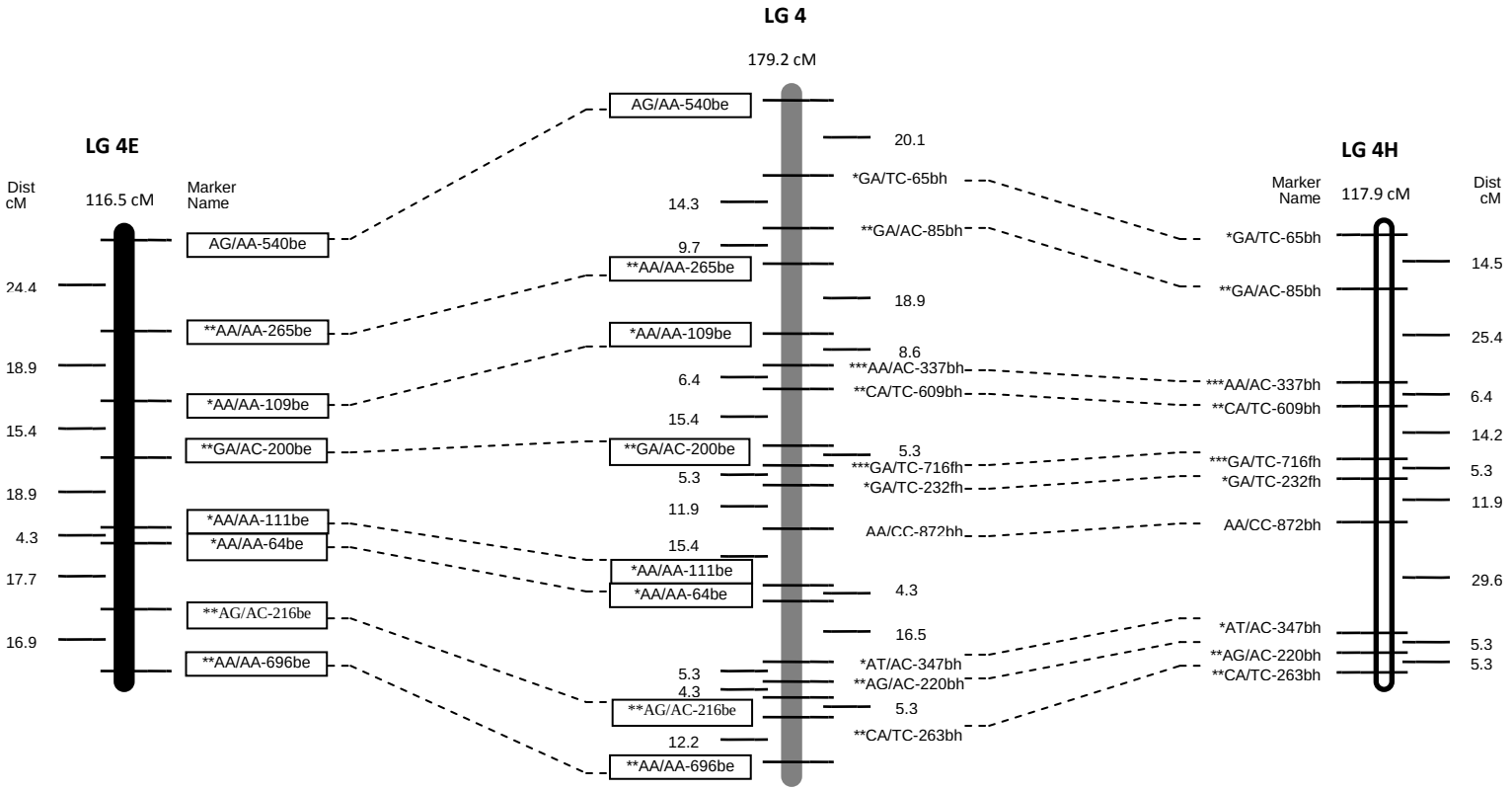

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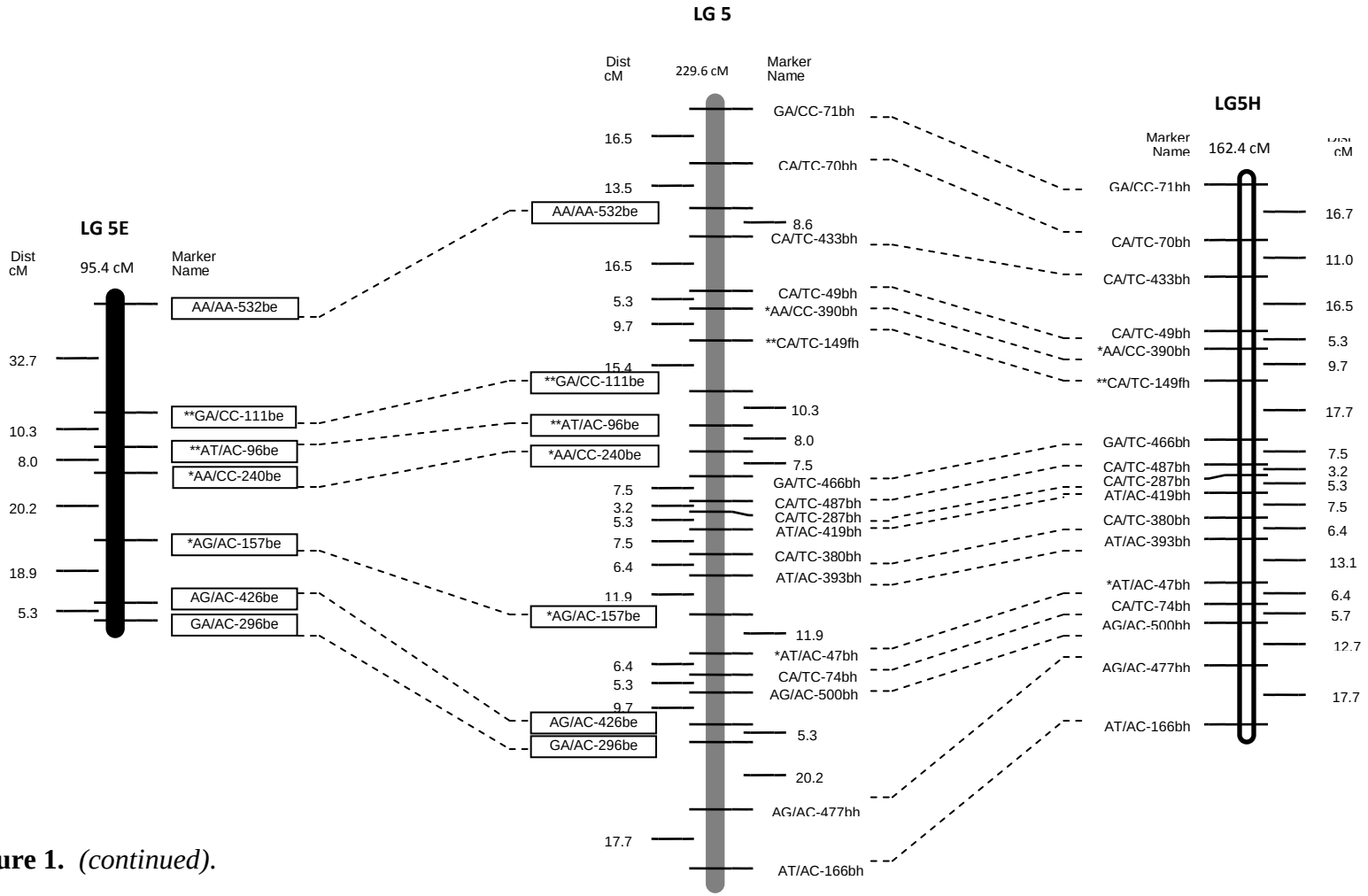

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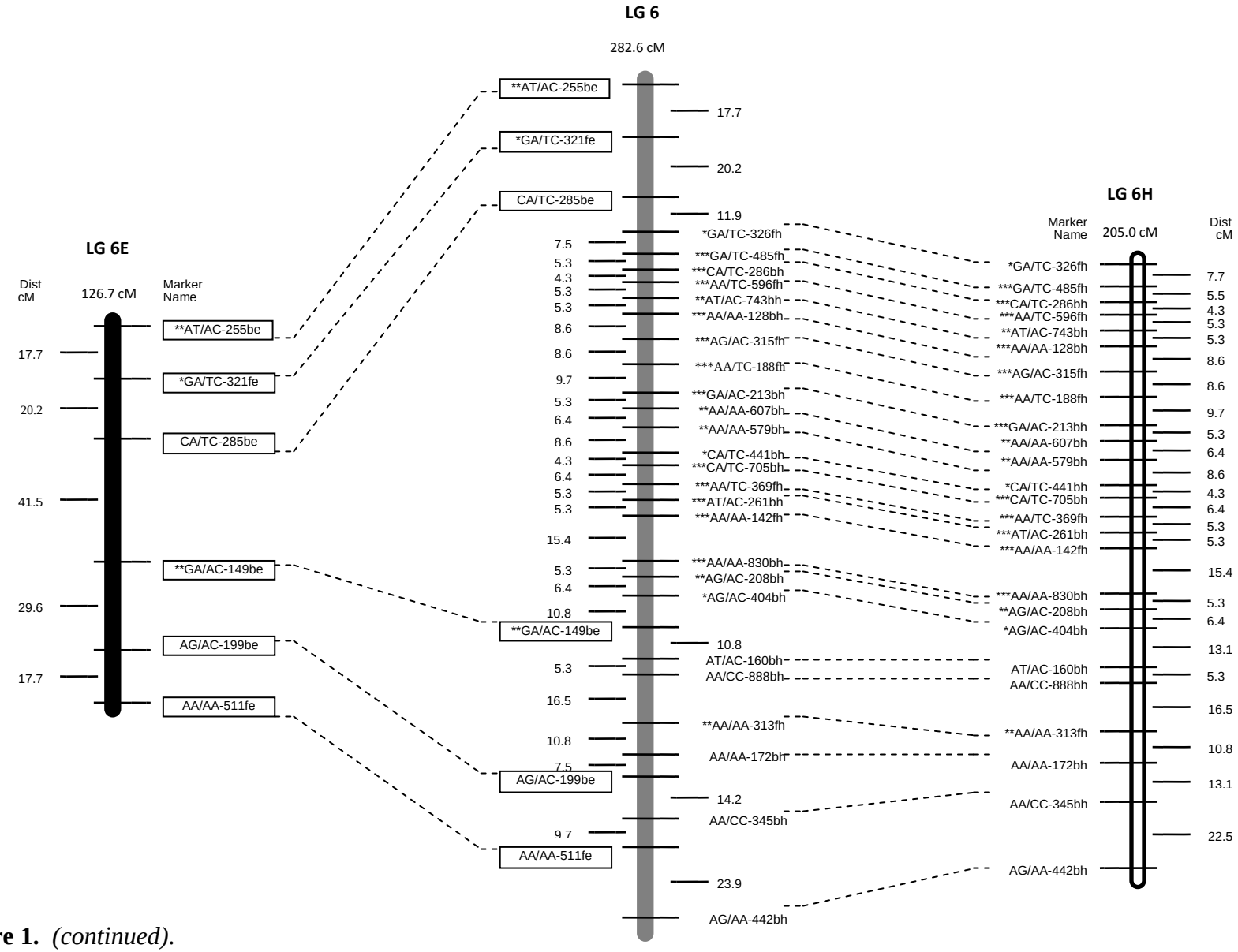

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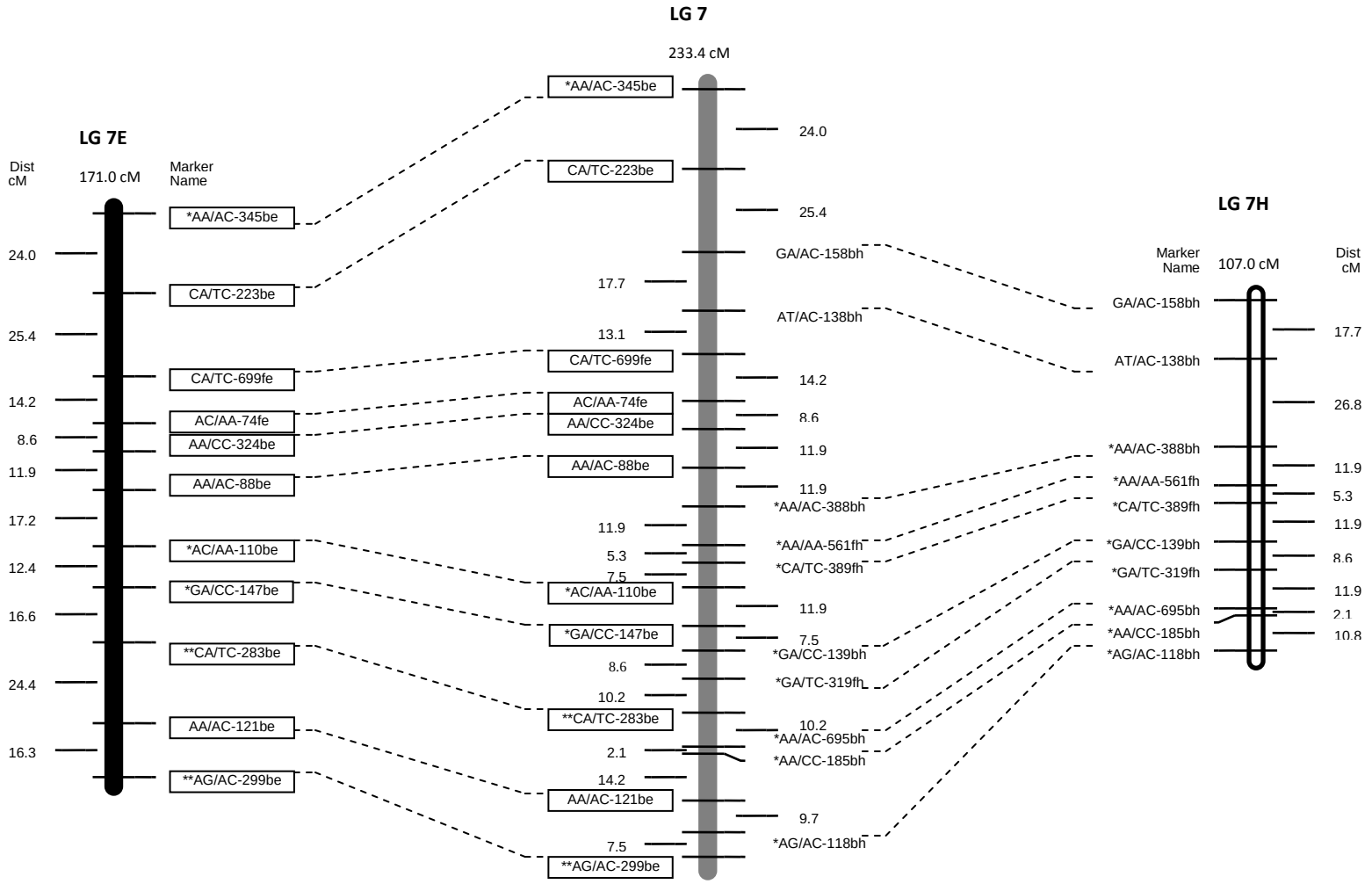

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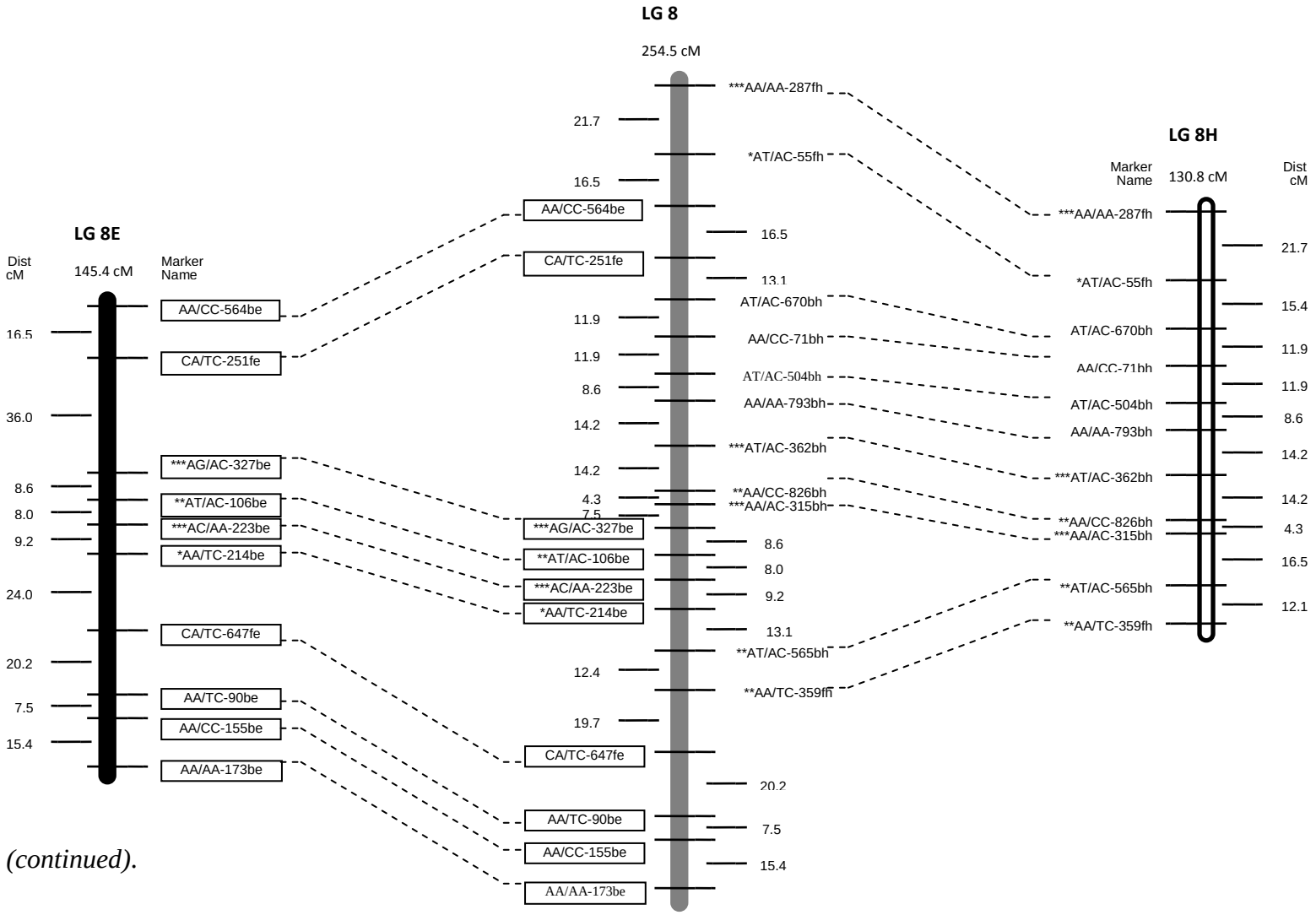

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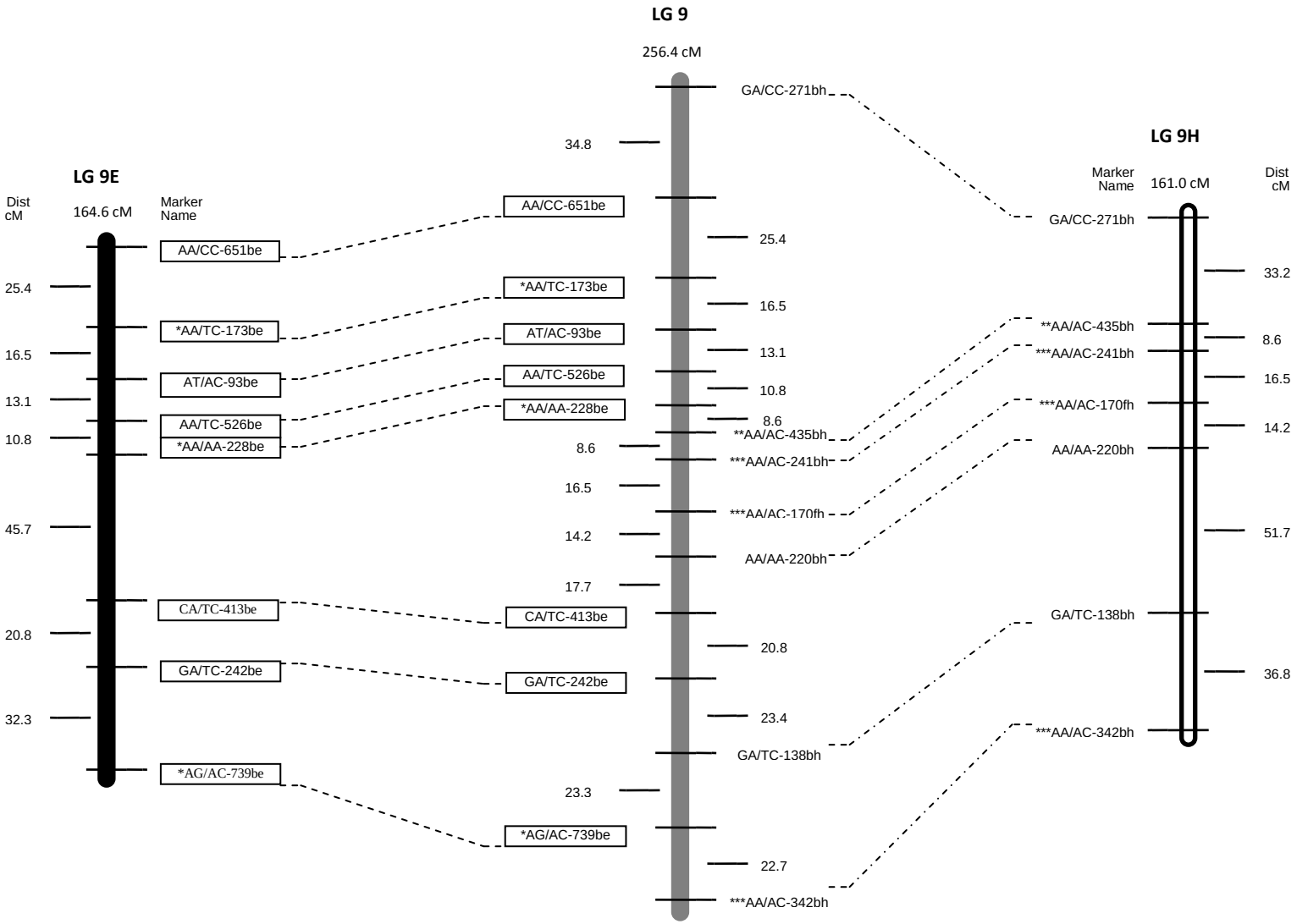

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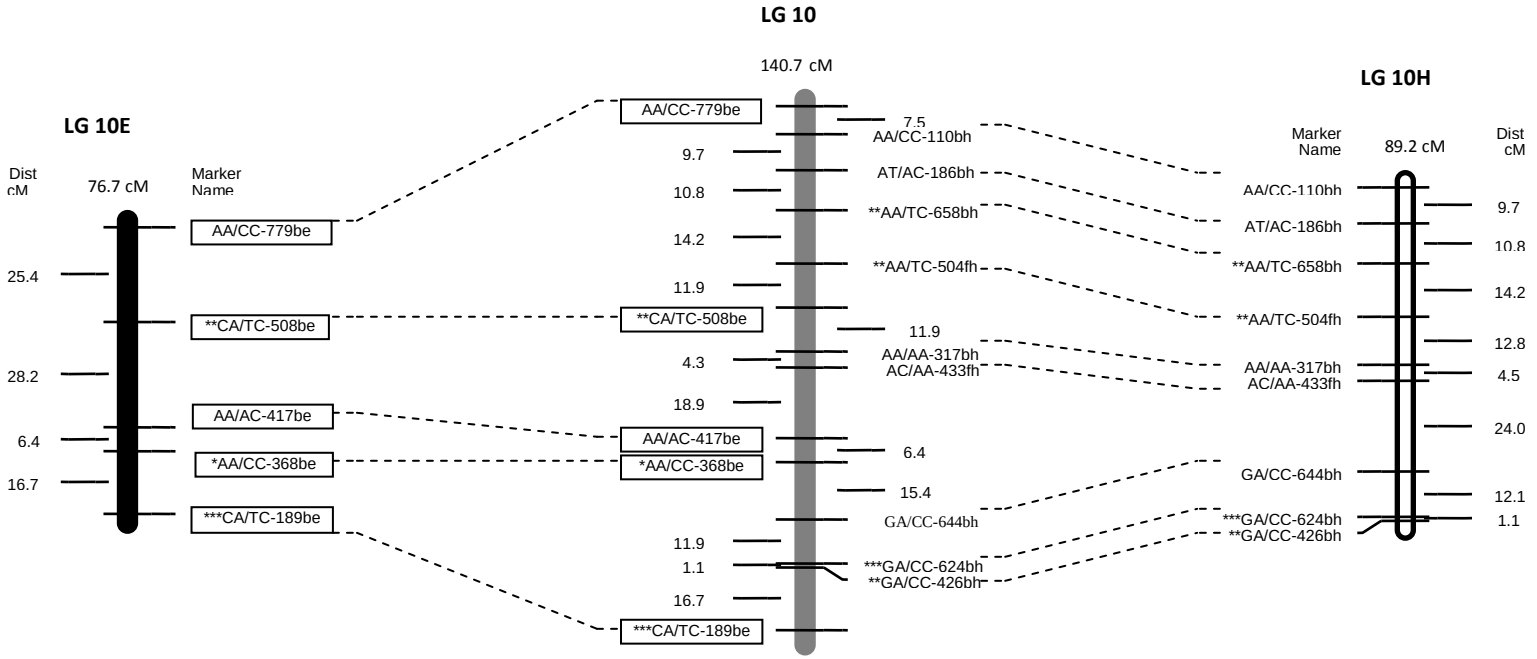

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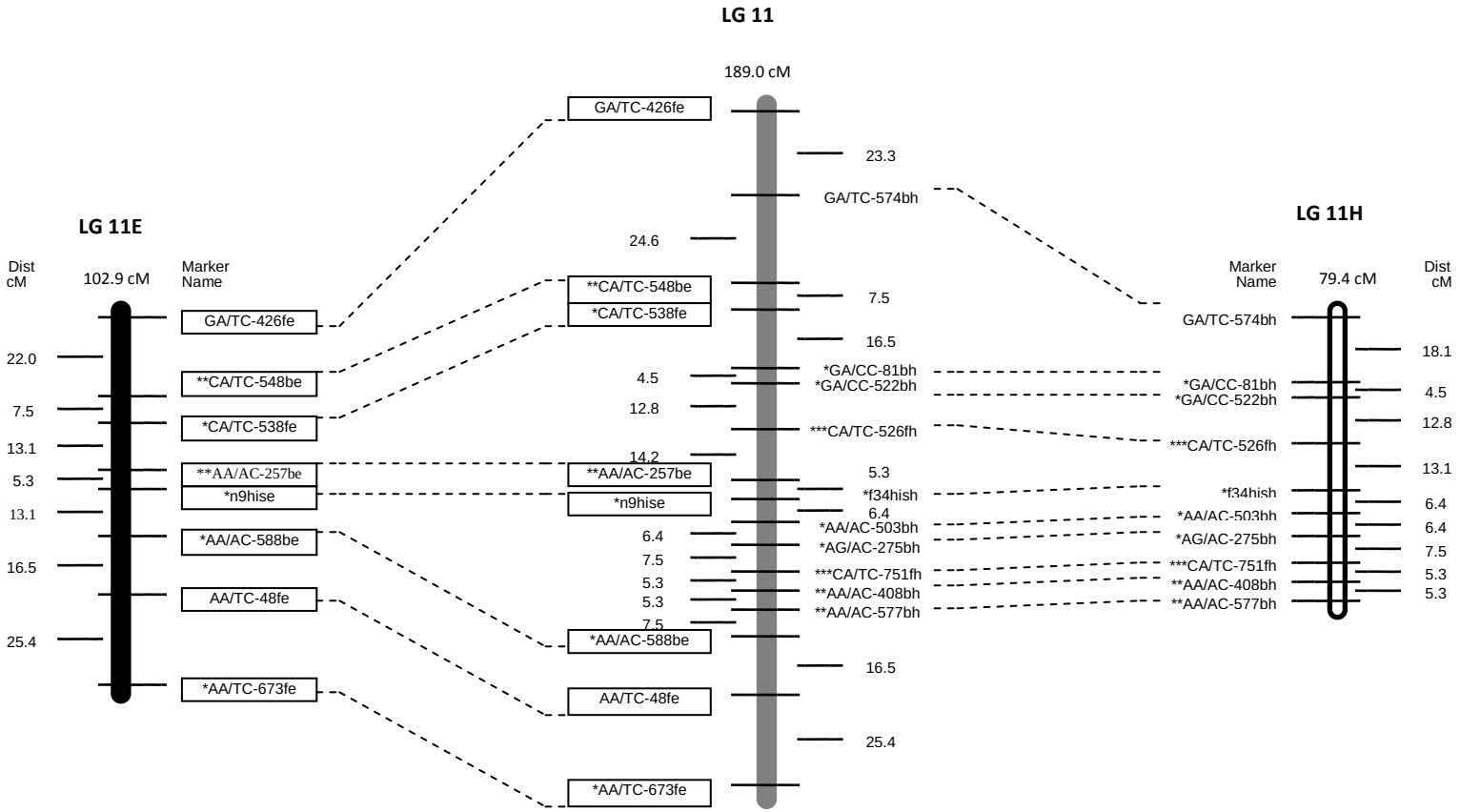

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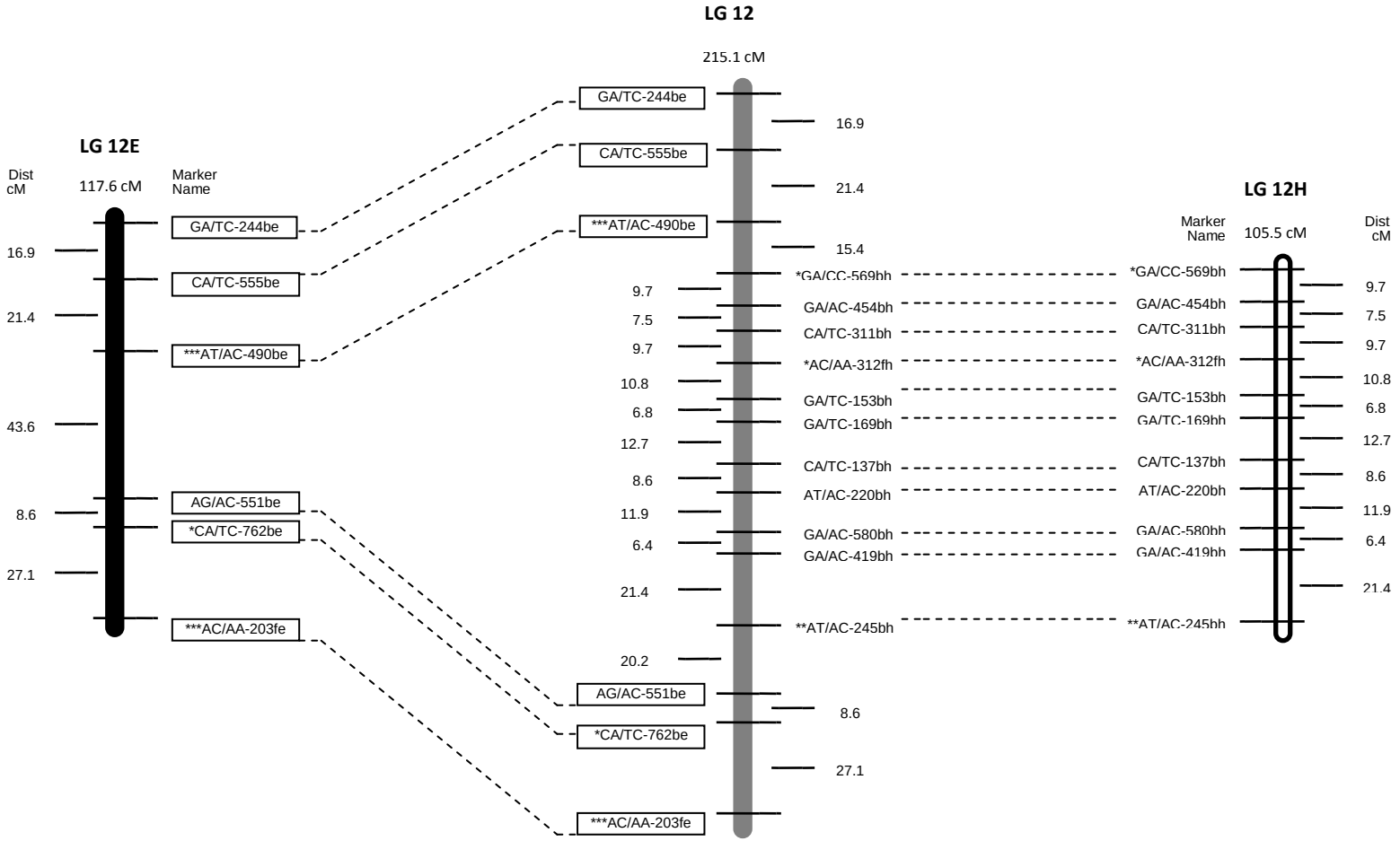

Figure 1. *(continued).*

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Table 1. Hosts, geographic origins and source of the F_1 parents used in this study.

