Population dynamics of Fusarium head blight causing species in South Africa

by

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Declaration

“I, Adré Minnaar-Ontong, do hereby declare that the thesis hereby submitted by me for the degree Philosophiae Doctor in Plant Breeding/Plant Pathology at the University of the Free State represents my own independent work and has not previously been submitted by me at another University/faculty.

I furthermore cede copyright of this thesis in favour of the University of the Free State.”

....

Adré Minnaar-Ontong Date

An inspiring poem

by

EE Cummings

Dive for dreams

dive for dreams
or a slogan may topple you
(trees are their roots
and wind is wind)
trust your heart
if the seas catch fire
(and live by love
though the stars walk backward)
honour the past
but welcome the future
(and dance your death
away at the wedding)
never mind a world
with its villains or heroes
(for good likes girls
and tomorrow and the earth)
in spite of everything
which breathes and moves, since Doom
(with white longest hands
neating each crease)
will smooth entirely our minds
-before leaving my room
i turn, and (stooping
through the morning) kiss
this pillow, dear
where our heads lived and were.

"Dive for Dreams," by E.E. Cummings is merely about taking numerous risks or chances throughout your life. Being able to trust in yourself/your heart and accomplish whatever it is that you may seek in your life. It is probably one of the most captivating and emotional poems of all.

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Phillipians 4:13

"I can do all things through Christ which strengthens me."

To God be the Glory, great things He has done. All we as humans can do is touch life at the edges, but with the presence of the Almighty, where knowledge fails, faith and love succeed.

G*od, this world's problems are so complicated,*
that I cannot make my way without your guidance.
Quiken my mind and deepen my understanding.
Help me hear your voice and heed your advice.
Keep me walking along the path behind you.
Amen

In: A guideposts outreach publication by Norman Vincent Peale

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Trust in the Lord with all your heart and lean not on your own understanding (Proverbs 3:5)
Behold, I have put before you an open door, which no one can shut (Revelation 3:8)

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List of abbreviations

3-DON	3-acetyldeoxynivalenol
α-tub	α -tubulin
AFLP	Amplified fragment length polymorphism
AMOVA	Analysis of molecular variance
ATP	Adenosine 5'-triphosphate
β-tub	β-tubulin
bp	Base pairs(s)
BSC	Biological species concept
CLA	Carnation leaf agar
CTAB	Hexadecyltrimethylammonium bromide
CYMMIT	International Maize and Wheat Improvement Centre
DDT	Dithiothreitol
DNA	Deoxyribonucleic acid
dNTP	2'-deoxynucleotide 5'-triphosphate
DON	Deoxynivalenol
EDTA	Ethylene-diaminetetraacetate
EF1-α	Elongation factor-1α
FHB	Fusarium head blight
f. sp.	*formae specialis*
F_{ST}	Genetic variation between sub-populations
g	Gravitational force
Ggt	*Gaeumannomyces graminis var. tritici*
G_{ST}	Fixation index for groups
HIS	Histone H3
IGS	Intergenic spacer region
ISSR	Intersimple sequence repeats
ITS 28S rDNA	Intertranscribed spacer 28S rDNA
K	"True" number of subpopulations
kb	Kilobase pair
KCl	Potassium chloride
LD	Linkage disequilibrium
MAP	Mitogen-activated protein

MAT	Mating type
MCMC	Monte Carlo Markov Chain
$MgCl_2$	Magnesium chloride
MJ	Median joining
MSC	Morphological species concept
NaCl	Sodium chloride
***nit* mutants**	Nitrate-nonutilising mutants
NIV	Nivalenol
NJ	Neighbour-joining
NTSYSpc	Numerical taxonomy and multivariate analysis system
PAGE	Polyacrylamide gel electrophoresis
PCA	Principal component analysis
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PHO	Phosphate permease
PIC	Polymorphic information content
PSC	Phylogenetic species concept
QTL	Quantitative trait loci
r	Goodness of fit
®	Reserved
RAPD	Random amplified polymorphic DNA
RED	Reductase
RFLP	Restriction fragment length polymorphism
RNAse	Ribonuclease
rRNA	Ribosomal ribonucleic acid
SA	South Africa
SAHN	Sequential agglomerative hierarchical nested cluster analysis
SNA	Spezieller Nährstoffarmer Agar
spp	Species
SRAP	Sequence related amplified polymorphism
SSR	Simple sequence repeat
Taq	*Thermus aquaticus*
™	Trade Mark
TBE	Tris- Cl/borate/EDTA