Isolate[a]	Host	Geographic origin	Source
MRC7828; Fst51[b]	*Zea mays* spp. *Mexicana*	Texcoco, Mexico	A.E. Desjardins
MRC7870; Fsp34[c]	*Pinus* spp.	California, USA	T.R. Gordon

[a] MRC = W.F.O. Marasas, Programme on Mycotoxins and Experimental Carcinogenesis (PROMEC), Medical Research Council, Tygerberg, South Africa.

[b] *Fusarium subglutinans* (mating population E)

[c] *F. circinatum* (mating population H)

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Table 2. AFLP primer combinations used in this study. In the first column, the primer combinations are given for the *Mse*I (M) selective nucleotides and the *Eco*RI (E) selective nucleotides. The value given in parentheses refers to the IRDye™ (LI-COR, Lincoln, NE) used for fragment analysis

Primer combination	No. polymorphisms[a]	% polymorphic bands/primer
M-AA + E-AA (700)	61	62.9
M-AA + E-CC (700)	44	56.4
M-AA + E-AC (700)	54	68.4
M-AA + E-TC (800)	55	70.7
M-GA + E-CC (700)	32	48.5
M-GA + E-AC (700)	40	62.5
M-GA + E-TC (800)	42	56.0
M-AC + E-AA (700)	26	26.5
M-AG + E-AC (700)	32	53.3
M-AT + E-AC (700)	45	70.3
M-CA + E-TC (800)	63	66.3
M-AA + E-TT (800)	45	48.9
M-AG + E-AA (700)	39	42.4
TOTAL	578	
AVERAGE	44.5 ± 11.3	56.4

[a]Only the markers used for framework map construction.

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Table 3. Summary of markers in the framework linkage maps

	F. subglutinans (paternal)	*F. circinatum* (maternal)	F_1 hybrid (combined)
Markers			
Total no. of markers	296	286	582
No. of markers showing transmission ratio distortion[a]	128 (43.2%)	164 (57.3%)	292 (50.2%)
Markers included in framework map	104 (35.1%)	148 (51.7%)	252 (43.3%)
No. of markers in framework map showing disortion[a]	54 (51.9%)	85 (57.4%)	139 (55.2%)
No. of accessory markers[b]	159 (53.7%)	112 (39.2%)	271 (46.6%)
No. of markers not mapped	33 (11.1%)	26 (9.1%)	59 (10.1%)
Framework maps[c]			
12 linkage groups each			
Average linkage group size (cM)	138.7	131.3	231.2
Average framework marker spacing (cM)[d]	18.1	11.6	11.6
Observed map length (cM)[e]	1664.4	1575.5	2774.4
Physical distance per unit of recombination[f]	32.5 kb/cM	34.4 kb/cM	19.5 kb/cM
Estimation of genome length			
Hulbert estimate of genome length (cM)			2331.7
Framework map coverage[g]			
Map coverage (c x 100%) at d = 20 cM			98.7%
Map coverage (c x 100%) at d = 10 cM			88.5%

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[a]5 % level of significance (α) used to determine the departure of markers from the expected ratio of 1:1 of a haploid cross.

[b]AFLP markers that were not placed in the framework maps were mapped to the framework map using the bin mapping function of MapPop. Terminal markers were placed using the 'Two Point/LOD' function of MAPMAKER.

[c]Distances are in centiMorgan (cM) Kosambi.

[d]Calculated by dividing the summed length of all the linkage groups by the number of framework marker intervals (number of framework markers minus the number of linkage groups).

[e]Based on the classical estimate of recombination (r).

[f]Xu *et al.* (1995) estimated the genome size of *F. subglutinans* to be 54.1 Mbp. This estimate of genome size was used to calculate the physical distance per unit of recombination.

[g]The Hulbert estimate of genome length was used to estimate the framework map coverage.

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Table 4. The number of F_1 individuals with parental types on each linkage group and the origin of framework markers in each linkage group

Linkage group	Intact parental linkage group[c]		Framework markers[f]	
	H[a]	E[b]	H[d]	E[e]
1	0	10**	12	16
2	6	13	12	10
3	9	19	14	9
4	4	17**	10	8
5	10	15	17*	7
6	6	11	25***	6
7	8	18*	10	11
8	4	19**	11	10
9	4	9	7	8
10	7	28***	9	5
11	10	24*	10	8
12	5	21**	11	6
Total	73	204***	148**	104

[a]Total number of intact linkage groups originating from *F. circinatum*.

[b] Total number of intact linkage groups originating from *F. subglutinans*.

[c]Significant deviation for progeny with an intact linkage group from each parent. Significant deviation is noted as follows: *5%, **1% and ***0.1%.

[d]Total number of framework markers originating from the *F. circinatum* parent.

[e]Total number of framework markers originating from the *F. subglutinans* parent.

[f]Significant deviation from the expected 1:1 marker frequency from each parent. Significant deviation is noted as follows: *5%, **1% and ***0.1%.

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CHAPTER 3

Transmission ratio distortion in an interspecific cross between *Fusarium circinatum* and *Fusarium subglutinans*

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Abstract

Previously, an interspecific cross between *Fusarium circinatum* and *Fusarium subglutinans* was used to generate a genetic linkage map. About 55% of the genotyped markers in this cross displayed genome-wide transmission ratio distortion (TRD). The working hypothesis for this study was that TRD would be nonrandomly distributed throughout the genome due to distorting loci. Using a genome-wide threshold of $\alpha = 0.01$, 79 markers displaying TRD were distributed on all twelve linkage groups. Eleven putative TRDLs (transmission ratio distortion loci), on eight linkage groups, were identified in regions containing three or more adjacent markers displaying distortion. No epistatic interactions were observed between these TRDLs. Thus, it is uncertain whether the genome-wide TRD was due to linkage between markers and genomic regions causing distortion. The parental origins of markers followed a non-random distribution throughout the linkage map, with linkage groups contained stretches of markers originating from only one parent. Thus, due to the nature of the interspecific cross, the current hypothesis to explain these observations is that the observed genome-wide segregation was caused by the high level of genomic divergence between the parental isolates. Thus, homologous chromosomes would not align properly during meiosis, resulting in aberrant transmission of markers. This would explain previous observations of the preferential transmission of *F. subglutinans* alleles to the F_1 progeny.

Introduction

The *Gibberella fujikuroi* species complex accommodates the sexual stage of *Fusarium* spp. collectively treated in the section *Liseola* (Leslie & Summerell, 2006). This complex includes some of the most ubiquitous and economically important fungal pathogens of plants. The biological species concept had been used to formally classify species in this complex into ten mating populations or biological species (Nirenberg & O'Donnell, 1988; Samuels *et al.,* 2001; Zeller *et al.,* 2003; Lepoint *et al.,* 2005). Species delineation, when applying the biological species concept, implies that individual species are reproductively isolated (Mayr, 1940;

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Dobzhansky, 1951). This is somewhat complicated in fungi where interspecific crosses can occur, such as those found between some taxa in the *G. fujikuroi* species complex (Desjardins *et al.*, 1997; Desjardins *et al.*, 2000; Leslie *et al.*, 2004b).

Several examples exist for interspecific crosses within this species complex. Certain isolates from *Fusarium fujikuroi* (mating population C) and *Fusarium proliferatum* (mating population D) are interfertile and produce viable progeny (Desjardins *et al.*, 1997; Desjardins *et al.*, 2000; Leslie *et al.*, 2004b), and a naturally occurring hybrid has been identified (Leslie *et al.*, 2004a). The current hypothesis is that genetic isolation between these two biological species is not complete, allowing reproductive barriers to be overcome (Leslie *et al.*, 2004b). Another example of a laboratory generated interspecific cross is one between *Fusarium subglutinans* and *Fusarium circinatum*, residing in mating populations E and H, respectively (Desjardins *et al.*, 2000; De Vos *et al.*, 2007; Friel *et al.*, 2007).

De Vos *et al.* (2007) constructed a genetic map for the interspecific cross between *F. circinatum* and *F. subglutinans.* These authors found that ca. 55% of the markers exhibited significant TRD (transmission ratio distortion) from the expected ratio of 1:1 of a haploid cross ($P < 0.05$). Ninety-six percent of the TRD markers were skewed towards the *F. subglutinans* male parent. There was also preferential transmission of alleles, as well as complete chromosomes, from the genome of *F. subglutinans*. The clear bias towards the transmission of *F. subglutinans* alleles led to the conclusion that the F_1 progeny that inherited *F. subglutinans* alleles exhibited a general fitness benefit (De Vos *et al.*, 2007).

Mendel's postulate of segregation dictates that during the formation of gametes, the paired unit factors segregate randomly such that each gamete receives one or the other with equal likelihood (Klug & Cummings, 1994). When deviations from the expected Mendelian ratio of segregation (TRD) occur, they are frequently observed in interspecific crosses (Zamir & Tadmor, 1986). It has been demonstrated that the larger the genetic divergence between the parental lines, the higher the levels of TRD (Grandillo & Tanksley, 1996; Lee *et al.*, 2009). Interspecific crosses in *Fusarium* display the same tendency. Thus, interspecific crosses display higher levels of

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segregation (Jurgenson *et al.*, 2002; Leslie *et al.*, 2004b), than intraspecific crosses (Gale *et al.*, 2005).

TRD has been attributed to linkage between markers and genetic factor(s) that affect the fitness of gametes leading to unbalanced transmission of parental alleles to the next generation (Zamir & Tadmor, 1986). This functions during the pre- and postzygotic stages of reproduction and can also affect the zygotic viability. The presence of non-random marker loci exhibiting TRD throughout the genome, would suggest the presence of a distorting genetic factor in that region of the genome (transmission ratio distortion loci; TRDL) (Jiang *et al.*, 2000; Myburg *et al.*, 2004). These loci form barriers that prevent recombination from occurring in these parts of the genome, leading to unbalanced transmission of parental alleles to the zygotes.

With the advent of high-throughput molecular markers such as AFLPs (Vos *et al.*, 1995), the construction of genetic linkage maps with high levels of map coverage has been possible. This has allowed for detailed examination of the transmission of these markers to the next generation, including markers displaying TRD. The working hypothesis for this study was that TRD in an interspecific cross between *F. circinatum* and *F. subglutinans* would be non-randomly distributed throughout the genome. The aim was, therefore, to determine the positions and effects of possible TRDLs, by means of a previously compiled genetic linkage map derived from an interspecific cross between *F. circinatum* and *F. subglutinans*.

Materials & Methods

Identification of TRD and putative TRDL

The direction and percentage of distortion of each marker from the genetic linkage map (De Vos *et al.*, 2007), was determined by employing the formula (allele frequency – 0.5) x 100% (Myburg *et al.*, 2004). Markers displaying TRD could have occurred by chance or by displaying linkage to genetic factor(s) that affect the fitness of gametes (Zamir & Tador, 1986). To distinguish between a "chance" TRDL or a "true" TRDL, a genome-wide significance threshold is needed (Myburg *et al.*, 2004). Using this method with results of De Vos *et al.* (2007), it was

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assumed that the 12 linkage groups identified correspond to the 12 chromosomes (n = 12 for *F. subglutinans*, Xu *et al.*, 1995). Assuming that each chromosome contains at least two independent segregating regions, there was an expectation of a minimum of 24 independently segregating regions. To acquire a genome-wide significance level of P = 0.05, a significance threshold of 0.05/24 ≈ 0.002 would be necessary. However, in order to include weak TRDLs in this study, a significance threshold of $\alpha = 0.01$ ($\chi^2 = 6.64$) was employed. All regions that displayed three or more distorted markers were noted. The most skewed marker in this region was considered the most likely position of the distorting factor (Lu *et al.*, 2002).

Epistatic interactions between the TRDLs

To determine whether epistatic interactions occur between pairs of TRDLs, the most distorted marker in each TRDL was used (*i.e.* the most likely position of the distorting factor). Marker scores were evaluated for each pair of markers using Fisher's exact test in Statistica (v. 10, StatSoft, Inc. 2011, www.statsoft.com).

Results

Identification of TRD and putative TRDL

Of the 252 markers that were placed on the genetic linkage map, 138 (55%) showed distortion at the 5% level of significance, 79 (31%) at the 1% level of significance and 37 (15%) at the 0.1% level of significance. Markers displaying TRD were distributed throughout the genome; all linkage groups had markers that deviated from the expected 1:1 ratio ($P < 0.05$, Figure 2). Sampling error was excluded as a possible reason for distorted frequencies, as at a 5% level of significance, only 29 markers would be expected to show TRD.

When comparing the transmission of markers on the linkage map, the distribution of the *F. subglutinans* genetic composition amongst the F_1 progeny showed a mean of 59.8% (Figure 1). This was significantly different (P = 0.049996) to the predicted value of 50%. Also, the

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distribution of the *F. subglutinans* genome in the progeny did not follow a normal distribution as expected (Shapiro-Wilk W-test; SW-W = 0.87, $P = 0.00$). The distribution showed two 'tails', with the second "tail" at the 90-100% genomic constitution of *F. subglutinans* (Figure 1). This is an indication that some (thirteen of ninety-four) F_1 progeny closely resembled the *F. subglutinans* parental isolate in their genetic constitution, compared to *F. circinatum* (De Vos *et al.*, 2011). In contrast, only one of the F_1 progeny showed a *F. circinatum* genomic constitution of >90%.

In determining the direction and percentage of distortion of each marker placed on the genetic linkage map, only 12 markers (4%) were skewed towards the *F. circinatum* parent (Figure 2). This directional distortion extended over whole linkage groups, except in LG 2, 5, 6, 8, 9 and 11, where isolated markers were skewed towards *F. circinatum* (one marker on LG 5, 8, 9 and 11 and two markers each on LG 2 and 6). The exception was LG 7, where four of six markers at the beginning of the linkage group were skewed towards *F. circinatum*.

Using the genome-wide significance threshold of $P = 0.01$, 79 markers displayed TRD (Figure 2). Eleven regions were identified that displayed three or more distorted markers ($P < 0.01$), with the marker displaying the highest distortion as the most probable area for the TRDL (Figure 2, indicated with arrows). TRD regions that displayed three or more distorted markers were all unidirectionally distorted towards the *F. subglutinans* parent. These were located on LG 1 (markers GA/CC-353be and GA/AC-523bh), on LG 2 (marker CA/TC-463fh), LG 4 (markers AA/AC-337bh and CA/TC-263be), LG 5 (between markers CA/TC-149fh and GA/CC-111be), LG 6 (markers GA/AC-213bh and AA/AA-142fh), LG 8 (marker AG/AC-315bh), LG 10 (marker CA/TC-189be) and LG 11 (marker AG/AC-751fh) (Table 1). Thus, the putative TRDLs were not evenly distributed across the linkage groups, with LG 1, 4 and 6 having two TRDLs, LG 2, 5, 8, 10 and 11 having one TRDL and LG 3, 7, 9 and 12 not containing any. These TRDLs covered a total of 396.5 cM which accounts for 14.29 % of the observed map length.

A haploid population has allelic frequencies that equal genotypic frequencies, so it was not possible to establish whether TRD was caused by gametic or zygotic selection. However, the TRDL effects can also be expressed as the differential viability, t ($0 < t < 1$), of gametes or

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zygotes with alternate genotypes to that of normal gametes or zygotes (Cheng *et al.*, 1998). The relative viability or fertilization ability of gametes or viability of zygotes affected by the TRDL ranged from 0.34 to 0.53 (Table 1), indicating the unidirectional skewing towards the *F. subglutinans* parent.

Epistatic interactions between the TRDLs

None of the 55 possible marker pairs displayed epistatic interaction at the 0.05 level. Thus, there was no interaction amongst the eleven TRDLs found in this study.

Discussion

In order to construct genetic linkage maps with a high level of confidence, molecular markers should be free of missing values, have no genotyping errors and display no segregation distortion (Hackett & Broadfoot, 2003). This is seldom the case, especially when examining interspecific crosses (Grandillo & Tanksley, 1996; Lee *et al.*, 2009). In this study, segregation distortion (or TRD) was analyzed in the progeny of an interspecific cross between *F. circinatum* and *F. subglutinans*. Eleven putative TRDLs were identified which spanned eight linkage groups and illustrated genome-wide TRD observed in this interspecific cross. It may have been due to linkage between AFLP markers and genomic regions (TRDLs) that ensured the preferential transmission of *F. subglutinans* alleles, or alternatively, prezygotic or postzygotic bias (Giruad *et al.*, 2008). Such bias can be explained by three factors: (1) chromosome loss or other local rearrangements (prezygotic; Zolan, 1995), (2) greater genomic divergence between the two parental species (Zamir & Tadmor, 1986), such as that found in interspecific crosses (pre- and post-zygotic), and (3) linkage to a lethal gene (postzygotic, Raju, 1994).

Chromosome loss would lead to linkage groups with extreme TRD at all loci, with one genotypic class absent in that linkage group. In the present study, TRD was observed throughout the genome, with no linkage group displaying the absence of a genotypic class. Thus, chromosomal loss would not account for the TRD observed in this study. Other chromosomal abnormalities,

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such as inversions, would only affect segregation distortion in that part of the genome, and would not be visible throughout the genome (Jurgenson *et al.*, 2002; Bowden *et al.*, 2008). In fungi, chromosome rearrangements in the form of chromosome length polymorphisms, translocations and gain/loss of nonessential sequences, are widespread (Zolan, 1995). Without fully assembled genomic sequence data, the presence of other local chromosomal rearrangements could not be excluded.

It is thought that the degree of TRD is related to the level of genomic divergence between taxa (Zamir & Tadmor, 1986; Jenczewski *et al.*, 1997; Whitkus, 1998; Jurgenson *et al.*, 2002; Leslie *et al.*, 2004b). Thus, TRD observed in this study could be linked to the presence of isolation mechanisms between these two species, *i.e.* barriers to gene flow (Giruad *et al.*, 2008). These barriers may be genic in nature (see below), or due to structural differences between homologous chromosomes of the two species. In the genetic linkage map investigated in this study, parental origins of markers were non-random, *i.e.* linkage groups contained stretches of markers originating from only one parent. The implication is that homologous chromosomes from the interspecific cross did not align along their entirety during meiosis, while only small parts of chromosomes were homologous. Chiasmata (crossovers) could only occur in these homologous regions. Also, preferential inheritance of complete *F. subglutinans* chromosomes by the F_1 progeny (De Vos *et al.*, 2007) provides further evidence for non-homology of chromosomes.

In the current study there was a bias towards the transmission of *F. subglutinans* alleles, suggesting a fitness benefit for F_1 progeny with these alleles. *Fusarium subglutinans* alleles could have had fewer negative interactions with the hybrid genetic background than those of *F. circinatum* (Burke & Arnold, 2001; Myburg *et al.*, 2004). This genic incompatibility is characterized by the proper functioning of alleles in their respective genetic backgrounds, but which are incompatible in the hybrid due to recombination (McDaniel *et al.*, 2007). The observed bias could also be due to increased viability of ascospores containing *F. subglutinans* alleles. Hybrids can produce abnormal meiotic products, which could lead to inviability of hybrid progeny (Giraud *et al.*, 2008). There could also be selection against certain recombinant gametes, due to co-evolved gene complexes that may be disrupted during recombination, leading

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to nonviable progeny (Burke & Arnold, 2001; Jurgenson *et al.*, 2002). Future tests of these hypotheses are warranted.

Linkage to a lethal gene acts as a meiotic drive element with only those markers linked to this gene displaying TRD. One such element found in the *G. fujikuroi* species complex is spore killer, identified in *F. verticillioides* (Kathariou & Spieth, 1982). Spore killer causes the postmeiotic abortion of ascospores that did not receive the killer element and can be observed using light microscopy (Raju, 1994). In the interspecific cross, viability of ascospores was high (90%, Desjardins *et al.*, 2000). Thus, the presence of the spore killer element in *F. circinatum* and *F. subglutinans* is uncertain.

In this study putative TRDL regions were identified that spanned eight linkage groups. The presence of these TRDLs is most likely not the reason for the preferential transmission of *F. subglutinans* alleles. Rather, the genomic divergence between *F. subglutinans* and *F. circinatum* was in all likelihood responsible for the genome-wide segregation of markers observed in the F_1 progeny. Thus, these regions that displayed significant distortion probably indicate extreme genomic dissimilarity between the two parental species. These differences could include 'niche-specific' genes that enable a particular species to be pathogenic to a specific host species. Increased understanding of the genetic determinants of TRDLs are needed to decipher its potential link to speciation and overall genome evolution of *F. circinatum* and *F. subglutinans* in the *G. fujikuroi* species complex.

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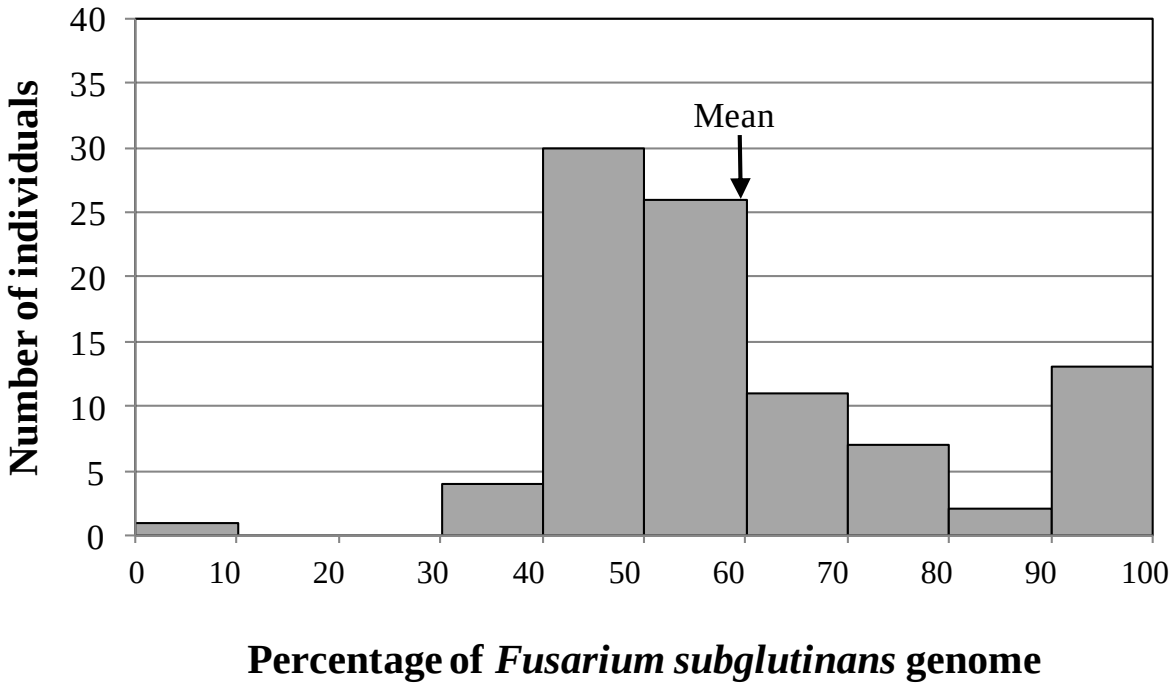

Figure 1. Distribution of the percentage of the *Fusarium subglutinans* parent genome present amongst the F_1 progeny. The y-axis is the number of individuals in each percentage class, whilst the x-axis is the percentage classes for *F. subglutinans* genome present among individual F_1 progeny.

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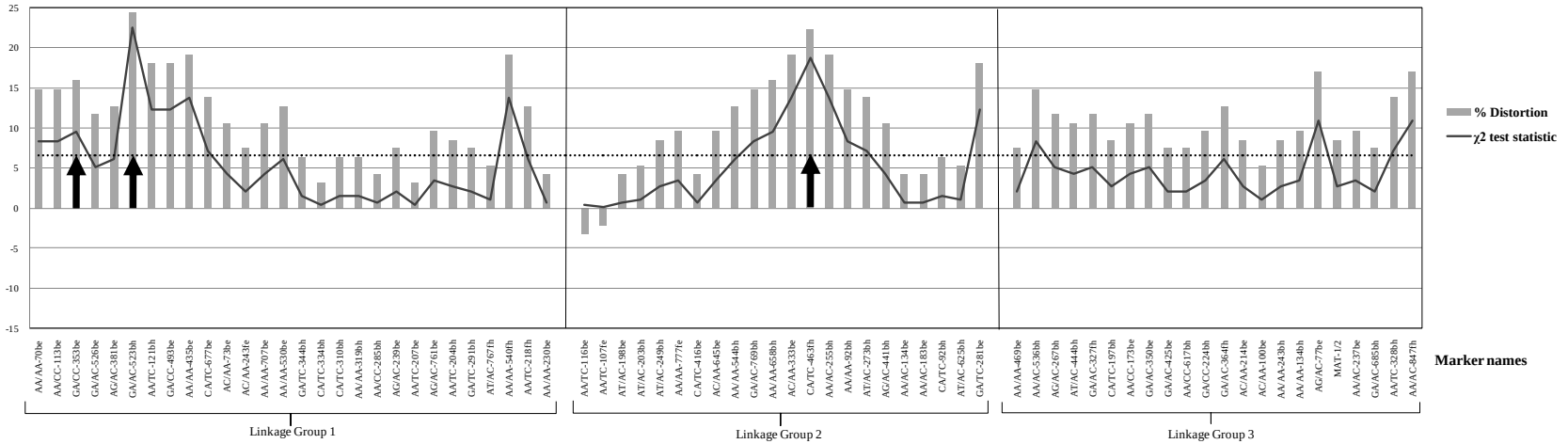

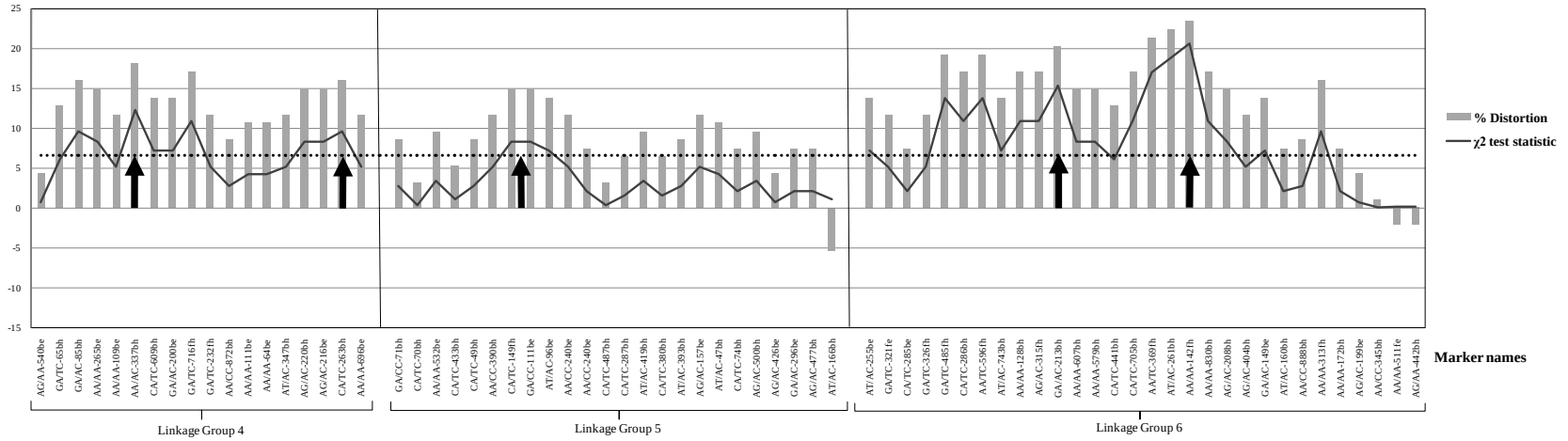

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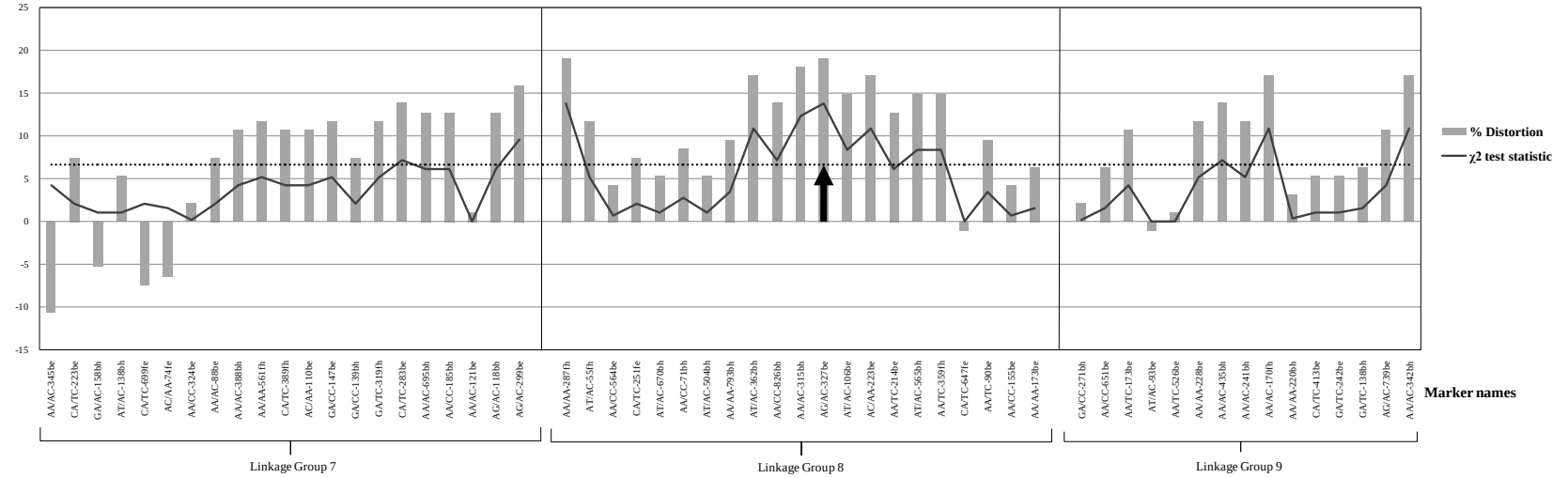

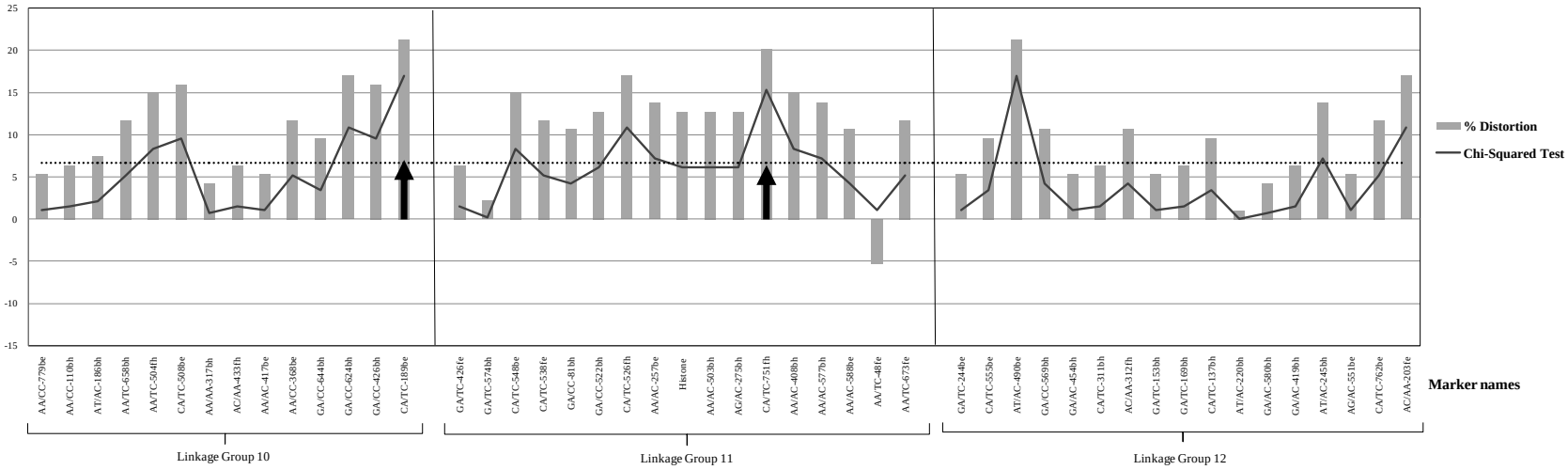

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Figure 2. The degree and direction of transmission ratio distortion of the F_1 hybrid. The vertical bars represent the percentage of distortion ([allele frequency – 0.5] x 100%) as well as the direction of distortion. The solid lines represent the χ^2 statistic for deviation from the expected 1:1 transmission ratio expected in a haploid F_1 cross ($P < 0.05$). The horizontal dotted line represents the χ^2 statistic at the 0.01 level of significance. Arrows represent the positions of the estimated TRDLs. The marker names are indicated on the x-axis. Marker names consist of the *Mse*I selective nucleotides followed by the *EcoR*I selective nucleotides and the molecular size (bp), followed by a b (bright) or f (faint) indicating the quality of the fragment, and an 'e' and 'h' indicating markers originating from either *F. subglutinans* and *F. circinatum*, respectively. Marker data was recoded so that the direction of distortion represents that of the paternal parent (*F. subglutinans*). Intermaker distances are not shown proportionally.

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Table 1. Summary of putative transmission ratio distortion loci (TRDLs).

TRDL	Linkage group	Map position (cM)[a]	*P*-value[b]	Deviation from expected 1:1[c] (%)	% of F_1 indivduals with allele[d]	Relative viability[e]
GA/CC-353be	1	62.1	0.002	15.96	66	0.52
GA/CC-523bh	1	121.4	2.09×10^{-06}	24.47	74	0.34
CA/TC-463fh	2	132.5	1.48×10^{-05}	22.34	72	0.38
AA/AC-337bh	4	71.6	0.00045	18.09	68	0.47
CA/TC-263bh	4	167	0.002	15.96	66	0.52
CA/TC-149fh and GA/CC-111be	5	77.8	0.0039	14.89	65	0.53
GA/AC-213bh	6	104.4	8.88×10^{-05}	20.21	70	0.42
AA/AA-142fh	6	139.6	5.67×10^{-06}	23.40	73	0.36
AG/AC-327be	8	140.4	0.00021	19.15	69	0.45
CA/TC-189be	10	140.7	3.70×10^{-05}	21.28	71	0.40
CA/TC-751fh	11	129	8.88×10^{-05}	20.21	70	0.42

[a] Distances in centiMorgan (Kosambi) from the top of the linkage group.

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[b] *P* values of the χ^2 test statistic was performed on all markers to test for departure from the expected Mendelian segregation ratio expected for a haploid cross (1:1).

[c] Percentage and direction of distortion of each marker was determined by employing the formula (allele frequency – 0.5) x 100% (Myburg *et al.*, 2004). All TRDL were skewed towards the *F. subglutinans* parent.

[d] Percentage of F_1 individuals with the TRDL out of a total of 94 F_1 individuals.

[e] The differential viability of gametes or zygotes calculated as the ratio of the frequency of the less frequent TRDL allele to the most frequent TRDL allele (Cheng *et al.*, 1998).

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CHAPTER 4

Genetic analysis of growth, morphology and pathogenicity in the F_1 progeny of an interspecific cross between *Fusarium circinatum* and *Fusarium subglutinans*[1,2]

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[1]De Vos, L., van der Nest, M.A., van der Merwe, N.A., Myburg, A.A., Wingfield, M.J., Wingfield, B.D. 2011. Genetic analysis of growth, morphology and pathogenicity in the F_1 progeny of an interspecific cross between *Fusarium circinatum* and *Fusarium subglutinans.* Fungal Biology 115, 902-908

[2]The text from this publication is reproduced in full. It has however, been reformatted to conform with the layout of the thesis.

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Abstract

Fusarium circinatum and *Fusarium subglutinans* are two distinct species in the *Gibberella fujikuroi* species complex. A genetic linkage map produced from an interspecific cross between these species was used to identify quantitative trait loci (QTLs) associated with variation in mycelial growth and morphology of colony margins (CM) in the 94 F_1 progeny. Mycelial growth was assessed by measuring culture size at 25˚C and 30˚C, while CM morphology was characterized in the parents and assessed in their F_1 progeny. In order to test the pathogenicity of the progeny, *Pinus patula* seedlings were inoculated and lesion lengths were measured after three weeks. Seven putative QTLs were associated with mycelial growth, three for growth at 25˚C and four at 30˚C. One highly significant QTL ($P < 0.001$) was present at both growth temperatures. For CM morphology, a QTL was identified at the same position ($P < 0.001$) as the QTL responsible for growth at the two temperatures. The putative QTLs accounted for 45 and 41% of the total mycelial growth variation at 25˚C and 30˚C, respectively, and for 21% of the variation in CM morphology. Only one of the 94 F_1 progeny was pathogenic on *P. patula* seedlings. This observation could be explained by the genetic constitution of this F_1 isolate, namely that ~96% of its genome originated from the *F. circinatum* parent. This F_1 individual also grew significantly faster at 25˚C than the *F. circinatum* parent ($P < 0.05$), as well as more rapidly than the average growth for the remaining 93 F_1 progeny ($P < 0.05$). However, no association was found between mycelial growth and pathogenicity at 25˚C. The highly significant QTL associated with growth at two temperatures, suggests that this is a principal genomic region involved in mycelial growth at both temperatures, and that the same region is also responsible for CM morphology.

Introduction

Fusarium circinatum and *Fusarium subglutinans* are distinct fungal taxa that reside in the *Gibberella fujikuroi* species complex (Nelson *et al.*, 1983; Nirenberg & O'Donnell, 1998). This complex includes economically important pathogens of crops and trees. Based on the biological species concept, *F. circinatum* resides in mating population H (Nirenberg & O'Donnell, 1998; Britz *et al.*, 1999) and *F. subglutinans* in mating population E (Nelson *et al.*, 1983).

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In a study of *F. subglutinans* isolates from maize and teosinte, one strain of *F. subglutinans* isolated from teosinte was moderately interfertile with a strain of the pine pitch canker pathogen, *F. circinatum* (Desjardins *et al.,* 2000). This interspecific cross was the basis for a study by De Vos *et al.* (2007), who used genetic linkage mapping to study the genetic differentiation of the two parental genomes. That study placed 248 AFLP markers and two gene-based markers (the mating type idiomorphs (*MAT*) and the histone (H3) gene) onto a genetic linkage map that was organized into 12 major linkage groups. Of these markers, 55% showed significant transmission ratio distortion from the expected 1:1 transmission ratio of a haploid cross ($P < 0.05$). All but 12 favoured alleles of the *F. subglutinans* parent. This unusually high percentage of markers displaying transmission ratio distortion could be attributed to various factors. One is the presence of linkage between markers and distorting genetic factor(s), which could affect the fitness of gametes leading to a biased transmission of parental alleles to the next generation (Zamir & Tadmor, 1986). Another contributing factor could be an association between the genetic divergence of the parental isolates and the levels of transmission ratio distortion (Paterson *et al.,* 1991; Grandillo & Tanksley, 1996). In this regard, interspecific crosses, such as those treated in this study, tend to display higher levels of segregation distortion.

Friel *et al.* (2007) also made an interspecific cross between the same parental isolates of *F. circinatum* and *F. subglutinans* (De Vos *et al.,* 2007) and showed that the *MAT* idiomorphs displayed no transmission ratio distortion, while this was present in the H3 gene. These results were consistent with those of De Vos *et al.* (2007) using the gene-based markers. Also, none of the F_1 progeny displayed pathogenicity on *Pinus radiata* and it was hypothesized that this could be due to a very low probability of finding viable F_1 progeny with all the *F. circinatum* genes necessary for pathogenicity to pines (Friel *et al.,* 2007). The bias against the genome of *F. circinatum* suggests a general fitness benefit for F_1 progeny that have inherited *F. subglutinans* alleles (De Vos *et al.,* 2007).

In the basidiomycete *Heterobasidion annosum* species complex, hybrid progeny placed on a substrate favouring only one parent were less competitive than this parental strain (Garbelotto *et al.,* 2007). When inoculated onto a substrate that is favourable to both parents, the hybrid progeny were as fit as the parental genotypes. The fact that none of the F_1 progeny from a cross between *F. circinatum* and *F. subglutinans* displayed pathogenicity to pines (Friel *et al.,* 2007) suggests that this was indicative of the effect that the substrate has on the fitness of

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fungal hybrids. Thus, *P. radiata* would represent an unfavourable substrate for the F_1 progeny of the cross between *F. subglutinans* that occurs on maize, and *F. circinatum*, a pathogen of pines. In the present study, we considered mycelial growth on agar, representing a substrate that is favourable to both parents. Mycelial growth in *Fusarium* spp. has been hypothesized to be correlated with isolate pathogenicity (Doohan *et al.*, 2003) where rapid growth is usually associated with high levels of pathogenicity. To test this hypothesis, we also investigated whether there might be an association between pathogenicity and fitness typified by mycelial growth at 25˚C.

The genetic linkage map of the *F. circinatum* x *F. subglutinans* interspecific cross can be used to identify QTLs for any quantitative traits that are polymorphic in the F_1 progeny. So far, only one study has reported on the mapping of QTLs in the genus *Fusarium*, where QTLs for pathogenicity and aggressiveness of *Fusarium graminearum* towards wheat was mapped (Cumagun *et al.*, 2004). In the present study we mapped regions of the genome involved in the expression of morphological traits such as mycelial growth and colony margin (CM) morphology. Mycelial growth was studied at two different temperatures, to consider differences in mycelial growths between the two parental species. In addition, this would make it possible to determine whether individual genes or a combination of genes are involved in the variation in growth observed in the F_1 population, and to determine the genomic origin of these QTLs. Furthermore, pathogenicity in the F_1 progeny of the interspecific cross was considered in order to verify whether there was a bias against the *F. circinatum* genome, as reported by Friel *et al.* (2007).

Materials and methods

Fungal isolates and mycelial growth studies

Isolates used in this study included the parents of an interspecific cross between *F. circinatum* and *F. subglutinans* (Desjardins *et al.*, 2000) and 94 isolates from the F_1 progeny. The progeny of this cross represented the same isolates used for genetic linkage analysis in De Vos *et al.* (2007). All isolates were grown on half strength PDA (potato dextrose agar; 20% w/v potato dextrose agar and 5% w/v agar). For mycelial growth and CM studies, a

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mycelial plug was removed from the edge of an actively growing culture for each isolate, and placed at the centre of a Petri plate (90 mm in diameter).

The two parental isolates were tested for growth at a range of temperatures, from 10˚C to 35˚C at 5˚C intervals, with five replicate plates for each isolate. After incubation in the dark for seven days, mycelial growth was measured along two perpendicular axes of the colonies at right angles to each other. Two growth temperatures, 25˚C and 30˚C, were identified at which the parental isolates displayed differential mycelial growth. Thereafter, growth was assessed at these temperatures for the 94 F_1 progeny with five replicate plates per isolate. In addition CM morphology was characterized for the parental isolates as well as for the 94 F_1 progeny when grown at 25˚C for 7 days in the dark. The two parents had an observable difference in CM morphology. *Fusarium circinatum* had a smooth colony edge and *F. subglutinans* had an irregular (laciniate) edge (Figure 1A and B). The progeny displayed either of these, or an intermediate morphotype (crenate) (Figure 1C). The phenotypes were scored as '1' for smooth, '2' for crenate and '3' for laciniate.

Pathogenicity studies

All 94 F_1 isolates were grown on ½ PDA for 7 days at 25˚C in the dark. Spores were washed from the cultures with 15% (v/v) glycerol. Spore concentration was determined using a haemacytometer and adjusted to 5 x 10^4 spores/ml for each isolate, using sterile distilled water.

Six month old *P. patula* seedlings were wounded by removing the growth tips and wounds were inoculated with a 10 µl drop of spore suspension. In order to minimize the effect of genetically variable seedlings on the pathogenicity of the fungus, ten biological replicates were used for each isolate. Ten seedlings were inoculated with sterile distilled water to serve as a negative control. Inoculated seedlings were allowed to grow in the greenhouse at 25˚C for three weeks, after which lesion lengths were measured from the point of inoculation along the seedling stem.

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Statistical analyses

Statistical analyses were performed using Statistica V8.0 (StatSoft, Inc.). The frequency distribution was determined and analysis of variance (ANOVA) performed for mycelial growth at 25˚C and 30˚C. Individual observed broad sense heritability (H^2), *i.e.* the proportion of genotypic to phenotypic variance ($H^2 = \sigma^2_G / \sigma^2_P$), was calculated for the *in vitro* studies. To determine whether there were significant differences ($P < 0.05$) in mycelial growth of parental isolates and the 94 F_1 progeny, *t*-tests were performed.

QTL detection

Map Manager QTXb15 V0.25 (Manly *et al.* 2001) was used to identify markers linked to mycelial growth at two temperatures and to the CM morphology. The "Hide redundant loci" option was chosen to remove markers that were associated with identical genotypes (duplicate markers) as well as those closer than 10 cM, in order to minimize interference due to background segregation of these markers. The cross type was selected as "Arbitrary cross" as this option allows for the most accurate analysis of haploid data. De Vos *et al.* (2007) included markers (55%) that displayed transmission ratio distortion ($P < 0.05$) in their map. Therefore, to allow Map Manager QTX to analyze data containing markers showing transmission ratio distortion, the "Allow for segregation distortion" function was chosen.

A permutation test (1000 permutations at 1 cM intervals using the additive model) was performed to empirically determine the experiment-wise significance levels for significant ($\alpha = 0.05$) and highly significant ($\alpha = 0.001$) QTLs. Analyses of QTLs for mycelial growth and CM morphology suggested that LOD values of 2.96 – 4.57 were significant ($\alpha = 0.05$) and those higher than 4.57 were highly significant ($\alpha = 0.001$) at 25˚C. Similarly, LOD values of 3.07 – 4.54 were significant and those higher than 4.54 were highly significant for QTLs at 30˚C. For CM morphology, LOD values of 3.07 – 4.72 were significant and values above 4.72 were highly significant. Simple interval mapping (SIM) was used to test for the presence of a putative QTL every 1 cM throughout the genetic linkage map. Composite interval mapping (CIM) was performed to control for the effect of background segregating QTLs, at the location of target QTLs. The marker from each linkage group with the highest association to a QTL was added as a background locus and mapping was performed to more

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precisely establish the interval position of the target QTL (Manly & Olson, 1999). QTLs were recorded when the SIM and CIM likelihood ratio (LR) values were equal to, or greater than, the experiment-wise significance levels for significant and highly significant QTLs. LR values were converted to Log-of-the-odds (LOD) values by using LR = 4.6 x LOD (Liu, 1998). Epistatic interactions were detected using the "Interactions" option.

Results

Mycelial growth studies and statistical analyses

The parental isolates, *F. circinatum* and *F. subglutinans*, have different hosts (*Pinus* spp. and teosinte, respectively). Therefore mycelial growth at a range of temperatures was expected to be different for the two species (Figure 2). In comparison to *F. circinatum*, *F. subglutinans* showed a greater range of temperatures at which it could grow. At 25˚C, *F. circinatum* grew significantly faster than *F. subglutinans* ($P = 1.08 \times 10^{-6}$) (Table 1). The opposite was true at 30˚C ($P = 3.58 \times 10^{-6}$), with *F. circinatum* growing significantly more slowly than *F. subglutinans* (Table 1). These two temperatures were thus selected for mycelial growth studies of the F_1 progeny of a cross between *F. circinatum* and *F. subglutinans*.

The average mycelial growth of the 94 F_1 progeny was significantly less than growth for *F. circinatum* ($P = 0.0088$), but not for *F. subglutinans* ($P = 0.46$), at 25ºC (Table 1). The average mycelial growth of the 94 F_1 progeny at 30˚C was not significantly different to that of *F. circinatum* ($P = 0.19$), but was different to that of *F. subglutinans* ($P = 0.015$) (Table 1). Mycelial growth at 25˚C was normally distributed ($P = 0.033$) and the broad sense heritability was 0.98. Similarly, at 30˚C the frequency distribution was normal ($P = 0.0497$) and the broad sense heritability was 0.99.

CM morphology of the progeny displayed either of the two parental species phenotypes, or an intermediate phenotype. Of the 94 F_1 progeny, 36.17% had smooth, 41.49% had laciniate and 22.34% had an intermediate (crenate) colony margin morphology. This was normally distributed ($P = 0.00$).

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Pathogenicity

Inoculation of pine seedlings with the 94 F_1 isolates revealed that only one isolate (FCC 2025; *Fusarium* Culture Collection, Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa) was pathogenic (11.80 ± 2.39 mm). The other 93 F_1 progeny were not significantly different from the *F. subglutinans* parental isolate or from the negative control (distilled water) ($P > 0.05$; results not shown). Therefore the lesion length data did not display a continuous distribution.

The pathogenic F_1 individual grew significantly faster *in vitro* at 25˚C in comparison to the two parental isolates. Growth in this isolate (66.90mm ± 2.28) was also more rapid than the average growth for the remaining 93 F_1 progeny ($P < 0.05$). However, no association was found between mycelial growth and pathogenicity at 25˚C as there were instances of other F_1 individuals that also grew significantly faster than the pathogenic F_1 at 25ºC, yet were not pathogenic.

QTL detection

Three QTLs were detected for mycelial growth at 25˚C (Figure 3A + B), four at 30˚C (Figure 3A + C), and only one for CM morphology (Figure 3A). Only one QTL, namely the one for mycelial growth at 30˚C that lies nearest to marker AA/TC-121bh, displayed transmission ratio distortion ($\chi^2 = 12.30$, $P = 0.00048$). No epistatic interactions were detected in mycelial growth, suggesting that these loci act independently. One QTL appeared in all three mapped traits, namely AT/AC-625bh on Linkage Group 2 at position 231cM (Figure 3A). This QTL was highly significant ($P < 0.001$) in all three cases.

For mycelial growth at 25˚C, the QTLs were located on two linkage groups (LG 2 and 12), and accounted for 45% of the total phenotypic variance (Table 2). Four QTLs were identified for mycelial growth at 30˚C. Three of these QTLs spanned LG1, and the fourth QTL was on LG2. Together, they accounted for 41% of the total trait variance. The QTL on LG2 was shared, indicating that for mycelial growth at 25˚C and 30˚C, this QTL is the only common factor. Only one QTL was identified for CM morphology. This QTL was present at the

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same location as the shared QTL for mycelial growth at 25˚C and 30˚C. This QTL accounted for 21% of the total phenotypic variance.

Discussion

The interspecific cross between *F. circinatum* and *F. subglutinans* (Desjardins *et al.*, 2000), and the genetic linkage map derived from it (De Vos *et al.*, 2007), provided a unique opportunity to determine the genetic basis of mycelial growth and pathogenicity of the F_1 progeny. Our results showed that only one isolate from 94 F_1 progeny was pathogenic, and that this result could be explained by the genetic constitution of this particular isolate. Mycelial growth was investigated at two temperatures (25˚C and 30˚C) and an area of the genome was found that was associated with variation in mycelial growth at both of these temperatures. One highly significant QTL ($P < 0.001$) was present at both growth temperatures as well as for CM morphology. This suggests that this QTL is involved in mycelial growth at both temperatures and that the same region is also involved in CM morphology. Furthermore, there was no association between mycelial growth and pathogenicity at 25˚C. This study is only the second after the study of *F. graminearum* to genetically map QTLs in the genus *Fusarium* (Cumagun *et al.*, 2004).

The fact that *F. circinatum* grew significantly faster at 25˚C than *F. subglutinans* and the opposite was observed for growth at 30˚C provided a useful basis for comparison of growth at these two temperatures. The high heritability (0.98 and 0.99 at 25˚C and 30˚C, respectively) gave an indication of the low environmental variation of mycelial growth in Petri dishes. Similar heritability values have been observed in growth studies with other fungi (Olson, 2006; van der Nest *et al.*, 2009). Furthermore, one highly significant QTL, positioned at marker AT/AC-625bh on LG 2, was detected at both growth temperatures, indicating that this genomic region is important for mycelial growth.

For CM morphology, only one QTL was identified and it was highly significant ($P < 0.001$). This QTL was present at the same location as the shared QTL for mycelial growth at 25˚C and 30˚C. As the putative QTLs only accounted for 45% and 41% of the total mycelial growth variation at 25˚C and 30˚C, respectively, and 21% for the variation in CM

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morphology, there are likely additional QTLs that are associated with these traits that were not detected. Possible reasons for this discrepancy could be the presence of additional small effect QTLs that were not detected, as has been shown by Olson (2006) in a *Heterobasidion* interspecific cross. QTLs that were not expressed under the growth conditions used in this study, as well as those that were not polymorphic in nature (and hence not detectable), could also account for additional QTLs.

In the genetic map of De Vos *et al.* (2007), a large proportion of the markers displayed transmission ratio distortion (55%, $P < 0.05$). Ninety-six percent of the markers exhibiting transmission ratio distortion were skewed towards the *F. subglutinans* parent. The estimated genome coverage of this map showed that 89% of loci were within 10 cM of a framework marker, so this bias was not due to genome coverage. In the present study, only one QTL, the one for mycelial growth at 30˚C that lies nearest to marker AA/TC-121bh, displayed transmission ratio distortion ($\chi^2 = 12.30$, $P = 0.00048$). This marker also displays bias towards the *F. subglutinans* genome (De Vos *et al*., 2007). The QTL lies in a 45.1cM area with four markers displaying highly significant transmission ratio distortion ($P < 0.001$). To account for the effect (beneficial or detrimental) of the distorting loci on QTL detection, MapManager QTX has an "Allow for segregation distortion" function. This allows the program to use the contingency analysis (G-statistic), which is not sensitive to the effects of segregation distortion (García-Dorado & Gallego, 1992).

In some basidiomycetes, an association between mycelial growth and the *MAT* locus has been given as a possible explanation of transmission ratio distortion at the *MAT* loci and markers surrounding them (Simchen, 1966; Larraya *et al.*, 2001; van der Nest *et al.*, 2009). In contrast, no association was found between a specific *MAT* idiomorph and mycelial growth for the *Fusarium* spp. used in this study. Also, the *MAT* locus did not display transmission segregation distortion in this study, which is similar to the results of Friel *et al.* (2007). The *Fusarium* spp. used in this study are ascomycetes and it appears that they do not display the same genetic determinants that influence mycelial growth and sexual recognition that have been shown for certain basidiomycetes (Simchen, 1966; Larraya *et al.*, 2001; van der Nest *et al.*, 2009).

The inoculation data for the F_1 progeny did not display a continuous distribution with only one individual (FCC 2025) pathogenic to *P. patula* seedlings. Therefore QTL analysis could

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not be performed for pathogenicity. When compared to the lesion length produced by the *F. circinatum* parent, the F_1 isolate FCC 2025 was equally pathogenic with no significant difference ($P = 1.00$) found between the two isolates. Doohan *et al.* (2003) hypothesized that faster growing *Fusarium* species on cereals are more pathogenic than those that grow slowly. In contrast, results of this study showed there was no association between mycelial growth and pathogenicity. This is similar to the results of a study using an interspecific cross between host specific species of *Heterobasidion* (Olson, 2006; Lind *et al.* 2007). Results of the present study add evidence to suggest that, mycelial growth and pathogenicity are traits apparently not controlled by the same loci.

It was unusual to find only a single isolate (FCC 2025) amongst the F_1 progeny that was highly pathogenic. This might be explained by the genetic constitution of the isolate. Data from the F_1 map of De Vos *et al.* (2007) were subjected to the Graphical GenoTyping program (GGT; Van Berloo, 1999). It was found that approximately 96.3% of this individual's genome was descended from *F. circinatum*, *i.e.* the maternal parent. The six putative QTLs identified in this study were not found in regions inherited from the *F. subglutinans* parent. Linkage Groups 2, 3, 5, 7, 9 and 11 (6/12 linkage groups) were intact (non-recombinant) linkage groups inherited from *F. circinatum* (Figure S1; see Table 4 of De Vos *et al.* (2007) for the number and origin of intact linkage groups). Interestingly, of the 94 F_1 progeny selected in this study, the F_1 isolate displaying a genomic constitution closest to the *F. circinatum* genomic constitution of the pathogenic isolate, was FCC 2020, with a *F. circinatum* genomic contribution of ~61.7% (Figure S1). Only $^{1}/_{94}$ F_1 progeny (FCC 2025) showed a *F. circinatum* genomic constitution > 90%, whereas $^{13}/_{94}$ F_1 progeny had a *F. subglutinans* genomic constitution > 90%. This could be explained by the fact that the interspecific cross showed a clear bias towards the transmission of *F. subglutinans* alleles, with F_1 individuals receiving an estimated 59.8% of their genomes from this parent (De Vos *et al.*, 2007). Although not tested in this study, we hypothesize that a greater number of progeny should be pathogenic on teosinte, as seen from the $^{13}/_{94}$ F_1 individuals having a genomic constitution of > 90%.

Friel *et al.* (2007) found that of 178 F_1 progeny isolates of the same cross, none were pathogenic on *Pinus radiata* trees. They speculated that the complete absence of pathogenicity in the F_1 progeny implied a bias towards the genome of the *F. subglutinans* (or nonpathogenic) parent. Our results suggest two possible reasons for this. One is that

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multiple genes may be required for pathogenesis, and these genes were possibly incompletely represented in the F_1 progeny, other than in isolate FCC 2025. This also implies that *F. circinatum* alleles that are essential for pathogenicity are located in the 35% of the *F. circinatum* genome present in FCC 2025, but absent in FCC 2020 (Figure S1). It is also possible that the gene-for-gene relationship could account for the lack of pathogenicity in the majority of the F_1 individuals (Flor, 1942). The F_1 individuals in this study (except FCC 2025) and in that of Friel *et al.* (2007) inherited various avirulence genes from the *F. subglutinans* parent. These were recognized by the host (*P. patula*), which resulted in resistance as seen by the inability of the F_1 individuals to cause disease.

In this study we identified QTLs involved in mycelial growth, CM morphology as well as a pathogenic F_1 isolate that displayed pathogenicity to *P. patula* due to its highly conserved genomic constitution to *F. circinatum*. These results are important to our understanding of the apparent lack of correlation between fitness traits such as pathogenesis and morphological traits such as mycelial growth. Specifically, variation in pathogenicity and mycelial growth variation may involve different genomic loci in *Fusarium* spp. To identify these genes as well as other genes of interest, the *F. circinatum* parental strain used in this study, has been sequenced with a 10X coverage (www.genomesonline.org). The potential applications of this genomic sequence are great and could, for instance, provide insights into *Fusarium circinatum* genomic architecture, the identification of host-specific genes and could aid in the elucidation of the molecular mechanisms of pathogenicity, as well as aid in studies regarding different *Fusarium* species.

Acknowledgements

We thank the University of Pretoria, members of the Tree Protection Cooperative Program (TPCP), the Mellon Foundation, the National Research Foundation (NRF) / Department of Science and Technology (DST), Centre of Excellence in Tree Health Biotechnology and the THRIP initiative of the Department of Trade and Industry (DTI) in South Africa for financial assistance.

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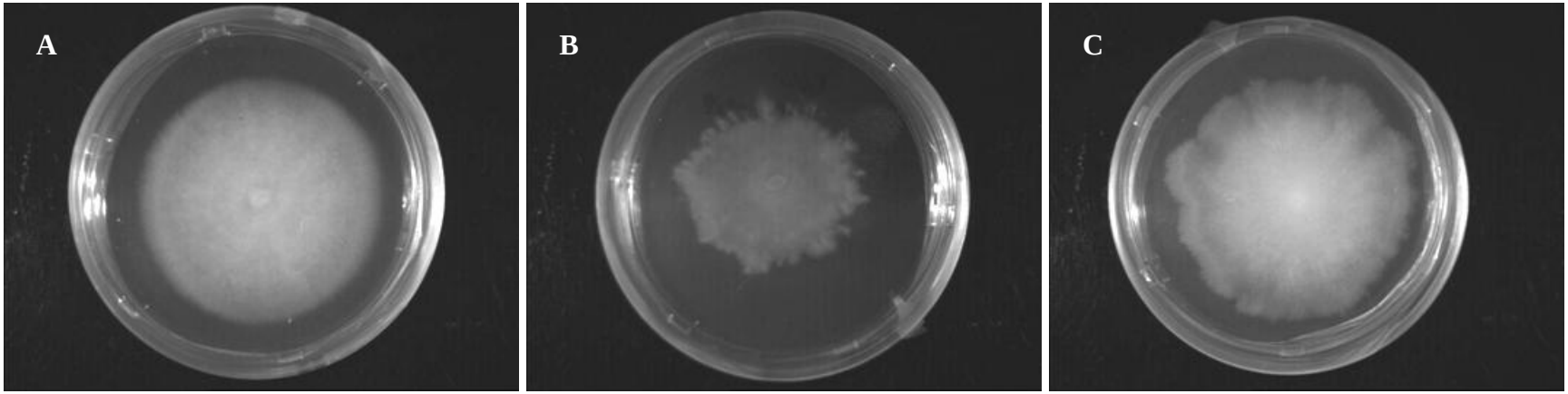

Figure 1. Colony margin morphology at 25˚C after seven days of growth in the dark. (A) *F. circinatum* displays a smooth edge. (B) *F. subglutinans* displays an irregular (laciniate) edge. (C) Example of F_1 isolate that showed an intermediate (crenate) edge morphology to (A) and (B).

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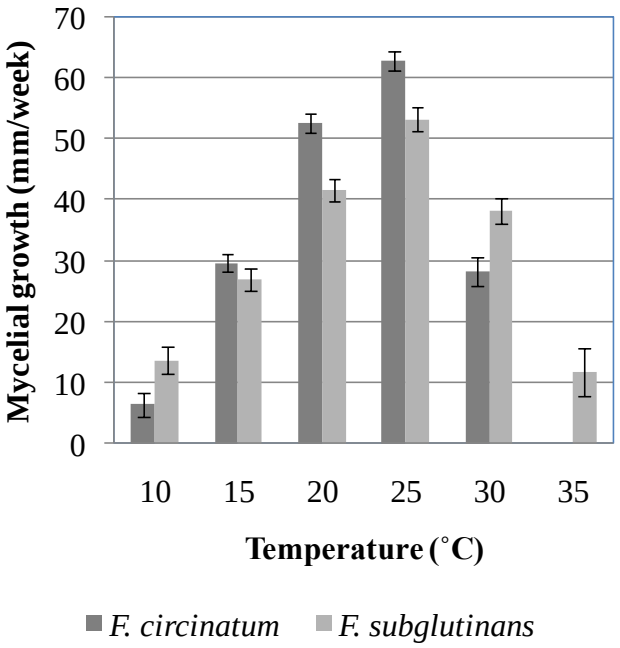

Figure 2. Measurements of the mycelial growth of *F. circinatum* and *F. subglutinans* (five replicates) at a range of temperatures for seven days in the dark on ½ PDA. Error bars represent the standard deviation.

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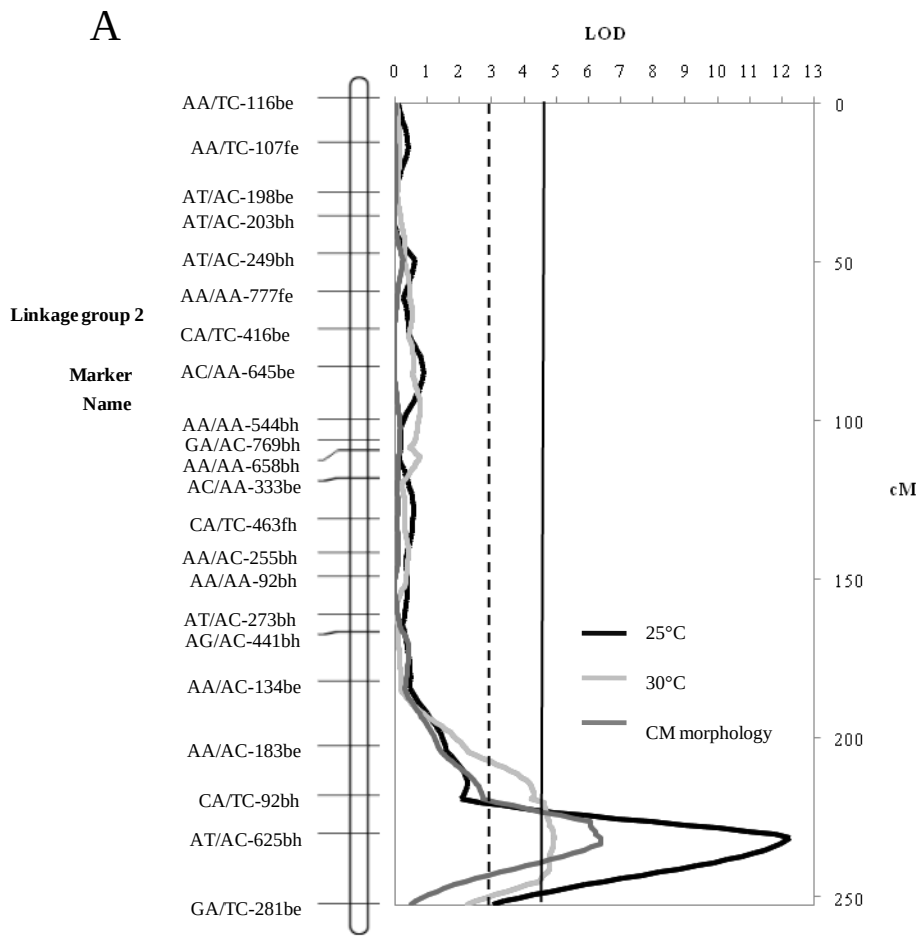

Figure 3. **(A)** Location of a QTL on Linkage Group 2. The LOD significance levels for significant (α = 0.05) and highly significant (α = 0.001) QTLs are indicated by a dashed and solid line, respectively. **(B)** Location of a QTL for mycelial growth at 25˚C on Linkage Group 12. The LOD significance level (α = 0.05) is indicated with a dashed line. **(C)** Location of a QTL for mycelial growth at 30˚C on Linkage Group 1. The LOD significance level (α = 0.05) is indicated with a dashed line.

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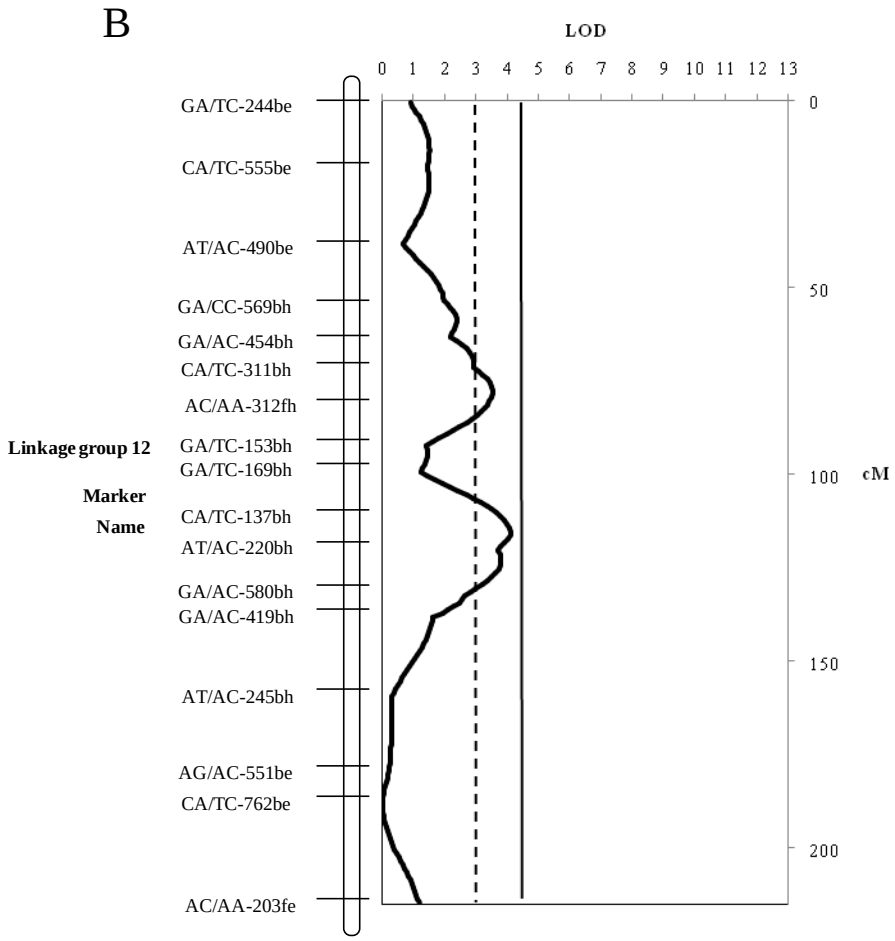

Figure 3. (*continued*)

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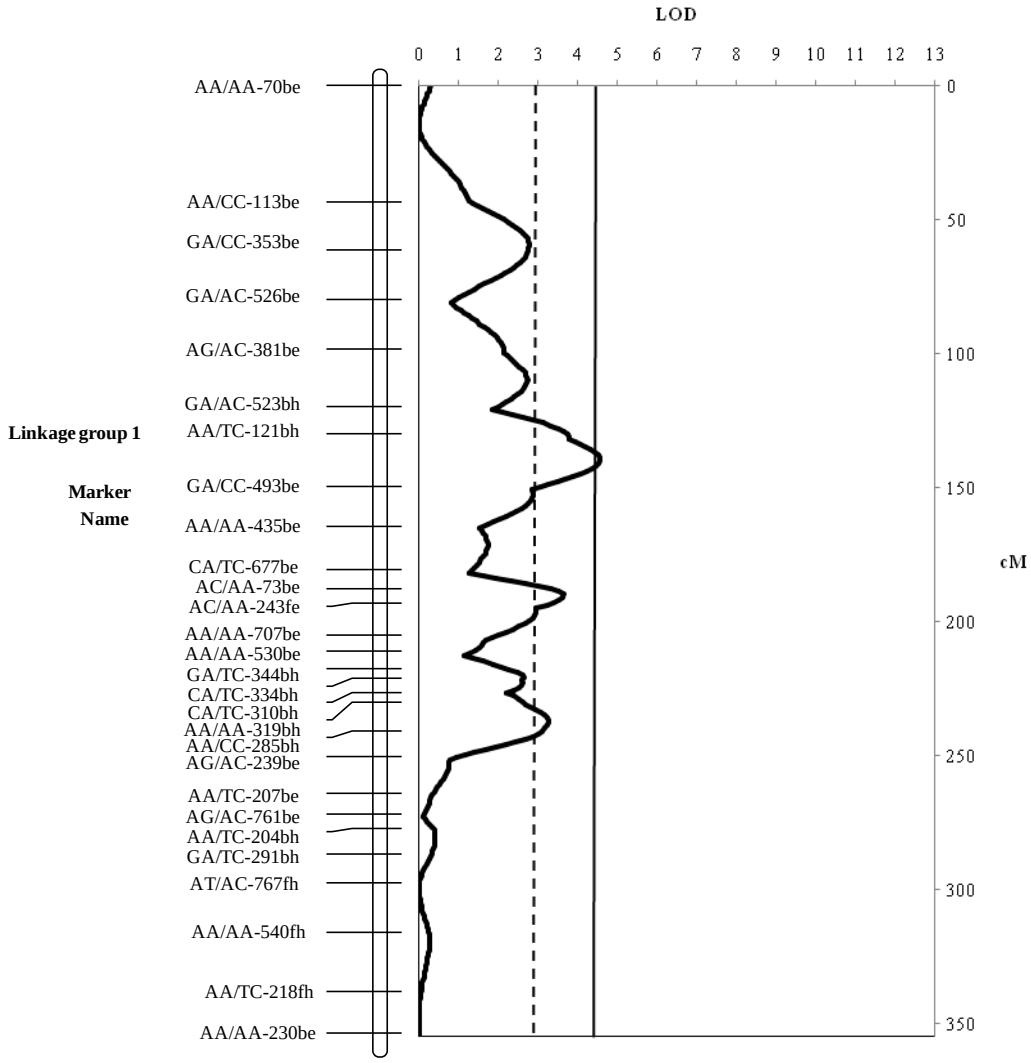

Figure 3. *(continued)*

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Table 1. Average mycelial growth of the parental isolates, 94 F_1 progeny and the F_1 isolate FCC 2025 at 25ºC and 30ºC for seven days in the dark on ½ PDA.

Isolate	25°C[a]	30ºC[b]
F. circinatum	62.90 ± 1.52 (a)	28.30 ± 2.41 (a)
F. subglutinans	53.30 ± 2.06 (b)	38.30 ± 2.06 (b)
94 F_1 progeny[c]	53.66 ± 12.28 (b)	31.22 ± 10.23 (a)

[a] *In vitro* mycelial growth at 25ºC measured in mm/week. Numbers followed by different letters in the same column are significantly different at $P = 0.05$.

[b] *In vitro* mycelial growth at 30ºC measured in mm/week. Numbers followed by different letters in the same column are significantly different at $P = 0.05$.

[c] Mycelial growth as measured for the mean of the 94 F_1 progeny.

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Table 2. QTLs for mycelial growth detected at 25˚C and 30˚C, as well as for colony margin morphology at 25ºC.

Trait	Linkage Group	Nearest upstream marker [a]	QTL position (cM)[b]	LOD[c]	R^2 [d]
25˚C	LG2	AT/AC-625bh	231	12.26**	32%
	LG12	CA/TC-311bh	77	3.52*	6%
		CA/TC-137bh	117	4.09*	7%
30˚C	LG1	AA/TC-121bh	148	4.48*	12%
		AC/AA-73be	190	3.48*	9%
		AA/AA-319bh	238	3.24*	9%
	LG2	AT/AC-625bh	236	4.96**	11%
CM	LG2	AT/AC-625bh	231	6.37**	21%

* Experiment-wise significance level of $P = 0.05$ determined using Map Manager QTX.

** Experiment-wise significance level of $P = 0.001$ determined using Map Manager QTX.

[a] Nearest marker upstream to QTL position on the map of De Vos *et al*. (2007).

[b] Based on De Vos *et al.* (2007). Values indicate the map position of the QTL towards the bottom of the linkage group.

[c] LOD values were obtained using the equation LR = 4.6 x LOD (Liu, 1998).

[d] The percentage of the total trait variance that can be explained by a QTL being present at this locus.

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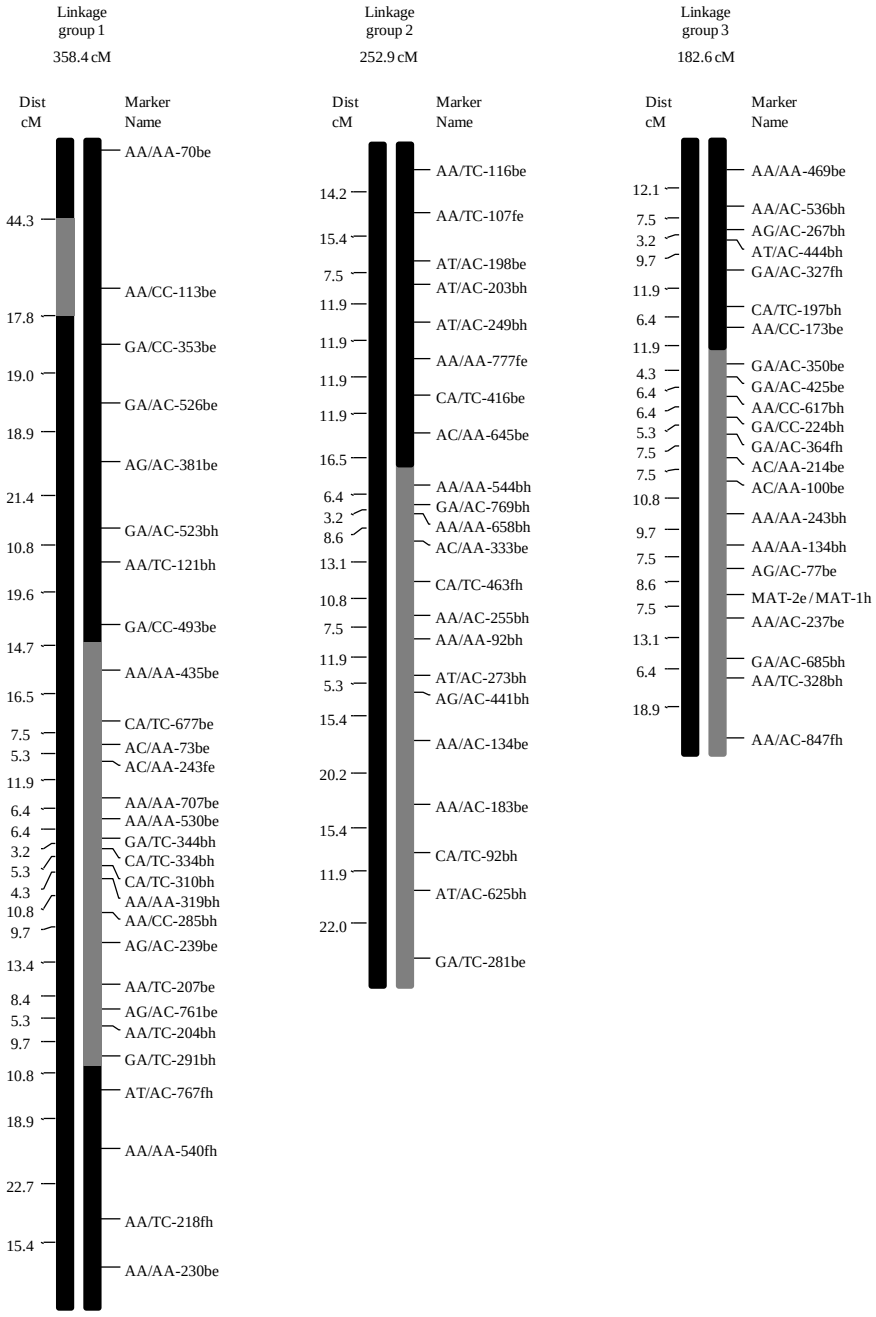

Supplementary Figure 1. Comparison of the graphical representation of the genome of FCC 2025 (linkage group to the left) and FCC 2020 (linkage group to the right). Twelve linkage groups are shown with the black bars representing the genome originating from the *F. circinatum* parent and the grey bars that originating from the *F. subglutinans* parent.

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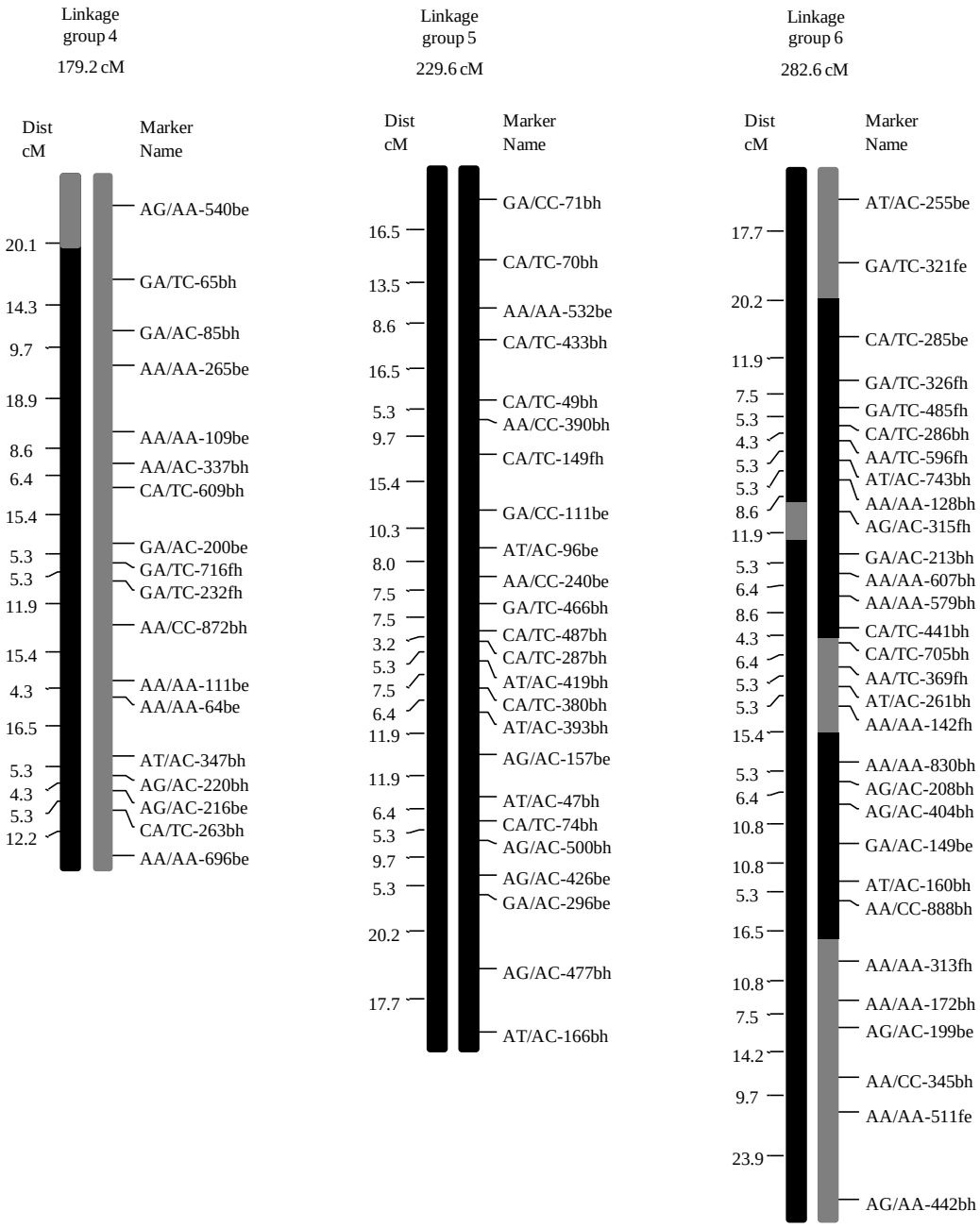

Suppl. Fig.1. (*continued)*

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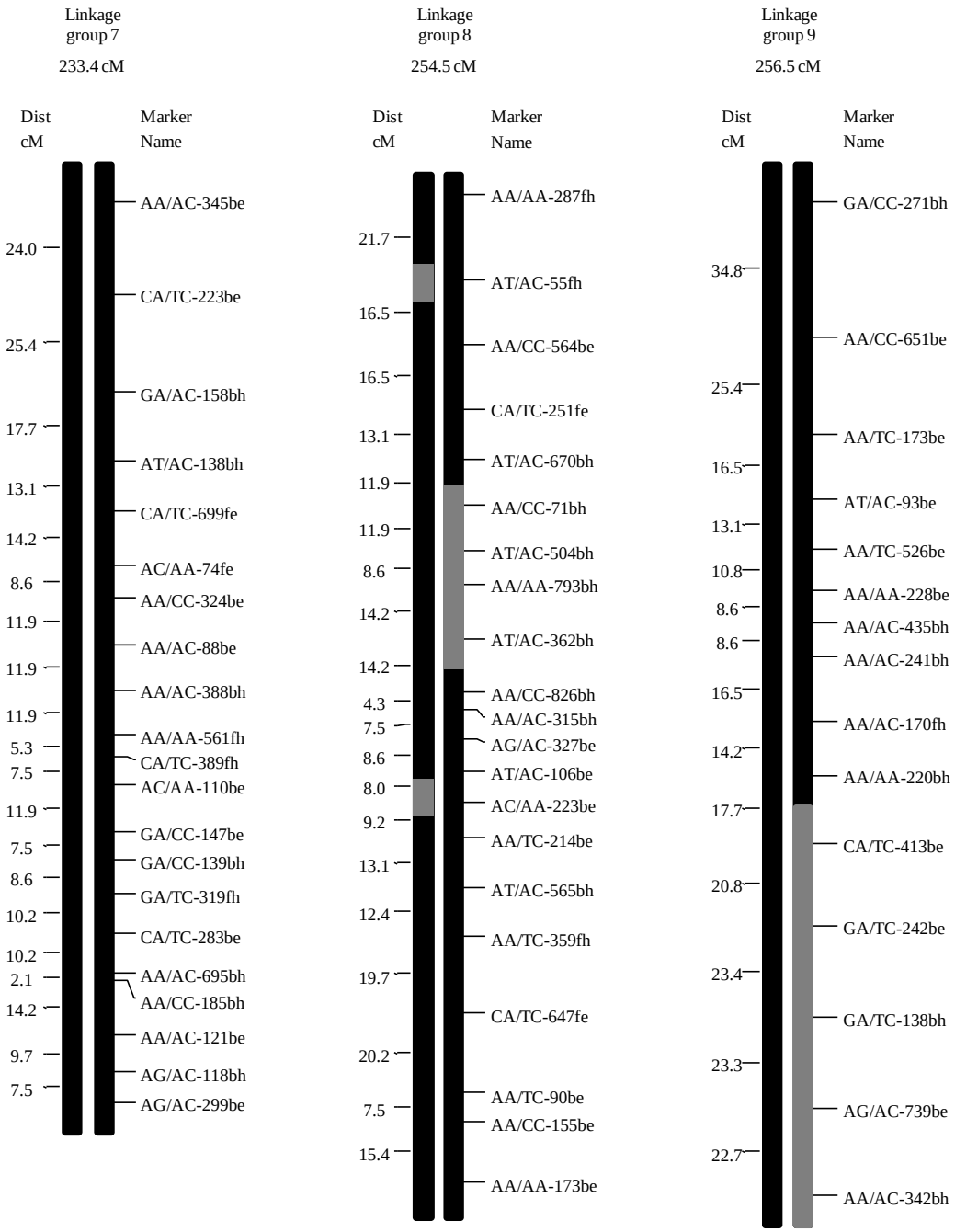

Suppl. Fig. 1. (*continued*)

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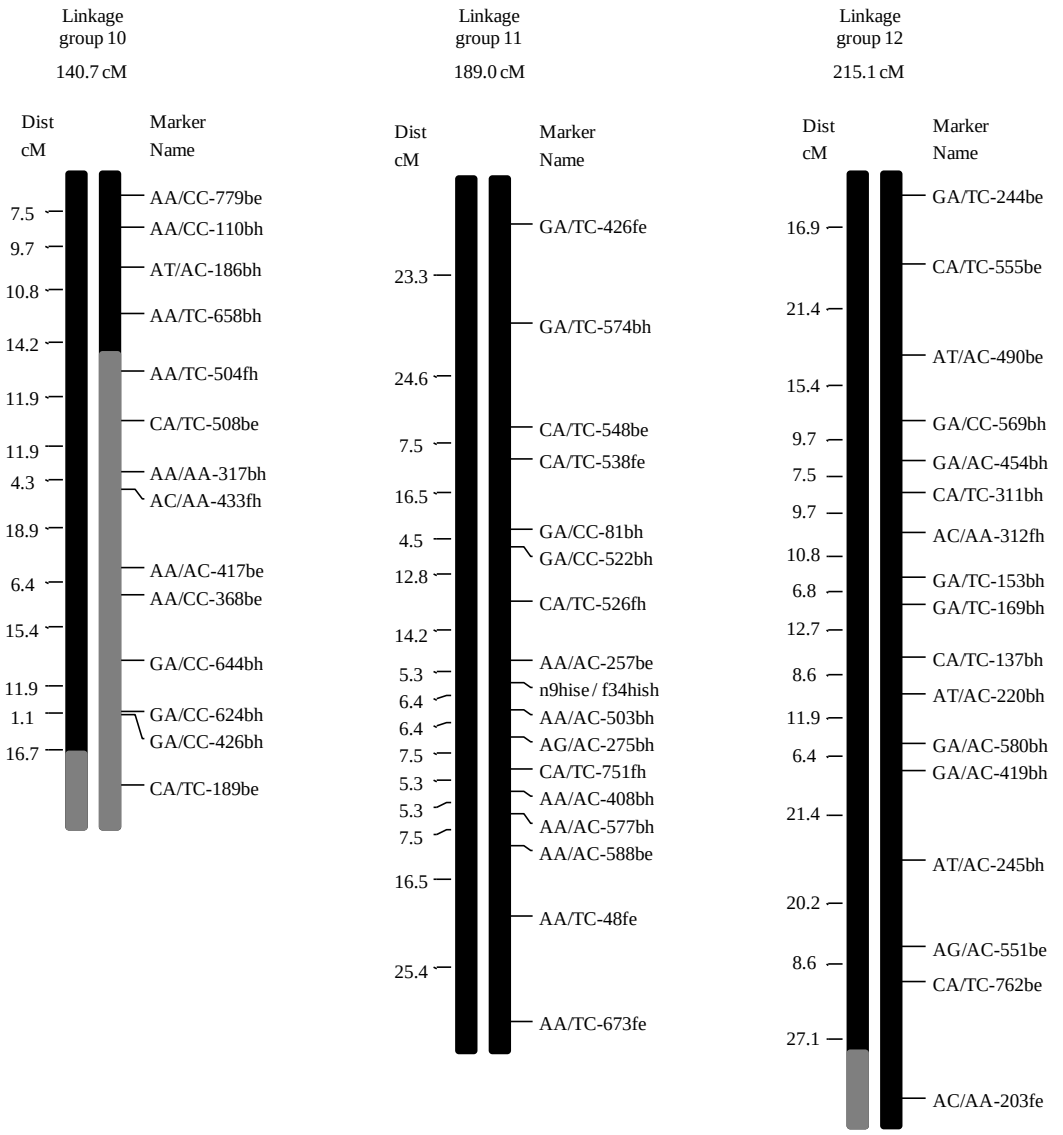

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CHAPTER 5

Macrosynteny within the *Gibberella fujikuroi* species complex allows for the identification of a putative reciprocal translocation

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Abstract

Fusarium circinatum, *Fusarium fujikuroi* and *Fusarium verticillioides* are members of the *Gibberella fujikuroi* species complex for which genomic sequences are available. In this study, a previously completed genetic linkage map of an interspecific cross between *Fusarium subglutinans* and *F. circinatum*, together with pyrosequenced AFLP fragments of these two species, helped identify synteny among the three *Fusarium* genomes. Using *in silico* generated AFLPs of the *F. circinatum* genome, and BLAST results from pyrosequenced AFLP fragments, 298 homologous regions were aligned to the genomes of *F. fujikuroi* and *F. verticillioides*, and used to order 275 contigs of the *F. circinatum* genome to that of *F. verticillioides*. By incorporating the genetic linkage map data with that of the *Fusarium* genomic sequences, a putative translocation between linkage group 4 and 7 was identified. Eleven of 12 linkage groups from the genetic linkage map were successfully mapped to the eleven chromosomes of *F. verticillioides*. A remarkable level of macrosynteny was observed between *F. verticillioides* and *F. fujikuroi*, which will aid in the genome assemblies of new *Fusarium* spp., including *F. circinatum*, in the *G. fujikuroi* species complex. A putative fungal transcription factor closely linked to a QTL previously shown to control mycelial growth in *F. circinatum*, was identified.

Introduction

Next generation sequencing has resulted in an explosion of genomic data and consequently genome assemblies for species of diverse life forms. This is also evident for fungi in the genus *Fusarium* where genome sequences are available for increasing numbers of species (Cuomo *et al.*, 2007; Ma *et al*. 2007; Coleman *et al.*, 2009; Gardiner *et al.*, 2011; Srivastava *et al.*, 2011; Wingfield *et al.*, 2012; http://www.fgsc.net/Fusarium/2011FusWkshp_Program.htm). This has allowed unparalleled whole-genome comparisons between different *Fusarium* spp. (Cuomo *et al.*, 2007; Coleman *et al.*, 2009; Ma *et al.*, 2010), with significant progress being made specifically in understanding genomic organization and the role of lineage-specific chromosomes, some of which are linked to pathogenicity.

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The *Gibberella fujikuroi* species complex reflects the sexual stage associated with strains of *Fusarium* within the section *Liseola*. It consists of over 50 distinct phylogenetic lineages (Kvas *et al.*, 2009), including the important pathogens of pine (*Fusarium circinatum*), rice (*Fusarium fujikuroi*) and maize (*Fusarium subglutinans*). Desjardins *et al.* (2000) reported on an interspecific cross between *F. circinatum* and *F. subglutinans*, which was subsequently used to generate a genetic linkage map of the progeny (De Vos *et al.*, 2007). In this map, AFLP markers spanned twelve major linkage groups. A large proportion of these markers (55%) showed transmission ratio distortion ($P < 0.05$) and there was a bias towards progeny receiving alleles from the *F. subglutinans* parent.

Phylogeographic studies have provided support for African, Asian and American clades in the *G. fujikuroi* species complex (O'Donnell *et al.*, 1998a, 2000). *Fusarium verticillioides* (Fusarium ear rot of maize) is represented in the African clade, *Fusarium fujikuroi* (bakanae disease in rice seedlings) in the Asian clade and *F. circinatum* in the American clade (Suppl. Figure 1). The genomes of all three species have been sequenced (*Fusarium* Comparative Sequencing Project; Wingfield *et al.*, 2012; http://www.fgsc.net/Fusarium/2011FusWkshp_Program.htm) thus enabling comparisons among three species in the three geographic clades of the *G. fujikuroi* species complex.

The *F. circinatum* genome is partially assembled and currently consists of 4509 contigs, which have not been ordered into scaffolds (Wingfield *et al.*, 2012). The *F. verticillioides* genome has been ordered into 11 chromosomes (*Fusarium* Comparative Sequencing Project) and the *F. fujikuroi* genome into 32 supercontigs (http://www.fgsc.net/Fusarium/2011FusWkshp_Program.htm). Utilizing *F. circinatum* AFLP markers from the genetic linkage map[1] (De Vos *et al.*, 2007) in conjunction with *in silico* generated AFLPs of *F. circinatum*[2] (Rombauts *et al.*, 2003), as well as a subset of pyrosequenced AFLP fragments of the interspecific cross[3], BLAST was used to directly compare data from the three genomes by way of homologous AFLP sequences. The objectives of this study were thus to determine the extent of synteny among the three *Fusarium* genomes, and whether significant synteny exists to order the contigs of the pine

[1] Hereafter referred to as AFLP map markers.
[2] Hereafter referred to as *in silico* generated AFLPs.
[3] Hereafter referred to as pyrosequenced AFLPs.

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pathogen, *F. circinatum*. An additional aim was to determine whether the AFLP sequences linked to previously identified QTLs involved in controlling mycelial growth (De Vos *et al.*, 2011) could be used to identify putative genes underpinning the QTL in the *F. circinatum* genome.

Materials & Methods

AFLP generation

DNA was extracted from the two parental isolates of the interspecific cross between *F. circinatum* and *F. subglutinans* following the protocol of De Vos *et al*. (2007). AFLP fingerprints were generated for the two parents (De Vos *et al.*, 2007; Vos *et al.*, 1995) whereby DNA was digested with the restriction endonucleases *Eco*RI and *Mse*I (Zeller *et al.*, 2000). These fragments were ligated to *Eco*RI and *Mse*I specific adapters. Adapter-specific primers with zero-base-addition selective nucleotides were used to perform pre-selective PCR amplifications. The final selective PCR amplification made use of adapter-specific primers with two additional selective nucleotides at the 3' end. Three selective-amplification primer combinations (of the 13 used by De Vos *et al.* (2007) to generate the linkage map) used in this study and were designated X, Y and Z (Table 1); they provided the best coverage of the twelve linkage groups.

PCR addition of sequencing adapters

After AFLP generation, all fragments carry the same sequences at their 5′ and 3′ ends, which correspond to the 16nt pre-selective PCR primers (Figure 1A). During a standard library preparation for sequencing on the GS FLX system (Roche Diagnostics, Basel, Switzerland), DNA is fragmented and the fragments are then "polished" or made blunt-ended so that the 'A' and 'B' pyrosequencing adaptors can be ligated (Margulies *et al.*, 2005). Because AFLPs had already been performed, these steps were not necessary. Secondly, the GS library protocol was further modified by the addition of modified 454 'A' and 'B' pyrosequencing adapters to the ends of the AFLP fragments, using PCR (Figure 1B). Primers complementary to the AFLP ends, and carrying the 19nt pyrosequencing adapter sequences on the 5' end were synthesized (*Eco*RI + A adaptor:

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GCCTCCCTCGCGCCATCAGGACTGCGTACCGAATTC and *Mse*I + B adaptor: TTACTCAGGACTCATCCTGGATGAGTCCTGAGTAATTAA). A PCR with these primers added the pyrosequencing adapter sequences to the AFLP fragments. PyroStart™ *Taq* polymerase (Fermentas Life Sciences, Ontario, Canada) was used because it is suitable when using large primers with very high annealing temperatures. This enzyme is able to extend at a range of temperatures, allowing annealing and extension to take place simultaneously. The following conditions, calculated following the manufacturer's instructions, were used for the amplification: One cycle of 95ºC for 60s, 25 cycles of 95ºC for 60s and 65ºC for 40s and one cycle of 72ºC for 20s. To confirm the addition of pyrosequencing adapters, the products were analyzed using a 6% polyacrylamide gel electrophoresis (PAGE) at 9V/cm. Silver staining (Bassam & Caetane-Annolés, 1993) was used to visualize the DNA fragments. As controls, the final amplification products from the AFLP reaction were run alongside the products of the adapter-addition reaction.

Pyrosequencing

DNA samples were concentrated by centrifugation in a vacuum centrifuge at 45ºC for 20min until dry, and re-suspended in deionised sterile water to a final concentration of 1300ng/ul. The three primer combinations were then pooled into a single sample for each isolate, which was frozen and submitted for sequencing. Pyrosequencing was performed by Inqaba Biotec (Pretoria, South Africa) using the GS FLX system. Because the 'A' and 'B' adapters were already present on the fragments, the customary library-preparation steps were omitted. Sequencing was performed from both ends of the fragments, using both pyrosequencing adapters as targets for sequencing primers. One lane was used for each isolate, with the three primer combinations pooled. The +2 selective nucleotides of the final amplification were used to differentiate the three samples from each other.

AFLP pyrosequence data analysis

Sequences were analyzed and edited using the Vector *NTI ADVANCE* ™ 9.0 software (Invitrogen Life Technologies, Midrand, South Africa). Pyrosequencing and AFLP adapters were trimmed from the ends using the ContigExpress utility (Figure 1C). AFLP sequences were recognized as starting and ending with the *Eco*RI and *Mse*I restriction sites, and the

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three primer combinations were distinguished from each other by the two selective nucleotides downstream of the restriction sites. Sequences of the same length and primer combination were aligned using the AlignX utility of the program.

This dataset was then BLAST searched (Altschul *et al.*, 1990) against the *F. circinatum* (Wingfield *et al.*, 2012), *Fusarium verticillioides* (*Fusarium* Comparative Sequencing Project) and *Fusarium fujikuroi* (http://www.fgsc.net/Fusarium/2011FusWkshp_Program.htm) genome sequences, using the 'Local BLAST' function of CLC Genomics Workbench (v4.8, CLC bio, Denmark). A cutoff E-value of $\leq 1 \times 10^{-10}$ was used to determine sequence homology. Sequences not displaying the necessary similarity to *F. verticillioides* and *F. fujikuroi*, but to *F. circinatum*, were subjected to a second round of BLAST. Here, the original length of the pyrosequenced fragment was substantially increased using data originating from the *F. circinatum* genome sequence, *i.e.* a 100bp fragment that showed similarity to *F. circinatum* but not to the other genomes was increased in length to 300bp by including sequence data from the flanking regions in the *F. circinatum* genome.

Fusarium circinatum marker placement

Using the genome sequence of *F. circinatum* (Wingfield *et al.*, 2012), *in silico* AFLPs were generated using the program, AFLPinSilico (v2, Rombauts *et al.*, 2003). This was performed for the 13 primer combinations (De Vos *et al.*, 2007). There were two reasons for generating *in silico* AFLPs were generated in addition to the pyrosequenced AFLP data. Pyrosequenced AFLP fragments were limited, due to the nature of the sequencing technology, to 250-500bp in length. AFLP map markers contained fragments ranging in size from ~50-1000bp and by using *in silico* generated AFLPs more AFLP map markers would be assigned to actual genomic sequence. Secondly, pyrosequencing was performed for only three primer combinations, of a total of thirteen (De Vos *et al.*, 2007).

The genetic linkage map (De Vos *et al.*, 2007) consisted of 148 *F. circinatum* AFLP markers of known size. With the use of *in silico* generated AFLPs, we attempted to assign each *F. circinatum* AFLP map marker to a corresponding AFLP in the genome of *F. circinatum*. BLAST search was then made of these AFLP sequences against the other two *Fusarium*

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genomes (see section on AFLP pyrosequence data analysis) to determine the position and presence of these sequences in these other genomes. Two gene-based markers included on the original genetic linkage map, the mating type idiomorph and histone (H3) gene, as well as these for translation elongation factor 1-α, β-tubulin and calmodulin genes, (Suppl. Materials) were also linked to the *Fusarium* genomes.

Using the program Genome Synteny Viewer (GSV, Revanna *et al.*, 2011), syntenous regions (corresponding to the 11 chromosomes of *F. verticillioides*) between the *F. verticillioides* and *F. fujikuroi* genomes were aligned, using the BLAST results from the homologous AFLP fragments. However, because only a draft *F. circinatum* assembly is available (Wingfield *et al.*, in 2012), it was not possible to directly compare this genome sequence with the other *Fusarium* spp. Using the AFLP linkage map, the order of the *F. circinatum* contigs could be determined. Hence, an annotation file was also prepared for the *F. circinatum* genome in GSV, which showed the syntenous regions corresponding to contigs of *F. circinatum*. These contigs were drawn relative to the *F. verticillioides* genome, which has the most comprehensively annotated genome.

Results

AFLP generation

DNA was successfully isolated from the *F. circinatum* and *F. subglutinans* parental isolates. AFLPs were generated for the two parental isolates using primer combination X, Y and Z (Table 1) for the final amplification. Primer X was represented on 10 linkage groups (LG), Y on 12 LGs and Z on 11 LGs of the genetic linkage map of De Vos *et al.* (2007).

PCR addition of sequencing adapters

The *Eco*RI + A and *Mse*I + B primers were used in a PCR to add the 454 'A' and 'B' pyrosequencing adapters to the AFLP fragments for pyrosequencing. To determine whether the PCR had successfully amplified all the AFLP fragments, samples were separated on a PAGE gel with the final amplification products as controls. Separation over the 25cm gel

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was insufficient to distinguish between individual bands, but the PCR products profiles matched those of the final amplification products (results not shown).

AFLP pyrosequence data analysis

In order to determine the sequences of the pyrosequenced AFLP fragments larger than 250 bp, sequencing was performed in two directions, using both the 'A' and 'B' adapters as priming targets (as opposed to using the 'B' adapter only). This raised the potential to yield contigs ranging from 250-500 bp in length. However, none of the sequences generated could be assembled to yield fragments greater than 250 bp in length. This could be due to the emulsion (EM) PCR reaction, a preparatory step in 454 sequencing, being optimized for fragments around 250 bp in length. Also, closer inspection of pyrosequenced AFLP fragments revealed that almost all of these carried the B adapter, suggesting that priming may have been less effective on the A adapter.

Two sets of sequences were generated, one for each parental isolate, each consisting of AFLP fragments from the three primer combinations (Table 2). The total number of sequence reads was 6104, with significantly similar numbers of reads from *F. circinatum* and *F. subglutinans* ($P = 0.12$). Incomplete and duplicate sequences were removed, yielding 337 AFLP sequences, equating to 5.52%, when compared to the original dataset. After removal of incomplete sequences, there was a bias towards AFLP fragments originating from the *F. circinatum* parent ($P = 0.0039$). This was also observed after removing duplicate sequences, although this was statistically more significant ($P = 0.00060$), although the gel bands observed on the LI-COR® gel were statistically similar between *F. circinatum* and *F. subglutinans* ($P = 0.58$). This inconsistency was most likely due to a partial failure of the AFLP procedure for the primer Z combination in *F. subglutinans* (Table 2, $P < 0.001$ for primer combination Z).

The unique AFLP sequences obtained (Table 2) were more numerous than the number of scored gel bands in the same size range (De Vos et al., 2007; $P < 0.001$). Fifty-nine (17.51%) of the pyrosequenced fragments had two or more different sequences that were identical in length. The average number of different sequences of any particular length was 1.22 ± 0.53. For those sequences that had one or more different partners of the same length, each sequence

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was compared to each of its counterparts. The percentage similarity was determined for each pair and these were plotted (Figure 2). Two clusters were clear on the graph (labeled "A" and "B"); the first was a major cluster (91.95% of pairs), averaged at 50.15% similarity, while the second smaller cluster (8.046% of pairs) averaged at 95% similarity. A 50% AFLP sequence similarity is expected if the sequences are unrelated. The highly similar cluster of sequences was not due to sequencing error, as these were BLAST searched and showed similarity to different genomic areas. The average GC content of the sequences (excluding the added AFLP adapters) was 43.31%. Thirty-seven monomorphic AFLP fragments were shared between *F. circinatum* and *F. subglutinans* (Table 2).

BLAST searches of the 300 pyrosequenced AFLP fragments to the genomes of *F. circinatum*, *F. verticillioides* and *F. fujikuroi* (Table 3) showed that approximately 60% of the fragments shared significant similarity with all three *Fusarium* genomes. Forty-nine of the fragments (16.33%) showed no similarity with any of the three genomes. Fifteen percent were unique to *F. circinatum* and were not found in *F. verticillioides* or *F. fujikuroi*. Furthermore, there were 25 fragments (8.33%) not shared between all three genomes, but only by two of the species, *i.e.*, 19 fragments were similar in sequence in both *F. circinatum* and *F. verticillioides*, and 6 fragments were similar in sequence in only *F. circinatum* and *F. fujikuroi*.

Fusarium circinatum marker placement

Using *in silico* AFLPs, an attempt was made to assign each *F. circinatum* AFLP genetic linkage map marker to a corresponding *in silico* generated AFLP from the genome of *F. circinatum*. The size of the AFLP fragments on the LI-COR® gel was estimated using the Saga^{MX} AFLP® Analysis Software package (De Vos *et al.*, 2007). Included in this estimate were the AFLP adaptors, which added an additional 24 bp to the size of the AFLP fragment. Thus, the 'true' size of the AFLP fragments on the genetic linkage map was the size (as indicated) minus 24 bp. This 'true' size was used when assigning these fragments to the AFLP *in silico* fragments. A slight discrepancy was found between the 'true' size as compared to the size obtained from the AFLP *in silico* generated from the actual genomic sequence (the 'true' size was underestimated by <5bp). One hundred and twenty-nine (91.49%) of the *F. circinatum* AFLP fragments were assigned to the genome of *F.*

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circinatum, and the corresponding homologous regions on the genomes of *F. verticillioides* and *F. fujikuroi* were found (Table 4). Because *F. verticillioides* was used as the reference genome, only the 11 chromosomes in this particular strain were used, even though members of the *Gibberella fujikuroi* species complex are believed to have 12 chromosomes (Xu *et al.*, 1995). For this reason, the designations of chromosomes by *F. verticillioides* (*Fusarium* Comparative Sequencing Project) were used to assign supercontigs from *F. fujikuroi* and contigs of *F. circinatum*, as well as linkage groups from the interspecific cross of *F. circinatum* and *F. subglutinans*, to specific chromosomes (Table 5).

Using the GSV program, sequences of 169 pyrosequenced AFLP fragments (of 181 (Table 3)) were incorporated to show synteny amongst *F. verticillioides* and *F. fujikuroi*. Likewise, the 129 AFLP map markers identified on the genetic linkage map using *in silico* analysis (De Vos *et al.*, 2007), were added to show a significant amount of conservation of AFLP fragment order between *F. verticillioides* and *F. fujikuroi* (Table 4 and Suppl. Figure 2). This reflects the macrosynteny between the genomes of *F. verticillioides* and *F. fujikuroi*. The genetic linkage maps of the interspecific cross between *F. circinatum* and *F. subglutinans* were also incorporated with the genomic sequence of *F. verticillioides* and *F. fujikuroi* (Table 5 shows these comparisons). Using only those AFLP markers (from the genetic linkage map) that could be placed on the *F. verticillioides* sequence, the relationship between the physical and genetic distance for *F. circinatum* was determined to be 14.34 kb/cM (Table 5). This was relative to the *F. verticillioides* sequence because the *F. circinatum* genome (Wingfield *et al.*, 2012) has not been completely assembled. Using only those *F. circinatum* contigs that contain more than one AFLP map marker, it was possible to approximate a value of 16.69 kb/cM for this species.

While there was overall macrosynteny between *F. verticillioides* and *F. fujikuroi* there was a size discrepancy between the corresponding contigs for chromosome 4 in *F. fujikuroi* (2343307 bp) and *F. verticillioides* (4234339 bp) (Suppl. Figure 2), with the *F. verticillioides* chromosome more than 2 Mb larger than the corresponding *F. fujikuroi* chromosome. This DNA sequence was not found elsewhere in the *F. fujikuroi* genome. The corresponding markers of the *F. circinatum* linkage group (LG 10), which is the homolog to chromosome 4, shows similarity only to the region in *F. verticillioides* that is apparently missing in *F. fujikuroi*.

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Evidence was found of a translocation between the distal ends of chromosome 8 and 11 of the genetic linkage map (Suppl. Figure 2 and Figure 3). This was evident between six *F. circinatum* markers on chromosome 8 and five from chromosome 11 (indicated by an asterix on Suppl. Figure 2 and Figure 3). To test whether a miscalculation had occurred in the original construction of LG 4 (chromosome 8) and LG 7 (chromosome 11), the original data were reanalyzed in MapMaker. These two linkage groups were shown to group separately, with no markers showing linkage between these two linkage groups.

The 298 markers incorporated into the GSV program were found on 275 contigs of *F. circinatum* (Table 6). This represents 6.10% of the total of 4509 contigs (Wingfield *et al.*, 2012). In some cases, more than one marker sequence was found on a contig.

The highly significant growth QTL identified in the study of De Vos *et al.* (2011) was positioned on a marker, AT/AC-625bh of LG 2 (chromosome 3). The sequence of this marker was identified using *in silico* generated AFLPs. Examination revealed that this marker was present on contig 02138 (position 9351 to 9953 bp) of the *F. circinatum* sequence and overlapped with a gene, FCIRG_04559 (complement position 9513 to 11824bp). This putative ORF was validated by BLAST comparison to an EST database (Wingfield *et al.*, 2012). This ORF does not give any significant BLAST results to the genomic sequences of *F. verticillioides* and *F. fujikuroi*. However, flanking regions to this ORF are present in the correct place on the corresponding supercontigs of these two *Fusarium* spp., which were included in the data for the GSV program. This ORF shows homology to a putative Zn_2Cys_6 fungal transcription factor (InterPro Scan, http://www.ebi.ac.uk/Tools/services/web/toolform.ebi?tool=iprscan). Only the translated protein shows homology to a *F. verticillioides* protein (FVEG_13162.3, *Fusarium* Comparative Sequencing Project), which is a hypothetical protein with a fungal specific transcription factor domain. This protein is, however, found on chromosome 6 (supercontig 3.22) and has a corresponding match in the *F. fujikuroi* sequence (supercontig 004). It has a corresponding match to contig 02646 in the *F. circinatum* genome (FCIRG_01652, E = 0.0). Other QTLs identified in the study of De Vos *et al.* (2011) could not be identified as they were not positioned on an AFLP map marker. In addition, the closest AFLP map marker to these QTLs originated from the *F. subglutinans* parent, the genome of which has not been sequenced.

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Discussion

This study served to demonstrate the usefulness of previously anonymous AFLP fragments, in conjunction with genomic sequence data, in synteny analyses between species in the *G. fujikuroi* species complex. The placement of sequence characterized AFLP fragments originating from *F. circinatum* and *F. subglutinans* revealed a considerable amount of macrosynteny between *F. verticillioides* and *F. fujikuroi*. Additionally, a putative translocation involving two chromosomes in the F_1 progeny of an interspecific cross was detected. A QTL associated with mycelial growth could be identified from the genome sequence of *F. circinatum*, and it was characterized as a putative transcription factor.

Pyrosequencing of AFLP fragments showed that *ca.* 16% of these fragments had no similarity to any of the genomic sequences of the three *Fusarium* spp. The possible reasons for this observation are two-fold. Firstly, in many cases the pyrosequenced fragments were short, resulting in numerous chance 'hits' to the reference genome. This complicated the assignment of these sequences to the correct homologous regions. Secondly, the pyrosequenced AFLP fragments originated from both *F. subglutinans* and *F. circinatum*. A proportion of these AFLP fragments are possibly unique to *F. subglutinans*, a species for which no genomic sequence data are available. Approximately 9% of the AFLP fragments were unique to *F. circinatum*, which suggests that an equivalent percentage of the AFLP fragments are likely to be unique to *F. subglutinans* and, as such, to *F. verticillioides* and *F. fujikuroi*. In fact, synteny studies showed that there is on average a 90% sequence identity between *Fusarium oxysporum* f. sp. *lycopersici* and *F. verticillioides* (Ma *et al.*, 2010).

The extent of synteny was considered for species in the *G. fujikuroi* species complex. This was achieved by making comparisons of marker homology between the genomes of *F. verticillioides* and *F. fujikuroi*, as well as to a previously generated genetic linkage map (De Vos *et al.*, 2007). The sequenced strain of *F. verticillioides* harbored only eleven chromosomes; no genetic markers were found for the twelfth chromosome in the sequence data (Ma *et al.*, 2010). However, based on a genetic linkage map of this *Fusarium* species, this chromosome is probably dispensable, *i.e.*, it is deleted in some progeny (Xu & Leslie, 1996; Jurgenson *et al.*, 2002). Other progeny were found that had duplications and deletions of parts of this chromosome. It is possible that the particular strain of *F. verticillioides* for

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which the genome was sequenced, did not harbor chromosome 12. The genetic linkage map of De Vos *et al.* (2007) revealed 12 linkage groups, with the assumption that they corresponded to 12 chromosomes (Xu *et al.*, 1995). In the present study, LG 1 to 11 correspond to 11 chromosomes, when compared to *F. verticillioides*. These LGs could also be assigned to corresponding supercontigs in *F. fujikuroi*. Since *F. verticillioides* was used as a reference to order the *F. fujikuroi* supercontigs and the contigs of *F. circinatum*, LG 12 of the interspecific cross could not be mapped to the corresponding genomes.

Colinearity between the physical location of markers and their genetic map location generally corresponded well, with the exception of 17 (5.70%) of the markers. Inversions in the linkage map marker orders were observed in chromosome 1, 5 and 6, in comparison to the genome sequences of *F. verticillioides* and *F. fujikuroi*. Whether or not these are inversions or errors in mapping is not certain and should be clarified in a subsequent study. Chromosome 5, 7 and 9, on which small inversions were evident, may have been subject to scoring errors. Thus, excluding these possible errors, a remaining 1.68% of markers did not display colinearity. In comparison, 1.28% of markers in *F. graminearum* were not collinear (Gale *et al.*, 2005), which is comparable with the results of this study.

Comparisons of the degree of synteny among the *Fusarium* spp. used in this study showed that there is conservation of gene order and content on homologous chromosomes. We refer to synteny as the conservation of genomic sequences (mostly AFLP sequences) and order. It was found that the conservation of AFLP fragments from *F. circinatum* and *F. subglutinans* follows a pattern of macrosynteny in comparison to *F. verticillioides* and *F. fujikuroi*. Macrosynteny is the synteny of genes at the chromosomal level, with the backbone of genes on a chromosome being colinear (Hane *et al.*, 2011). Exceptions to macrosynteny were observed in single instances on chromosomes 1, 3 and 8, as evidenced by small inversions between two adjacent AFLP sequences. On chromosome 11, macrosynteny was not observed to the same extent as on other chromosomes. On this chromosome, a larger inversion was observed between three AFLP sequences which span 20.82% and 15.39% of the chromosomes of *F. verticillioides* and *F. fujikuroi* respectively. Thus, large regions of the compared genomes conform to various levels of synteny (cf. Waalwijk et al. 2004).

Recently, mesosynteny has been found in the filamentous ascomycete fungi (Hane *et al.*, 2011). Mesosynteny relates to macrosynteny without colinearity. Gene content is conserved

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without the conservation of gene order. Mesosynteny is prevalent only in the Pezizomycotina, and in this sub-phylum it is more evident between species in the Dothideomycetes (Goodwin *et al.*, 2011, Hane *et al.*, 2011). Mesosynteny was not observed in the three *Fusarium* spp. considered in this study. Although no genomic sequence is available for *F. subglutinans*, it would presumably follow the same pattern of macrosynteny shown in the species studied here. Thus, if the observed macrosynteny correlates to other species in the *G. fujikuroi* species complex, it could aid in identifying similar candidate genes using *in silico* sequence homology to other *Fusarium* spp. in this complex with ease (*e.g.* Parkin *et al.*, 2002). Also, genome assemblies of new *Fusarium* spp. would be assisted with the knowledge that macrosynteny is common.

A putative reciprocal translocation was observed on the genetic linkage map of the interspecific cross between *F. circinatum* and *F. subglutinans*. This region was found to be translocated relative to the genomic sequence of both *F. verticillioides* and *F. fujikuroi*. A translocation is a balanced structural rearrangement that is associated with a high risk of unbalanced gametes, resulting in abnormal progeny. This should occur more frequently in crosses between different species than in those where the parents belong to the same species (Zolan, 1995), as was found for this study. In the interspecific cross of this study, viability of ascospores was high (90%, Desjardins *et al.*, 2000), although there was a bias towards the transmission of *F. subglutinans* alleles (De Vos *et al.*, 2007).

The current study represents the first report of a translocation within the *G. fujikuroi* species complex. A translocation was observed previously in *Fusarium oxysporum* f. sp. *lycopersici* relative to *F. verticillioides* (Ma *et al.*, 2010), and has also been documented in a variety of other fungal species (*e.g.* Andersen *et al.*, 2011; Singh *et al.*, 2010). Structural chromosomal rearrangements, such as translocations, could cause distorted segregation of markers (Gale *et al.*, 2005). The section of LG 7 involved in the translocation, as analyzed by De Vos *et al.* (2007), does not display any segregation distortion, whilst that of LG 4 displayed segregation distortion. Additionally, AFLP marker AA/AC-337bh was identified as a putative transmission ratio distortion locus, but evidence of translocation events was still lacking. The distorted segregation of markers on the translocated region of LG 4 is most probably due to divergence between the genomes of *F. circinatum* and *F. subglutinans*, i.e. non-homologous regions of the chromosome, and not due to a distorting genetic locus in that region of the genome.

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The question remains whether the detected translocation event represents an ancestral or recent state. When considering the genetics of *F. circinatum* and *F. subglutinans* (Suppl. Materials), it becomes clear that the translocation detected in the current study must have been present in both the parental isolates. A translocation that is present in both *F. circinatum* and *F. subglutinans*, but not *F. verticillioides* or *F. fujikuroi*, would imply a translocation event that is ancestral to the divergence of *F. circinatum* and *F. subglutinans*. This ancestral translocation would have to be basal to the American clade lineage of the *G. fujikuroi* species complex (Suppl. Figure 1). This hypothesis should be confirmed through whole genome sequencing of additional members of the American clade.

A highly significant QTL for mycelial growth (De Vos *et al.*, 2011) was identified in the genome of *F. circinatum*. A single ORF on chromosome 3, which is homologous to a putative Zn_2Cys_6 fungal transcription factor, was also associated with this QTL. These transcription factors are known pathway-specific regulators that target and up-regulate the expression of one specific gene cluster, which in turn regulates a variety of cellular and metabolic processes (Todd & Andrianopoulos, 1997). Further work is needed to test the hypothesis that this gene is involved in mycelial growth in *F. circinatum* and *F. subglutinans*. However, it is intriguing that the gene does not have an exact homolog in *F. verticillioides* and *F. fujikuroi*. Rather, the nearest homolog in these species seems to be related to its exact counterpart in *F. circinatum* on chromosome 6. Thus, the gene associated with the mycelial growth QTL in *F. circinatum* may represent a translocation of a gene that was originally duplicated in species of the American clade of *G. fujikuroi*.

In this study it was shown that there is a syntenic relationship between a previously published genetic linkage map and the genomic sequences of *F. verticillioides* and *F. fujikuroi*. Macrosynteny was widespread for the genomic sequences of *F. verticillioides* and *F. fujikuroi*, and this conservation was also observed in the genetic linkage map. Furthermore, a QTL for growth was identified as a putative transcription factor, by using AFLP fragments generated *in silico*. This technique was surprisingly effective in assigning sequences to AFLP markers (91.49%). A subset of the *F. circinatum* contigs was also aligned to the *F. verticillioides* genome to assist in building scaffolds of the *F. circinatum* genomic sequence. This study illustrates the utility of a genetic linkage map, together with a genomic sequence, to attain a high level of confidence in contig and genome assembly.

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Table 1. AFLP primer combinations used in this study.

Primer combination	Description[1]	Number of polymorphic bands[2]	Number of linkage groups covered[2]
X	AA/AA	61	10
Y	CA/TC	63	12
Z	AG/AC	32	11

[1]AFLP primer combinations consist of the *Mse*I selective nucleotides followed by the *Eco*RI selective nucleotides.

[2]Results from De Vos et al. (2007).

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Table 2. Summary of AFLP pyrosequence data.

Primer combination	Total number of pyrosequence reads		AFLP sequences[1]		Unique AFLP sequences[2]		Total sequences[3]	Actual gel bands observed[4]	
	H[5]	E[6]	H[5]	E[6]	H[5]	E[6]		H[5]	E[6]
X			330	300	59	53	98	36	26
Y			251	238	77	63	124	40	43
Z			254***	183***	64***	21***	78	30	29
Subtotal			835*	721*	200***	137***		106	98
Total	2992	3112	1556		337		300	204	

[1]Carrying AFLP adapters at both ends.

[2]After filtering duplicate sequences.

3After filtering out the homologous sequences (*i.e.* monomorphic bands) present in both *F. circinatum* and *F. subglutinans*.

[4]Results from De Vos et al. (2007). These are the actual gel bands visible on the LI-COR® gel in the range 20 bp to 250 bp.

[5]Originating from *F. circinatum.*

[6]Originating from *F. subglutinans*.

*Significant deviation between markers originating from *F. circinatum* (H) and *F. subglutinans* (E). Significant deviation is noted as follows: * 5%, ** 1% and *** 0.1%.

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Table 3. Summary of AFLP pyrosequenced fragments showing sequence homology to *F. circinatum* (Fc), *F. verticillioides* (Fv) and *F. fujikuroi* (Ff).

Primer combination	Total number of sequences	No homology	Homology to Fc, Fv and Ff	Homology to Fc	Homology to Fc and Fv	Homology to Fc and Ff
X	98	7	70	11	7	3
Y	124	30	66	20	7	1
Z	78	12	45	14	5	2
Total	300	49	181	45	19	6

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Table 4. Markers that could be aligned to the genomic sequences of *F. verticillioides* and *F. fujikuroi* and their distribution over the chromosomes.

Chromosome[1]	AFLP fragments[2]	Linkage map[3]	Total
1	39	12 (12)	51
2	25	12 (12)[5]	37
3	24	12 (12)	36
4	7	8 (9)	15
5	13	22 (25)	35
6	14	12 (16)[6]	26
7	7	14 (17)	21
8	17	5 + 6[4] (11)[7]	28
9	9	10 (10)	19
10	3	7 (7)	10
11	11	4 + 5[4] (10)	20
Total	169	118 + 11 (141)	298

[1]Based on the *F. verticillioides* genome (*Fusarium* Comparative Sequencing Project).

[2]AFLP pyrosequenced fragments that could be linked to the genomes of both *F. verticillioides* as well as *F. fujikuroi*.

[3]Based on data from De Vos *et al.* (2007). Numbers in parentheses are the total number of *F. circinatum* markers on each chromosome from the interspecific linkage map. Numbers not in parentheses are those that could be linked to the genomes of either/both *F. verticillioides* and *F. fujikuroi*.

[4]Included are the markers from the inversion between chromosome 6 and 8.

[5]Included to the original data from De Vos *et al.* (2007) is β-tubulin.

[6]Included to the original data from De Vos *et al.* (2007) is elongation factor.

[7]Included to the original data from De Vos *et al.* (2007) is calmodulin.

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Table 5. Genomic comparisons between *F. verticillioides*, *F. fujikuroi* and the genetic linkage map of *F. circinatum* and *F. subglutinans.*

Chromosome[1]	Linkage group[2]	*F. verticillioides* supercontig[3]	*F. fujikuroi* supercontig	kb/cM[4]
1	1	3.13, 3.1	001	21.28
2	8	3.7, 3.4	003	19.54
3	2	3.20, 3.36, 3.10, 3.6	002	13.69
4	10	3.5, 3.18, 3.19	011	6.80
5	6	3.3, 3.12	005	17.00
6	3	3.22, 3.2	004	12.23
7	5	3.8, 3.17	006	12.42
8	4	3.9, 3.25, 3.23	012, 010	16.78
9	11	3.16, 3.14	007	15.32
10	9	3.24, 3.11	008	10.07
11	7	3.21, 3.15	009	12.60
			Average	14.34

[1]Assigned to the *F. verticillioides* genome (*Fusarium* Comparative Sequencing Project).

[2]Linkage groups numbers from De Vos *et al.* (2007).

[3]From the *F. verticillioides* genome as well as validated y the current research.

[4]Physical distance per unit of recombination of the *F. circinatum* markers relative to the *F. verticillioides* genome.

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Table 6. Position of *F. circinatum* contigs in relation to *F. verticillioides* chromosomes. Contigs are shown in order as they appear on the *F. verticillioides* chromosomes.

Chromosome[1]	Contig	Size (bp)	Orientation[2]	Contig	Size (bp)	Orientation[2]	Chromosome[1]	Contig	Size (bp)	Orientation[2]	Contig	Size (bp)	Orientation
1	02425	63320	+	02289	16746	+	2	04330	6864	+	02420	2086	-
	02035	35069	-	01474	10627	+		02667	17392	+	01286	56279	+
	00726	94641	-	00272	25305	+		02610	10128	-	00326	23451	-
	01389	15703	+	00658	18369	+		01315	6421	+	02523	40557	+
	02595	30204	+	03273	38500	-		01194	10314	-	01249	12045	-
	01059	7009	+	02121	5120	-		01277	4320	+	03236	25907	-
	02177	39054	+	00370	53262	-		00562	40847	+	00886	8636	+
	00392	112639	+	00762	52230	+		02036	26873	-	02275	19207	-
	00443	21292	+	01028	21269	-		02165	26761	-	00595	23577	+
	02136	31176	+	00779	24332	+		01685	4910	-			
	03629	31779	-	01110	12144	+		02834	16341	+			
	02716	24421	+	02034	18046	-		02599	10475	-			
	00870	12888	-	00146	3946	-		00547	20120	-			
	00881	17626	-	02106	7937	-		01399	12407	+			
	00813	11602	-	01328	22128	+		01329	34675	+			
	01101	16382	-	00218	35820	-		01542	28076	-			
	02207	32317	+	03178	13697	+		00869	92925	-			
	02720	32303	+	02433	16142	+		02384	16996	-			
	02624	19915	-	00750	25247	-		00177	6441	+			
	00873	22488	-	01077	10002	-		02143	34907	+			
	00698	27425	-	00879	13905	-		00198	2671	+			
	01132	11170	-	00598	38426	+		00215	64907	-			
	02708	24428	+	00051	70832	+		00224	33895	-			
	00193	18196	-	02215	44300	-		00400	50469	-			
	00973	17496	-	03027	9249	-		03841	31450	-			

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Table 6. (*continued*)

Chromsome[1]	Contig	Size (bp)	Orientation[2]	Contig	Size (bp)	Orientation[2]	Chromosome[1]	Contig	Size (bp)	Orientation[2]	Chromosome[1]	Contig	Size (bp)	Orientation[2]
3	02138	36466	+	00290	21366	-	4	02727	14305	+	5	02660	30315	+
	00747	12965	-	02077	10910	-		00684	20315	-		02198	22439	+
	02723	24850	-	00888	11704	+		00857	34582	-		02093	58407	+
	00622	31597	+	01196	55834	+		00511	31666	-		02244	36847	+
	00678	100656	+	00480	63914	-		01997	24020	+		01203	3706	+
	01754	3193	-					02533	20422	-		02237	34911	+
	01641	7004	-					01174	33638	-		02046	38361	+
	02270	24047	+					00395	74817	-		03118	33666	+
	00887	26538	+					00412	15865	-		01009	18861	+
	00804	36551	-					02147	14376	+		02719	43330	-
	01400	12230	-					00490	83075	-		01594	10600	-
	02279	10771	-					02116	46287	+		00445	42134	+
	02577	28083	+					00253	11631	-		00274	14317	-
	02902	65940	-					00669	101466	+		04074	50469	-
	00200	32447	-					02686	39933	-		00221	28158	+
	02423	65117	+					02105	11779	+		00922	9453	+
	00423	14670	+					04175	7608	+		02126	40664	-
	00556	28337	-					00411	72776	+		02155	22012	+
	00350	48597	-					02449	8142	+		00953	39669	+
	00655	12281	-									02907	6963	-
	00133	9307	+									01102	17679	+
	00954	44203	-									00152	29250	+
	03227	19523	-									02682	72766	+
	01438	12018	-									01098	3262	-
	02093	63713	-									01222	24151	+
	00544	29691	-									01050	2589	-

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Table 6. (*continued*)

Chromosome[1]	Contig	Size (bp)	Orientation[2]	Chromosome[1]	Contig	Size (bp)	Orientation[2]	Chromosome[1]	Contig	Size (bp)	Orientation[2]
5 (*continued*)	02617	34272	+	6	00156	31144	-	7	00838	51451	+
	00642	85015	+		00894	39653	+		02508	7812	+
	00701	54631	+		01133	51443	-		03001	15150	+
	01362	38577	+		01288	10803	+		00008	58820	-
					01501	1751	+		02206	52854	+
					00012	29335	-		02361	18672	-
					00576	25789	+		02182	17571	+
					00182	34853	-		02519	59428	-
					00541	16660	-		00908	10837	-
					00269	30858	+		00348	44261	-
					00328	5328	-		01761	2945	-
					00903	27302	-		00561	46681	+
					00482	41874	-		02509	7445	+
					01463	6681	+		02305	13272	-
					01135	29679	+		02151	104676	+
					03176	25631	-		02205	27945	+
					01695	7453	-		00720	18211	+
					00448	10682	-		00368	31959	-
					02268	13732	-		00640	49487	-
					01184	27013	+		01093	20005	+
					01589	733	+		04186	16422	-
					02286	5351	+		01937	9949	-
					01403	14230	-				
					00528	29491	-				
					00249	48091	-				

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Table 6. (*continued*)

Chromosome[1]	Contig	Size (bp)	Orientation[2]	Chromosome[1]	Contig	Size (bp)	Orientation[2]	Chromosome[1]	Contig	Size (bp)	Orientation[2]	Chromosome[1]	Contig	Size (bp)	Orientation[2]
8	02863	15880	+	9	03621	5148	-	10	00289	38195	+	11	253*	28752	-
	03589	70372	+		01233	35353	+		00264	12257	-		00405	82185	+
	00425	83653	+		00641	11564	-		03007	29482	+		104*	8245	-
	02441	12687	-		02831	26696	+		02725	24057	+		376*	72569	+
	02629	45807	+		01653	40772	+		03023	5504	-		00376	72569	-
	02753	9054	+		01979	19709	-		02689	55654	-		87*	160192	-
	77*	72418	-		02213	7061	+		00418	36855	-		276*	116336	-
	02429	16699	+		00169	50767	+		00769	38666	+		00434	11297	-
	00225	7719	+		03304	27629	+		04174	47245	+		02877	22899	-
	01312	21539	+		00716	43321	+		00145	52384	-		00430	110286	+
	01371	11964	-		02280	27407	-		02211	7934	-		02152	1489	+
	02134	31548	+		03988	87280	-		02405	8680	+		00554	66503	+
	00728	10004	+		02603	6146	-		00976	13925	+		01563	8184	+
	02148	49210	+		03302	11209	-						02763	22220	-
	01039	100705	-		02018	7239	+						02142	74928	+
	01212	28234	-		01521	4948	-						00312	23097	-
	02530	29488	-		02273	30251	-								
	01034	29567	-												

[1] Assigned to the *F. verticillioides* genome (*Fusarium* Comparative Sequencing Project).

[2]Orientation of the *F. circinatum* contigs relative to the *F. verticillioides* genome.

*Contigs originating from new alignment.

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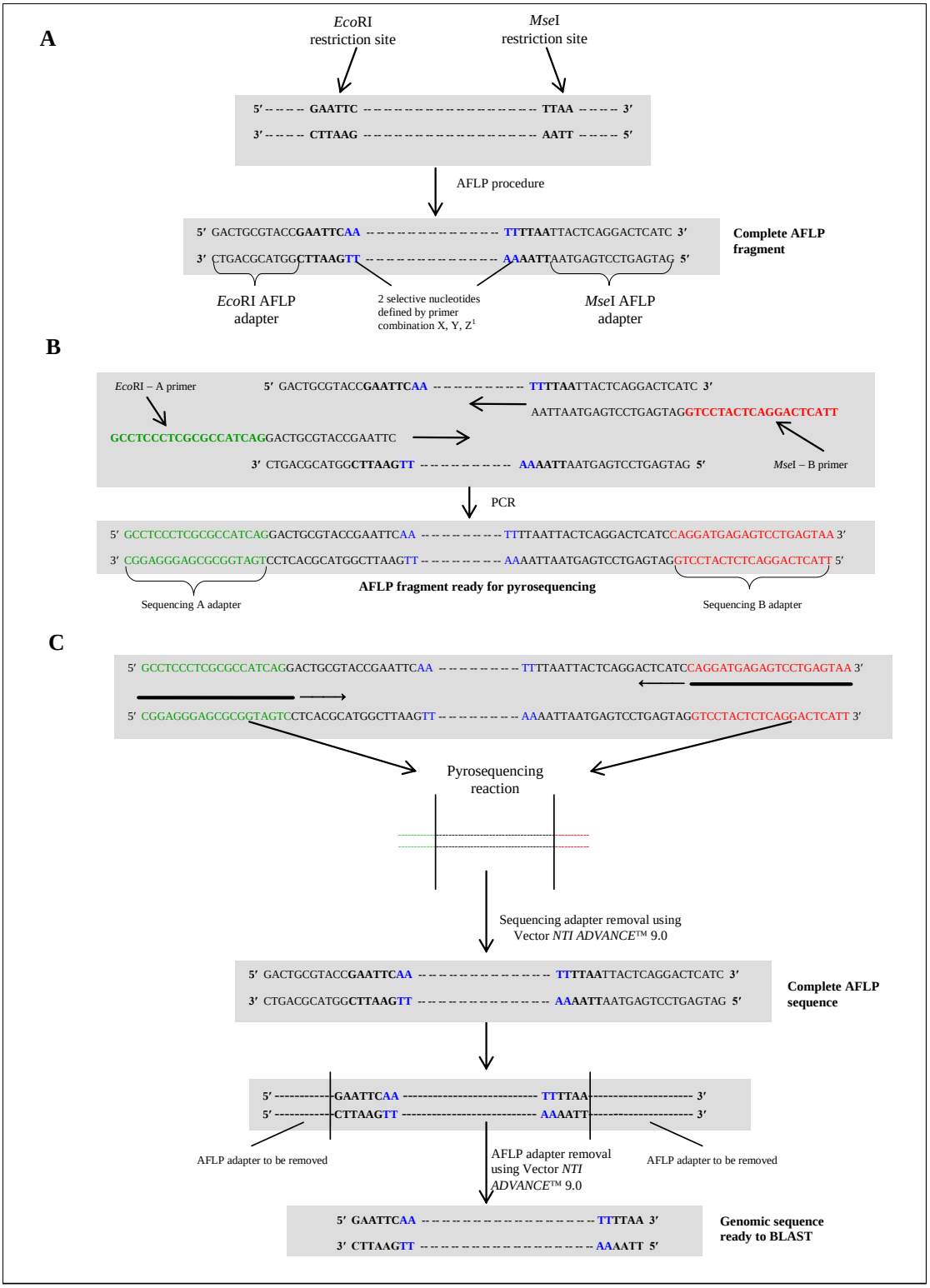

[1] X = AA/AA, Y = CA/TC and Z = AG/AC, AFLP primer combinations from De Vos *et al.*, (2007).

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Figure 1. AFLP generation, pyrosequencing and analysis. (A) Production of AFLP fragments by digesting genomic DNA and ligating adapters to the restriction sites. (B) Addition of pyrosequencing adapters through PCR. (C) "Sequencing-by-synthesis" reaction using primers on both adapters, and trimming of sequences to yield complete AFLPs sequences ready to BLAST.

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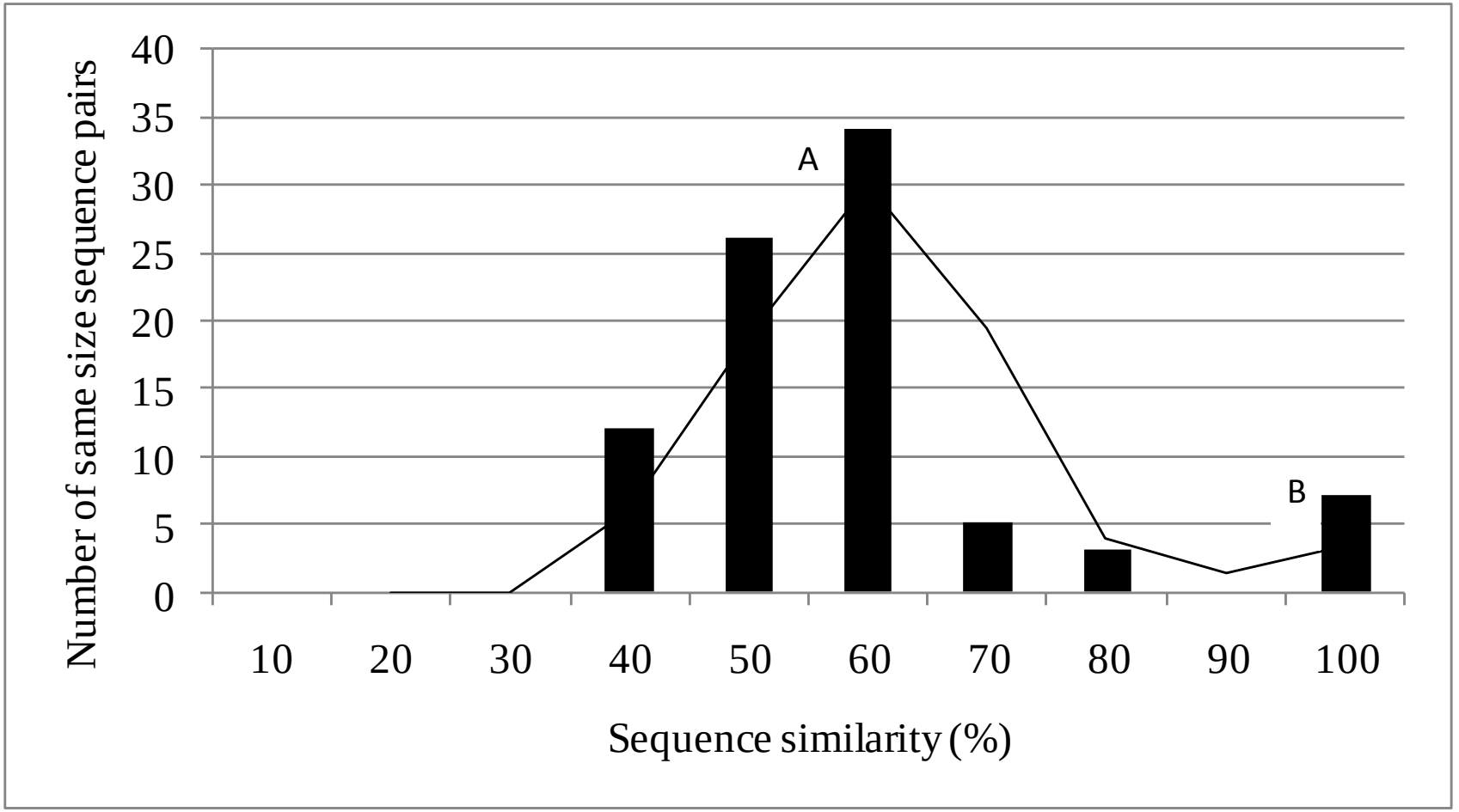

Figure 2. Histogram of sequence similarity between all pairs of same-sized sequences. A moving-average trendline has been added.

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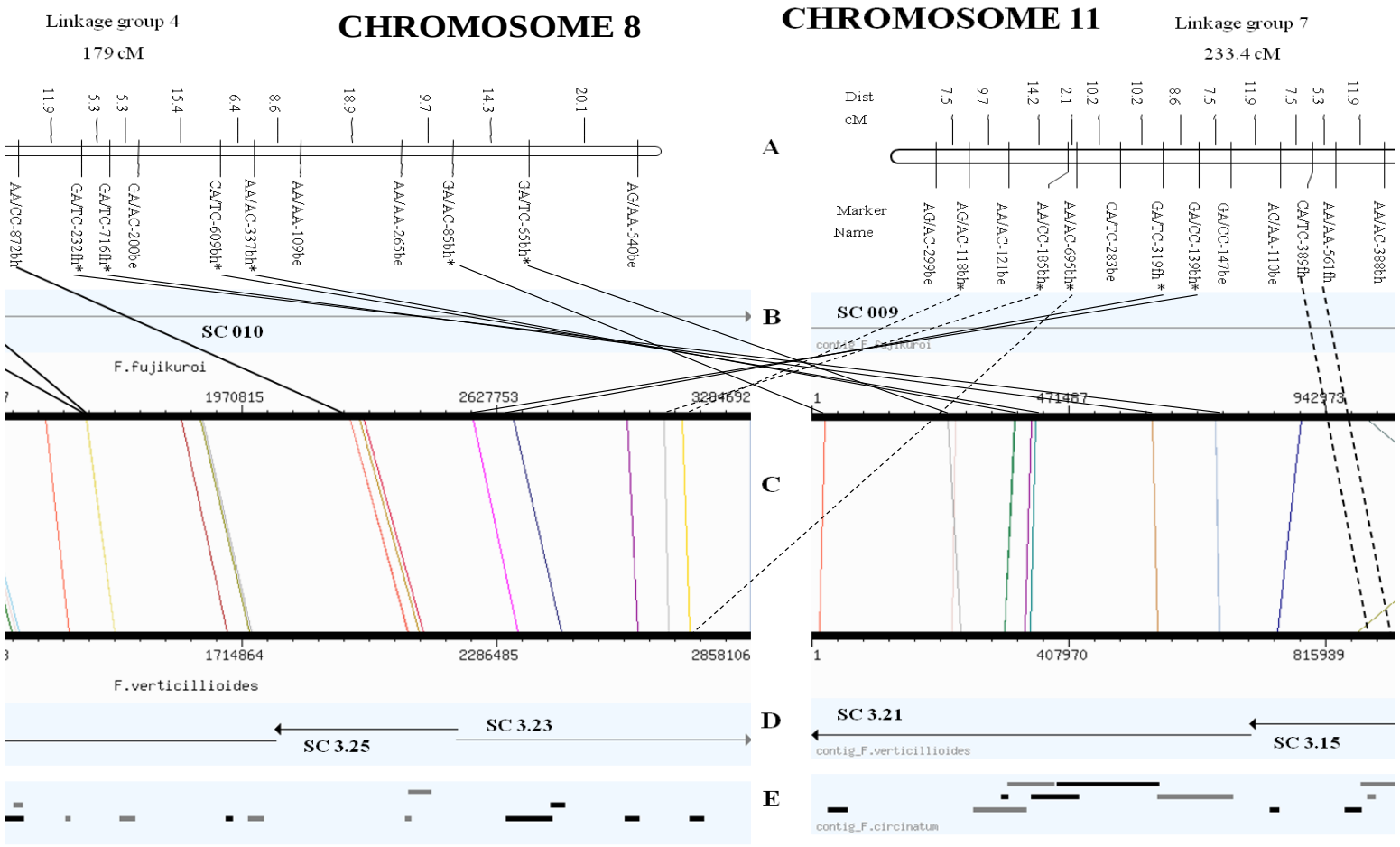

Figure 3. The translocation between chromosomes 8 and 11.

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Supplementary Materials

PCR-RFLP analysis

PCR amplification of three gene regions was performed on the two parental isolates. The translation elongation factor (EF) 1-α region was amplified with primers EF-1 and EF-2 (O'Donnell *et al*., 1998b), while the β-tubulin (BT) gene was amplified using primers T1 and T2 (O'Donnell & Cigelnik, 1997). Both these gene regions were amplified using the conditions described by Geiser *et al*. (2005). A portion of the calmodulin gene (Cal) was amplified using primers CL1 and CL2A (O'Donnell *et al*., 2000), using the conditions of White *et al*. (1990). PCR amplicons were purified with ethanol precipitation (Sambrook *et al*., 1989) and cloned into the pGEM®-T Easy vector (Promega, Madison, WI) according to the manufacturer's instructions. Sequencing was performed using the T7 and SP6 primers and the ABI PRISM BigDye Terminator v3.0 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) on a 3730 DNA Analyzer. Sequences were analyzed on BioEdit v7.0.5.3 (Hall, 1999). PCR-RFLPs were designed using this program to distinguish between the two parents for the EF, BT and Cal gene regions (Suppl. Table 1). These were used to screen the 94 F_1 progeny to determine the parental origin of the parental alleles. The results were scored as '0' for band absent and '1' for band present.

The PCR-RFLP dataset was imported, together with the original framework linkage marker set of De Vos *et al*. (2007), into MapMaker MACINTOSH v2.0 (Lander *et al*., 1987). The 3 gene regions were distributed into linkage groups using an LOD of 9.0, as the dataset separated into 12 linkage groups at this threshold. Using the 'First Order" command, the most probable placement of each gene was determined. Using MapMaker, EF was placed on linkage group 3, β-tubulin on linkage group 8 and calmodulin on linkage group 4, of the map by De Vos *et al.* (2007). The mating type idiomorph and the histone (H3) gene regions fell on linkage group 3 and 11, respectively (De Vos et al., 2007).

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Putative translocation

Three possibilities exist to account for this translocation (Suppl. Figure 3). First, if the translocation was only present in only one of the parents, the expectation was to find progeny with markers originating from only one parent on the translocated portions of the two chromosomes (Suppl. Figure 3A). This was not observed (De Vos *et al.*, 2007). Second, if the translocation was present in both the parental isolates, normal pairing of homologous chromosomes would happen, with recombination occurring between the translocated regions between these two species (Suppl. Figure 3B). Progeny would thus have a mixture of markers originating from both parents on the translocation. This was in fact found to be true (De Vos *et al.*, 2007). Third, if the translocation was as a result of meiosis, it would be an indication that chromosome 8 and 11 did not pair with their correct homologues during meiosis, instead chromosome 8 of *F. circinatum* paired with chromosome 11 of *F. subglutinans*, and *vice versa* (Suppl. Figure 3C). This is probably due only to a small homologous region shared between these two chromosomes. The expectation would be to have progeny with no recombination in large portions of those respective chromosomes as well as having progeny that contains the non-recombined translocation. This was not observed (De Vos *et al.*, 2007).

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Supplementary Table 1. PCR-RFLPs of three gene regions in *F. circinatum* and *F. subglutinans*.

Gene region	Size (bp)		Restriction	Restriction fragment (bp)	
	H[1]	E[2]	enzyme	H[1]	E[2]
Translation elongation factor 1-α	706	705	*Dde*I	416, 182, 55, 53	276, 182, 139, 55, 53
β-tubulin	605	604	*Nla*IV	350, 147 84, 24	276, 147 84, 73, 24
Calmodulin	756	754	*Cfo*I	308, 282 164, 2	446, 308

[1]Size of fragment originating from *F. circinatum*.

[2]Size of fragment originating from *F. subglutinans.*

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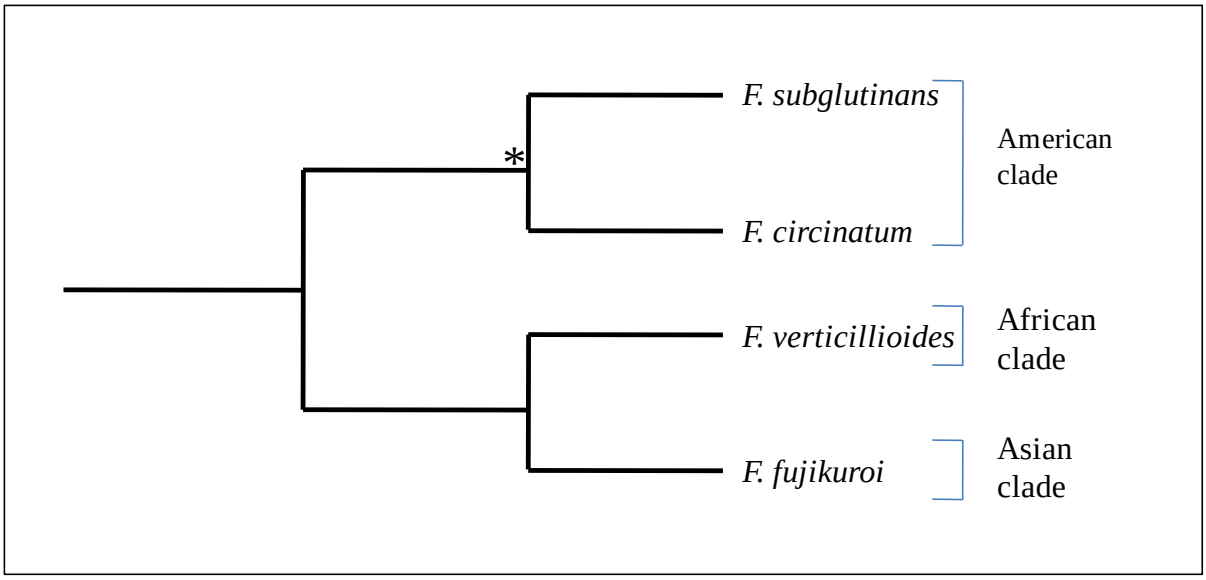

Supplementary Figure 1. Phylogenetic tree of the *G. fujikuroi* species complex (adapted from O'Donnell *et al.*, 1998a). The asterix refers to the putative translocation event.

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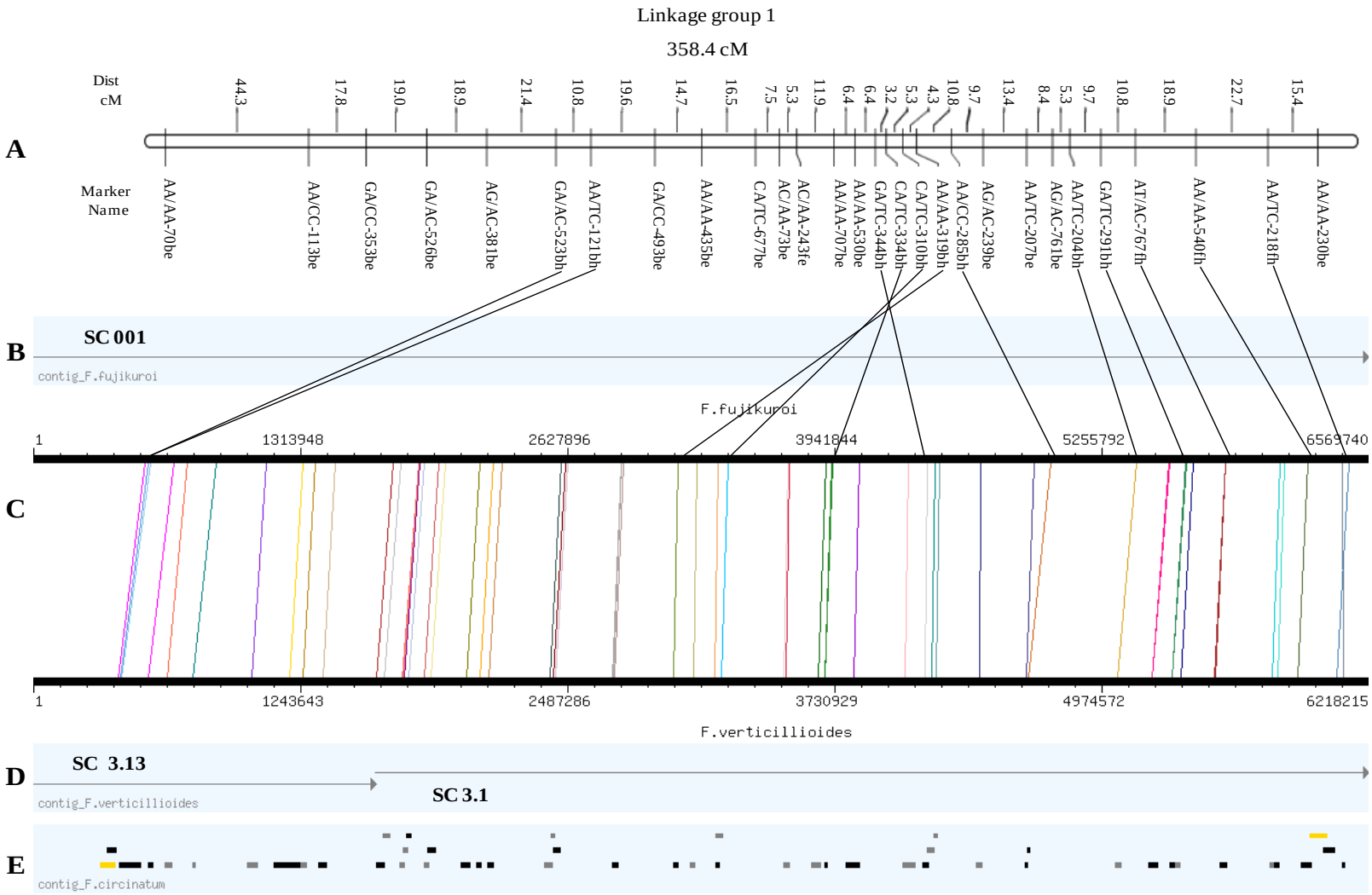
CHROMOSOME 1



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Supplemental Figure 2. Integration of the genetic linkage map of the cross between *F. circinatum* and *F. subglutinans* (A) with the genome sequence of *F. verticillioides* and *F. fujikuroi* (C). Solid lines joining A to C indicate syntenic regions between the genetic linkage map and *F. fujikuroi* and *F. verticillioides*. Dotted lines are indicative of synteny between the genetic linkage map and either *F. fujikuroi* or *F.* verticillioides. The *F. fujikuroi* and *F. verticillioides* supercontig(s) that corresponds to the indicated chromosome are indicated by B and D, respectively. Grey supercontigs indicate a forward orientation, and black a reverse orientation (SC 3.19 on chromosome 4 is shown in blue and designated in the forward direction, as no syntenous regions were found in *F. fujikuroi* to orientate this supercontig). C designates the synteny between *F. fujikuroi* and *F. verticillioides*, as indicated by the vertical lines. The corresponding contigs of *F. circinatum* are illustrated in E. Grey bars indicate contigs following a forward orientation relative to D, black bars the reverse orientation. Pyrosequenced AFLP fragments showing only synteny to the genomic sequence of *F. circinatum* and *F. verticillioides*, are indicated in E in red and yellow. These contigs are shown as yellow for the forward orientation and red in the reverse orientation.

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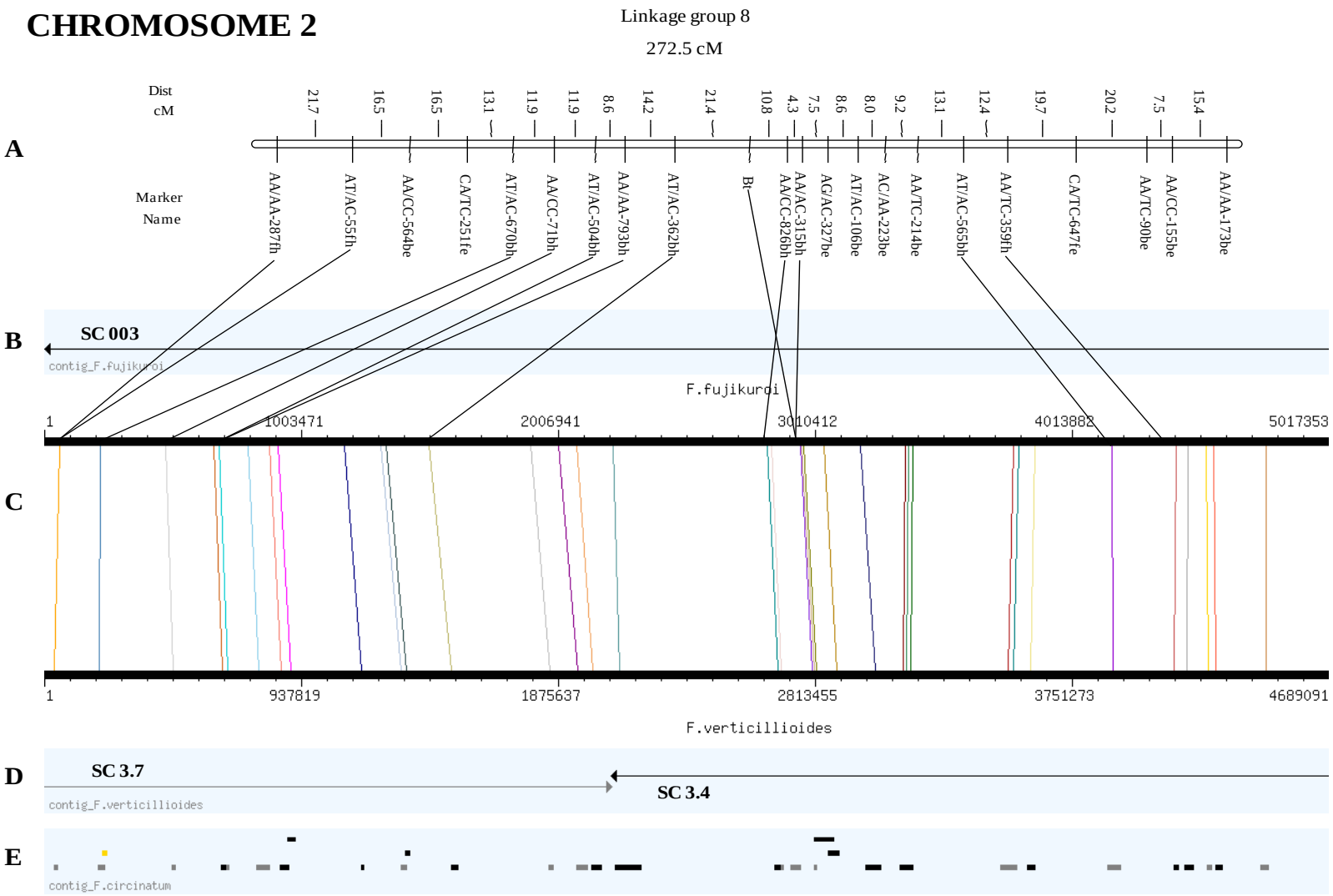

Suppl. Fig. 2. (*continued*)

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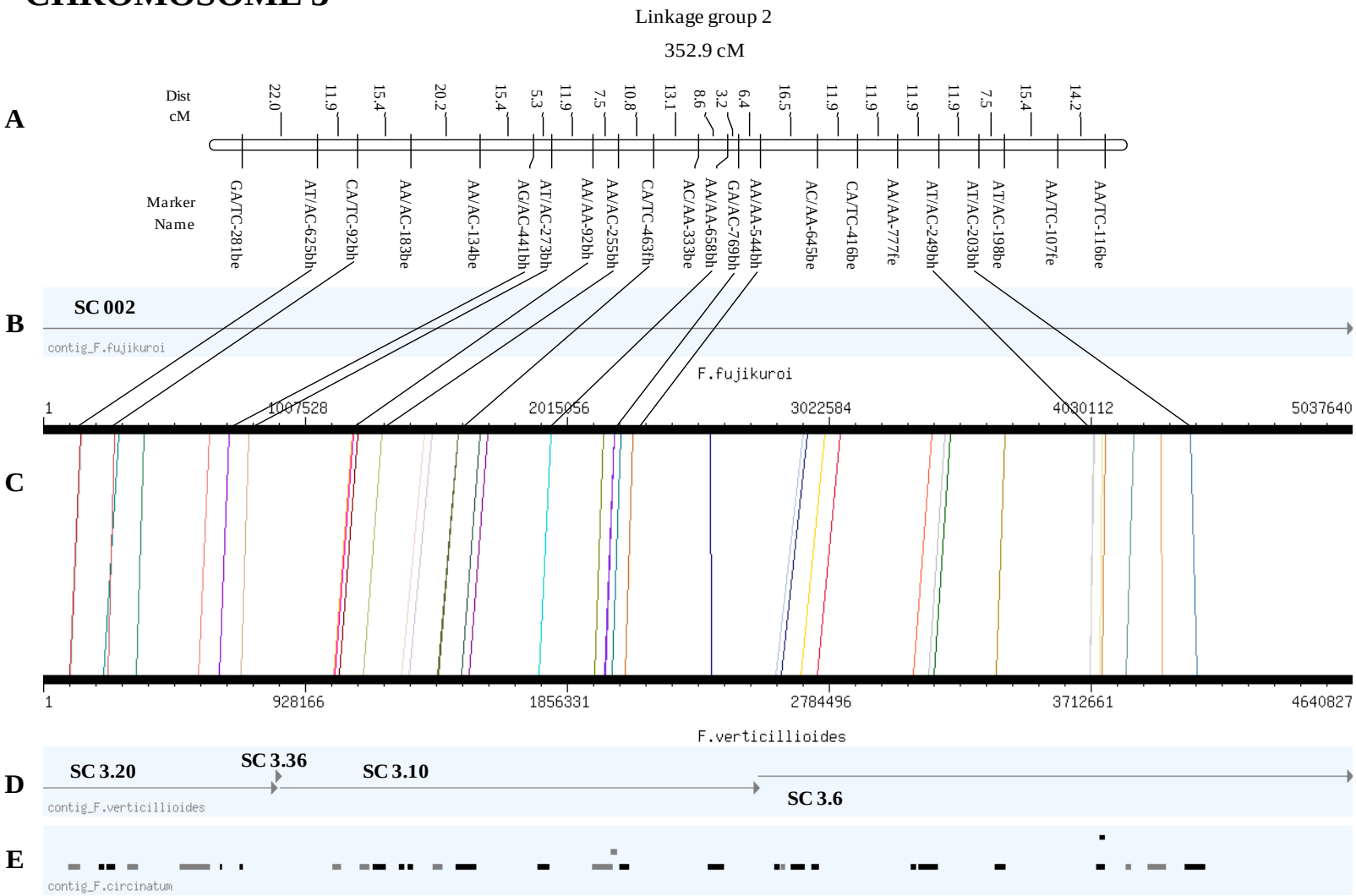
CHROMOSOME 3



Suppl. Fig. 2. (*continued*)

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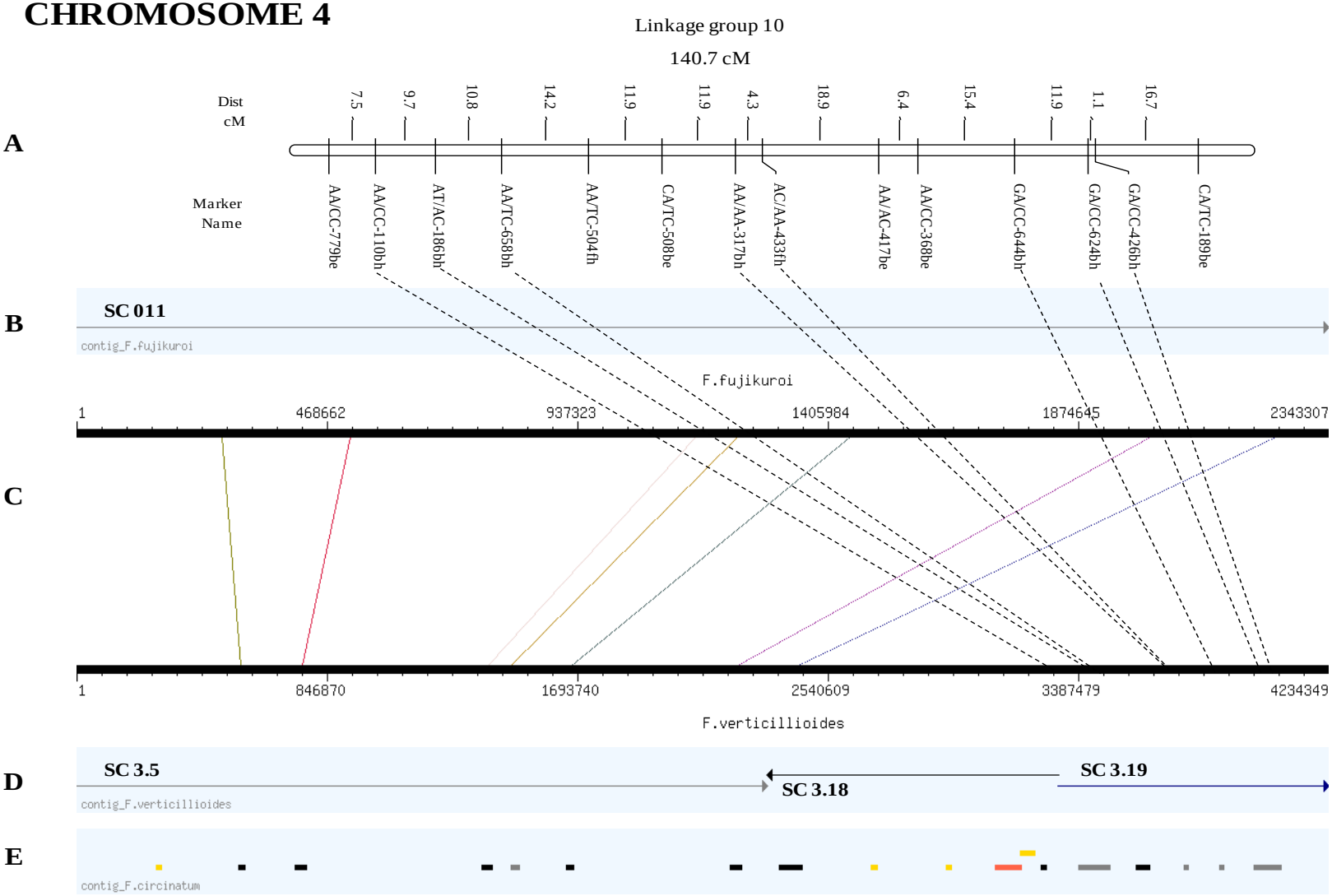
CHROMOSOME 4



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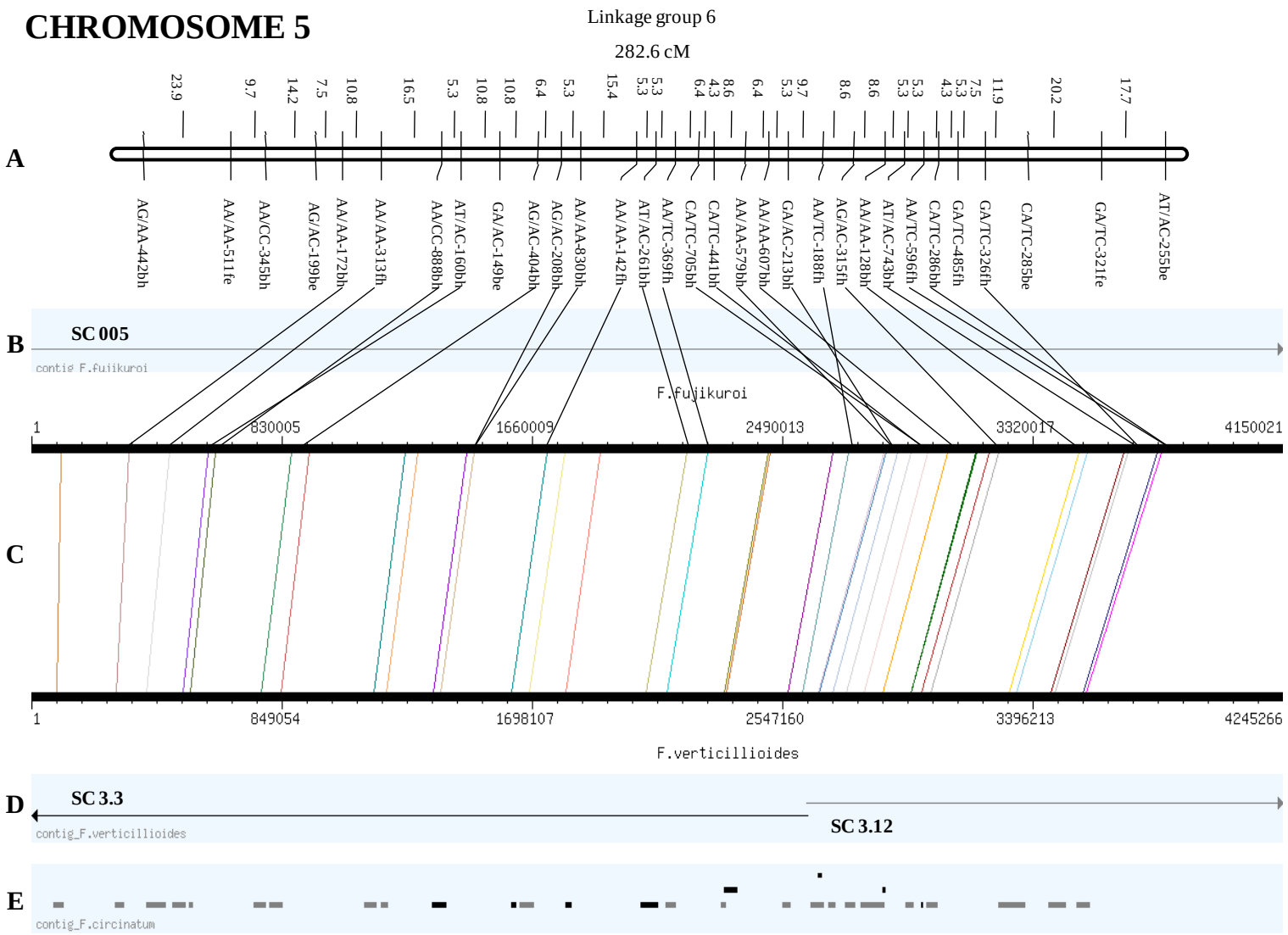

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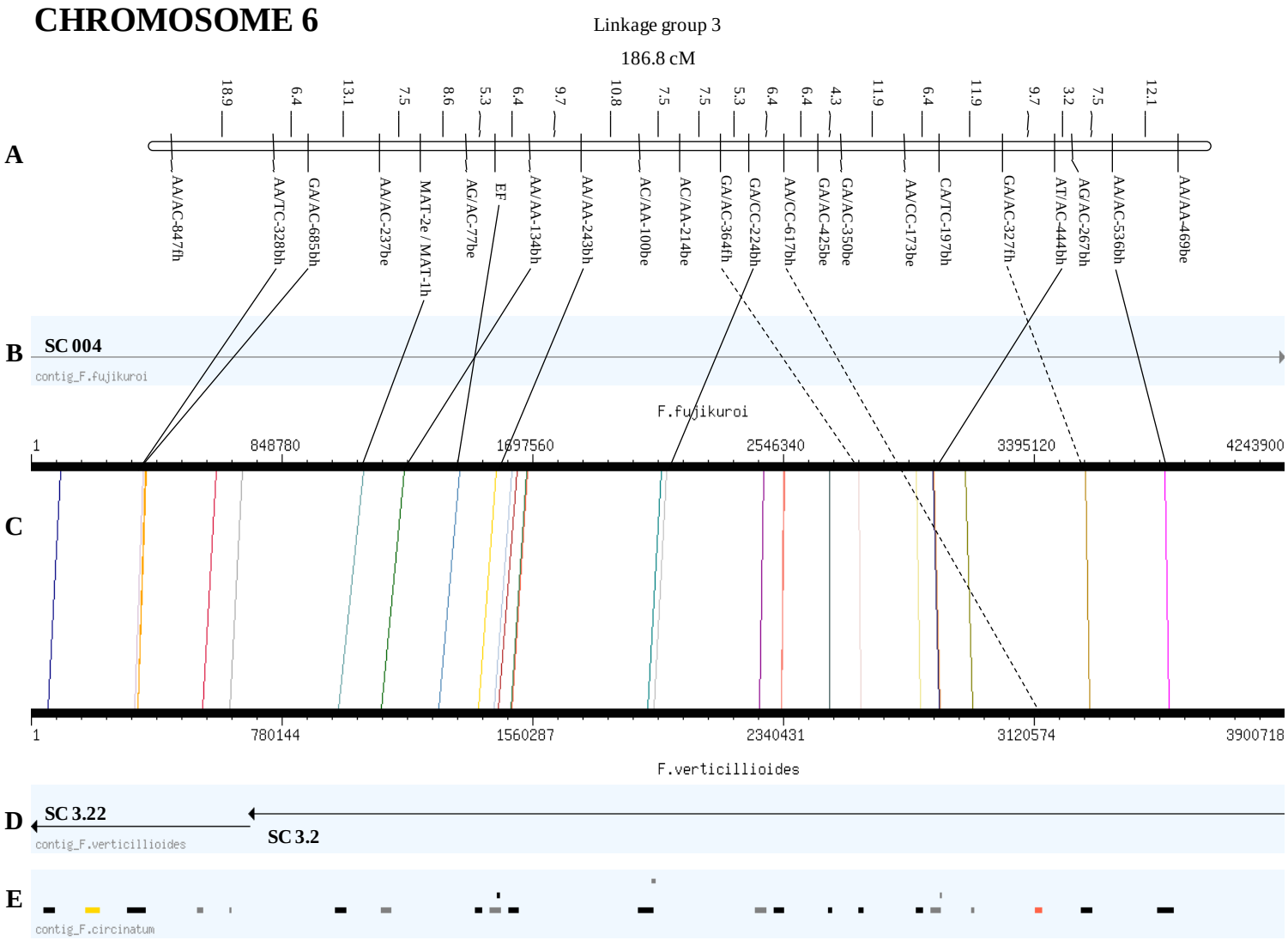

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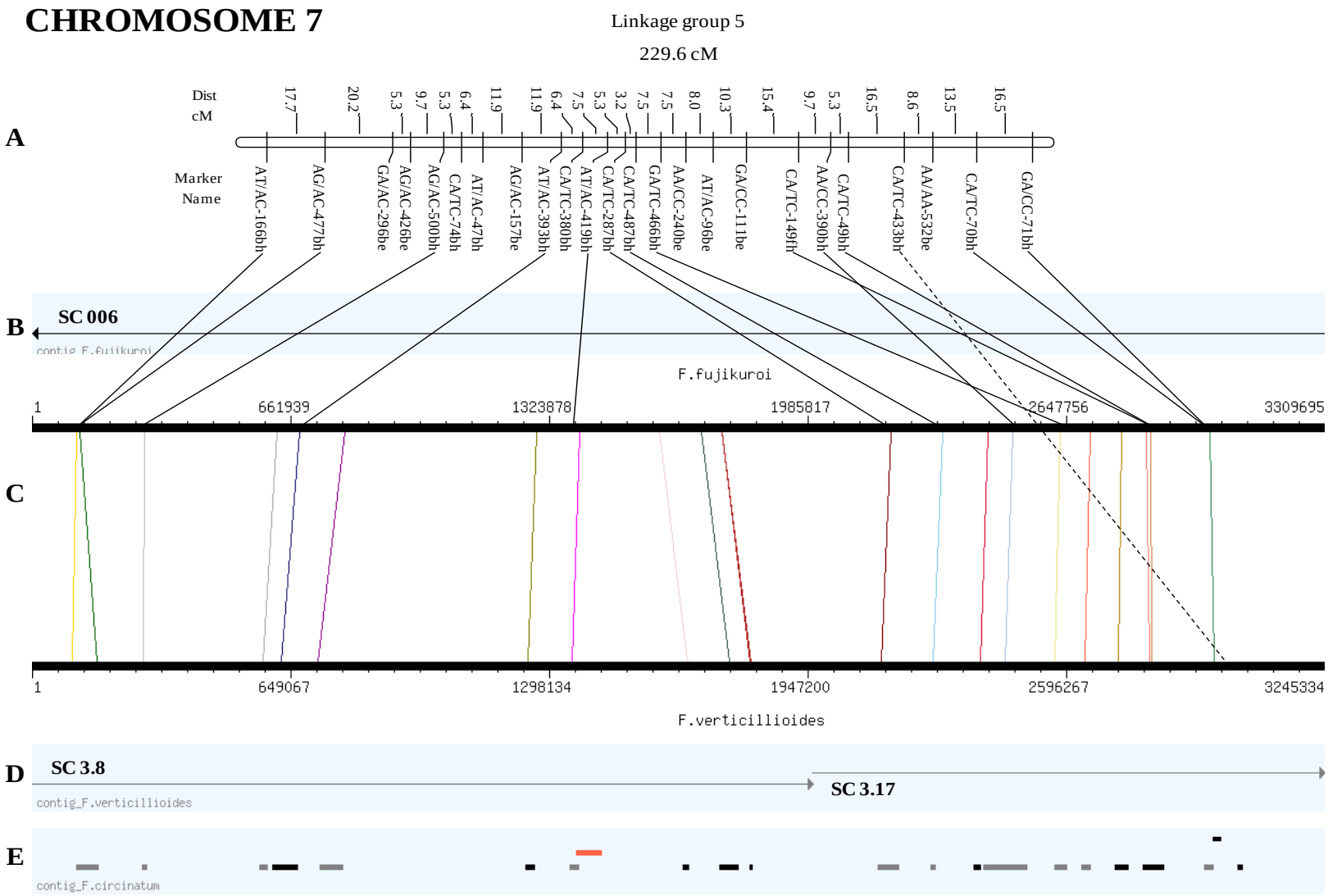

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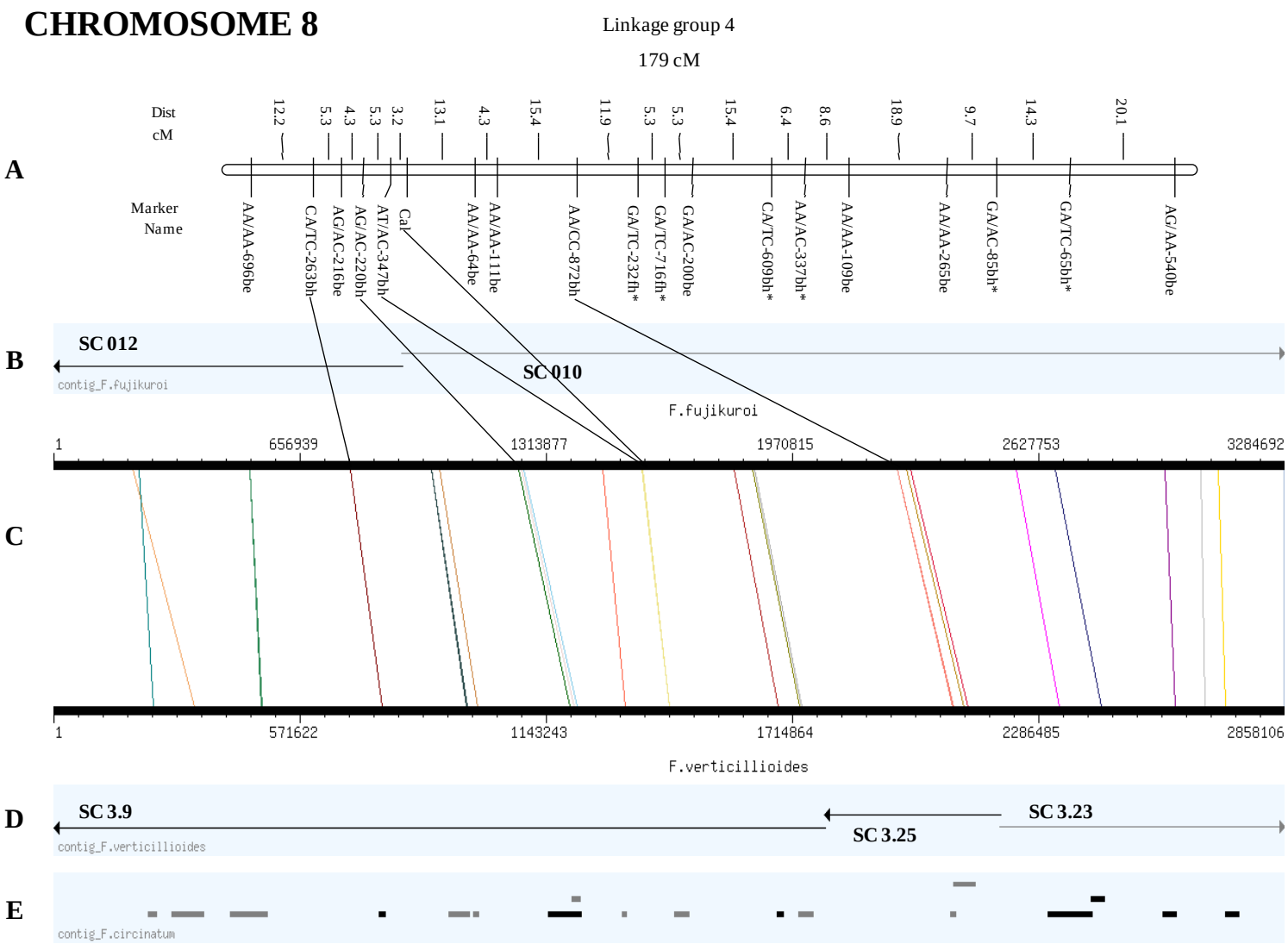

Suppl. Fig. 2. (*continued*)

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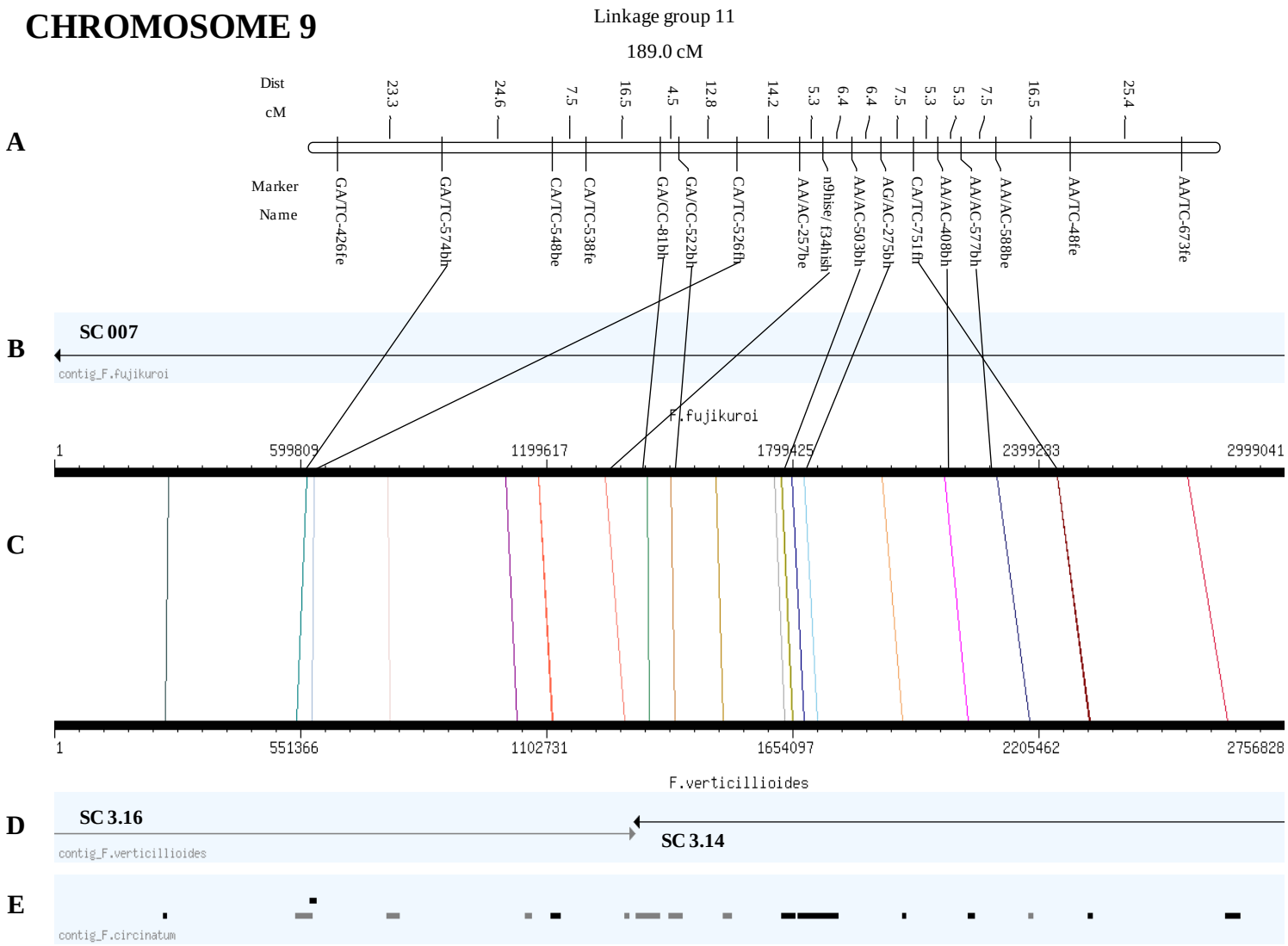

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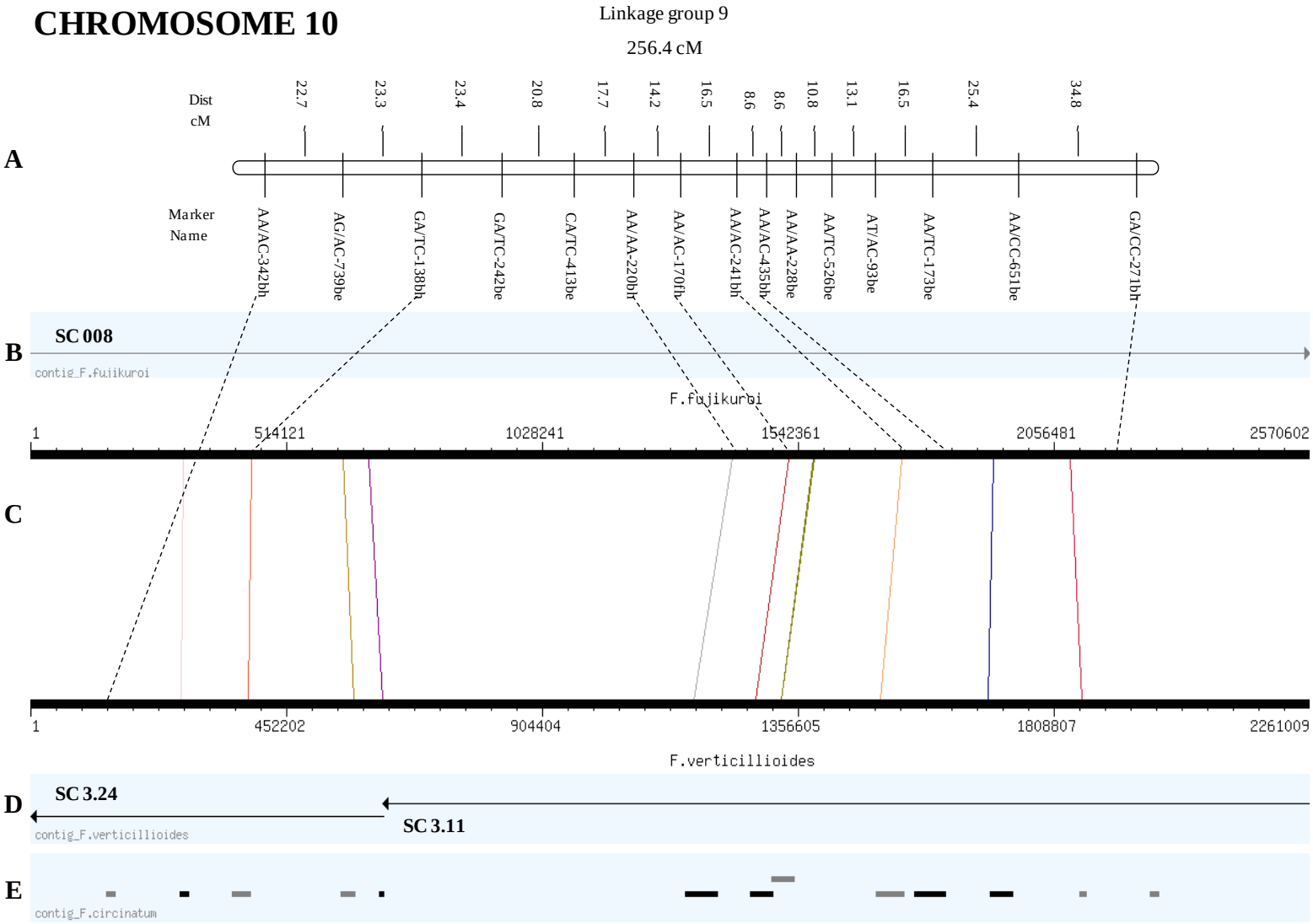

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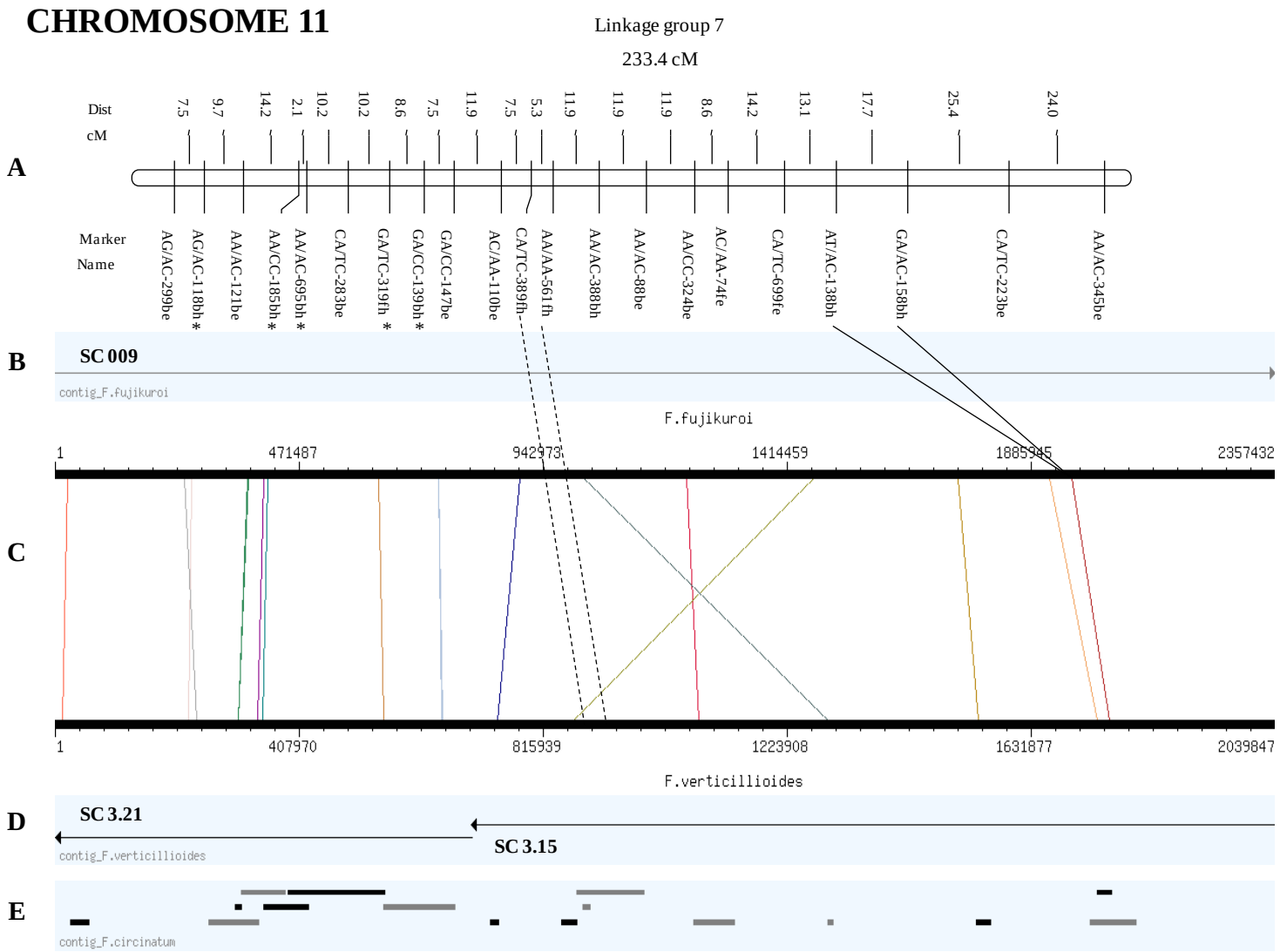

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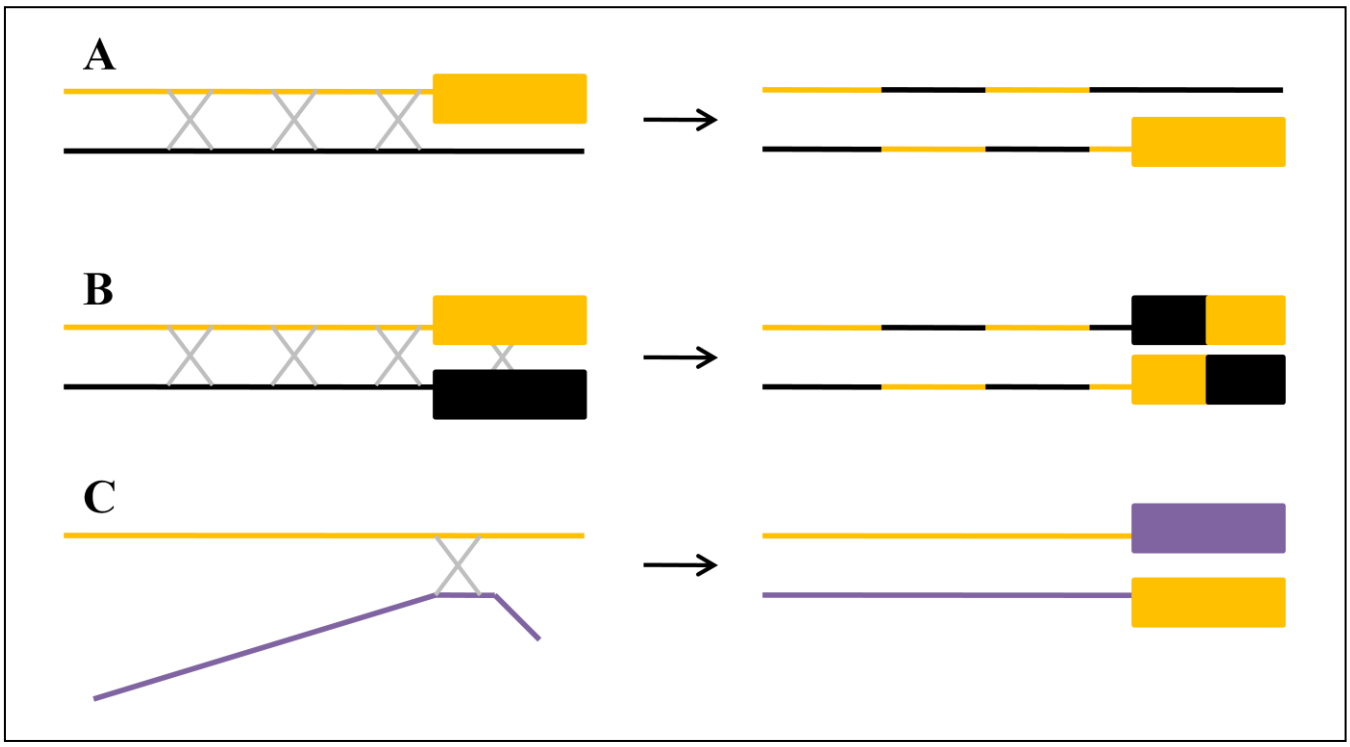

Supplemental Figure 3. Origin of the translocation. (A) If the translocation is present in only one parent, half of the progeny will have the non-recombinant translocation. (B) If the translocation is present on both parents all progeny will have the translocation which would have undergone recombination. (C) If none of the parents had the translocation, non-homologous chromosomes would pair up during meiosis and all progeny would receive the non-recombinant translocation. Blocked areas indicate the translocation and gray crosses are representative of chiasmata.

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Summary

The *Gibberella fujikuroi* species complex accommodates *Fusarium* spp. in the section *Liseola* that are unified by a common *Gibberella* sexual state. These are ubiquitous and economically important pathogens of plants, resulting in crop losses and the production of health-threatening mycotoxins. In this thesis, an interspecific cross between *Fusarium circinatum* and *Fusarium subglutinans*, both members of the *G. fujikuroi* species complex, were examined. A genetic linkage map was produced using Amplified Fragment Length Polymorphisms (AFLPs) and twelve linkage groups were identified. Distortion of the expected segregation patterns of these markers was observed in half of the AFLP markers genotyped. Distorted markers were found distributed throughout the genetic linkage map, leading to the hypothesis that distortion was caused by the high level of genomic divergence between the parental isolates. This was evident in that distorted alleles displayed genome-wide skewing towards the *F. subglutinans* parental genotype, as well as the differential transmission of *F. subglutinans* alleles to F_1 individuals. Investigations of Quantitative Trait Loci (QTL) involved in mycelial growth of the F_1 progeny at 25 and 30˚C, suggested that a principle genomic region was involved. Localization of this QTL to the genomic sequence of *F. circinatum* revealed a putative fungal transcription factor. The presence of this transcription factor was detected only in *F. circinatum*, and not in *F. verticillioides* or *F. fujikuroi*, the only additional species in the *G. fujikuroi* species complex for which genomic data are available. The AFLP map allowed investigation of synteny among chromosomes from *F. circinatum*, *F. verticillioides* and *F. fujikuroi*. A significant level of macrosynteny was observed between representatives of the African, American and Asian clades of the *G. fujikuroi* species complex, respectively. Evidence was also found for a putative reciprocal translocation present only in *F. circinatum* and *F. subglutinans*. This region was found to be translocated relative to the genomic sequence of both *F. verticillioides* and *F. fujikuroi*. Jointly, these results provided considerable insight, at the genomic level, for species within the *G. fujikuroi* species complex. Importantly, the utility of a genetic map together with genomic sequence data was demonstrated, and the genetic basis of a growth QTL determined.

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Conclusions and Future Prospects

Fusarium includes ubiquitous and economically important pathogens globally. This has spurred a great deal of research into this genus, with the field of genomics affording a closer look at the mechanisms of genomic organization. In this thesis, the genomics of species within the *Gibberella fujikuroi* species complex were examined with an interspecific cross between *Fusarium circinatum* and *Fusarium subglutinans*. The interspecific cross was investigated by means of a genetic linkage map (**Chapter 2**). This provided a framework for further study, throughout this thesis, to investigate the genomic architecture of the two parental genomes.

Genomic sequence data has revolutionized the field of fungal genomics. Comparative studies between related organisms, has advanced our understanding of host-pathogen interactions in *Fusarium* spp., as discussed in **Chapter 1**. In the context of this thesis, genomic sequence data from three *Fusarium* spp., in combination with the genetic linkage map, was examined. These species are representatives of the three clades in the *G. fujikuroi* species complex. *Fusarium fujikuroi* is a representative of the Asian clade, *F. circinatum* of the American clade and *F. verticillioides*, the African clade. To date, this is the first genomic comparison amongst these three clades.

Classically, genetic linkage mapping was used to determine the order (arrangement) of genes or markers on a chromosome. Fundamental questions about the genomic organization could also be answered. Furthermore, any quantitative trait loci (QTLs) that were polymorphic in the F_1 progeny could be positioned on these maps. With the advent of high-throughput sequencing of genomes, construction of these maps would seem to be redundant. However, as this thesis has shown, linkage maps can play an integral role in genomic studies. This significance will be discussed in context of the current thesis.

The genetic linkage map of the interspecific cross (**Chapter 2**) revealed a bias towards the transmission of *F. subglutinans* alleles and chromosomes to the F_1 generation. Also, there was significant segregation distortion of genotyped alleles in the genetic linkage map. Due to phylogenetic differences in the parents of this interspecific cross, deviations in the

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transmission of alleles to the next generation, was expected. This bias, with respect to *F. subglutinans*, occurred throughout the genome (**Chapter 3**). Impediments to genomic introgression between *F. circinatum* and *F. subglutinans* were most likely due to homologous chromosomes not aligning properly during meiosis. This resulted in the preferential transmission of *F. subglutinans* alleles, as well as chromosomes, to the next generation.

Regions identified that displayed significant distortion, are probably indicative of extreme dissimilarity between the two parental species, at the genomic sequence level. Thus, the most prominent differences between species should exist in the regions that display dissimilarity. These regions could potentially harbor 'niche-specific' genes, that make a particular species pathogenic to a specific host. Syntenic studies within the *G. fujikuroi* species complex should assist the identification of these regions.

Genetic linkage maps enable the identification of any quantitative trait loci (QTLs) that are polymorphic in the F_1 progeny (**Chapter 4**). Unfortunately, pathogenicity amongst the F_1 progeny did not display a continuous distribution and thus, could not be mapped. All, save one of the progeny, were not pathogenic on *Pinus patula* seedlings on which the *F. circinatum* parent is pathogenic. The pathogenicity of the exceptional isolate could be explained by its genetic constitution, in that it was almost, identical to the *F. circinatum* parent. It is thus reasonable to hypothesize that multiple genes are required for pathogenicity, both in the case of *F. circinatum* and *F. subglutinans*, and that the genes for pathogenicity to *P. patula* are incompletely represented in the F_1 progeny. However, it is also possible that the pathogenic progeny inherited various avirulence genes from the *F. subglutinans* parent that enabled the *Pinus* host to recognize the progeny, resulting in incompatible, resistance mechanisms being triggered. Further studies will be necessary to distinguish between these two scenarios.

A principal genomic region involved in mycelial growth, in both *F. circinatum* and *F. subglutinans*, was mapped to the genetic linkage map (**Chapter 4**). This was identified to be a putative fungal transcription factor in *F. circinatum* (**Chapter 5**). Future work should involve confirming the presence of this open reading frame in *F. subglutinans*, as well as disruption/deletion of the transcription factor to confirm the role in mycelial growth. Although no system for genetic manipulation currently exists in *F. circinatum* or *F. subglutinans* it could be developed based upon extensive work in *F. gramnearum*.

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Syntenic relationships among species within the *G. fujikuroi* species complex were examined to study the genomic architecture of members of this complex. It was found that the conservation of AFLP fragments from *F. circinatum* and *F. subglutinans* follows a pattern of macrosynteny, in comparison to *F. verticillioides* and *Fusarium fujikuroi* (**Chapter 5**). The unexpected level of macrosynteny will aid in the genome assemblies of new *Fusarium* spp. within this species complex, including the assembly of *F. circinatum*, which is incomplete. At present, four strains of *F. circinatum* have been sequenced (data not presented in this thesis), so studies into the intra- and interspecific variation amongst genomes within the *G. fujikuroi* species complex could be examined. Additionally, the syntenous AFLP regions identified in this study could be employed to study the phylogeny of *F. circinatum*, *F. verticillioides* and *F. fujikuroi*. This phylogenomic approach could provide answers to the divergence of these species on a genome-wide scale, something that has never been attempted in the species complex.

The reciprocal translocation detected in *F. circinatum* and *F. subglutinans*, represents an event that is ancestral to their divergence (**Chapter 5**). This ancestral translocation could have been basal to the American clade lineage of the *G. fujikuroi* species complex. This hypothesis could be confirmed through whole-genome sequencing of additional members of the American clade.

The percentage of pyrosequenced AFLP fragments that were unique to *F. circinatum* (ca. 9%), was similar to that found between *F. verticillioides* and *Fusarium oxysporum* f. sp. *lycopersici* (10% based on DNA sequence dissimilarity, **Chapter 5**). The position of these AFLP fragments in the *F. circinatum* genome could not be determined due to *F. circinatum* having, at present, a partial genome assembly. Currently, *Fusarium* spp. are postulated to have a 90% homologous 'core' genome, the remaining 10% of which would correspond to species-specific sequences. As discussed previously, regions identified displaying significant distortion (**Chapter 3**) are probably indicative of extreme sequence dissimilarity between the two parental species. If these correspond to the hypothesized 10%, their investigation could explain the divergence of these species.

In summary, the genetic linkage map and genomic sequence data, in this thesis, has validated the accuracy of each other. They have helped show macrosynteny among the three clades in the *G. fujikuroi* species complex, which will aid in the genome assembly of *F. circinatum*.

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Completion of the genome assembly of *F. circinatum* would be critical to help answer questions raised here. The utility of the genetic linkage, in examination of species within the *G. fujikuroi* species complex, has been demonstrated in this thesis. Together with genomic sequence data, analysis of the four species demonstrated genetic conservation in the *G. fujikuroi* species complex. Additional phenotypic traits could be easily mapped to the genetic linkage map, and localized in the respective genomic regions. The observed macrosynteny in this species complex, would aid in identifying similar candidate genes using *in silico* sequence homology, to other *Fusarium* spp. in this complex. This thesis provides a better understanding of the genomics of *Fusarium* spp., particularly those in the *G. fujikuroi* species complex.

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