TE	Tris-Cl/EDTA
***Tri*5**	Trichothecene 5 gene in trichothecene cluster
TRI101	Trichothecene 3-O-acetyltransferase
TRI-cluster	Trichothecene cluster
Tris-Cl	Tris (hydroxymethyl) aminomethane
UK	United Kingdom
UPGMA	Unweighted pair-group method using arithmetic averages
URA	UTP-ammonia ligase
USA	United States of America
UV	Ultraviolet
var	Variety
VWA	Van Wyk's agar
WA	Water agar
ZEN	Zearalanone

List of SI units

°C	Degrees Celsius
g	Gram(s)
h	Hour(s)
km	Kilometre
M	Molar(s)
min	Minute(s)
mg	Miligram(s)
ml	Millilitre(s)
mm	Millimetre(s)
mM	Millimolar(s)
ng	Nanogram
pH	Power of hydrogen
pmol	Picomole(s)
r/s	Revolutions per second
s	Second(s)
U	Unit(s)
µg	Microgram(s)
µl	Microlitre(s)
µm	Micrometre(s)
µM	Micromolar(s)
V	Volt(s)
v/v	Volume per volume
W	Watt(s)
w/v	Weight per volume

CHAPTER 1
General introduction

In South Africa, wheat is the second most important grain crop produced in all nine provinces. The Western Cape, Northern Cape and Free State lead the way with approximately 83% of the total domestic wheat production. Almost 20% of the total area planted to wheat is irrigated, with the rest cultivated under dry land conditions (http://www.nda.agric.za/docs/AMCP/WheatMVCP2009-2010.pdf). Wheat production in South Africa can be limited by numerous factors, including diseases, which may reduce yield and grain quality.

Fusarium head blight (FHB) is one of the most important diseases of wheat, especially under centre pivot irrigation. Economic losses can occur due to yield and grain quality reduction. Infected grain may contain mycotoxins that can reduce food safety along with human and animal health. FHB was first reported in South Africa in 1980 in the North West province (Scott *et al.,* 1988). Further FHB disease reports in South Africa include farms in the southern Cape, KwaZulu Natal, eastern Free State and the Northern Cape (De Jager, 1987; Boshoff, 1996; Boshoff *et al.*, 1999; Kriel and Pretorius, 2006).

This disease depends on the quantity of inoculum that survives until the next growing season (Goswami and Kistler, 2004). Disease incidence and severity can increase when wheat is sown in the residue of a previous infected host crop e.g., maize (*Zea mays* L.), followed by favourable conditions during anthesis (Windels, 1999; Stack, 2000; Goswami and Kistler, 2004). Ascospores, conidia and chlamydospores that act as agents of inoculum survive on residue of the previous crop and soil surfaces where saprophytic colonies of the FHB causing species occur. These spores are considered the primary inoculum source due to their dispersal by wind and water (Sutton, 1982; Bai and Shaner, 1994; Parry *et al.*, 1995). Control strategies for FHB are limited to fungicides (Pirgozliev *et al.,* 2003) and partial resistance in some cultivars (Browne and Brindle, 2007) present in some parts of the world. These control strategies are not applicable to FHB in South Africa, because no fungicides against FHB are registered for wheat and no resistant cultivars have yet been identified or released.

FHB can be caused by several *Fusarium* species globally, but *Fusarium graminearum* Schwabe [anamorph, *Gibberella zeae* Schwein (Petch)] is the dominant causal species in the warmer humid regions along with *F. culmorum* (WG Smith) Saccardo and *F. crookwellense* Burgess, Nelson and Tousson in the less temperate regions. In Europe, *F. graminearum* began to dominate in the less temperate regions as well, probably due to increased maize production in the crop rotation (Waalwjik *et al.,* 2003; Jennings *et al.*, 2004; Brennan *et al.*, 2005). Prior to 1999, *F. graminearum* was divided into two groups based on disease symptoms. Group one was later described as *F. pseudograminearum* Aoki and O' Donnell (Aoki and O'Donnell, 1999) with group two retaining the name *F. graminearum.* During the past decade, O'Donnell and his colleagues have split *F. graminearum* into at least 13 distinct lineages/phylogenetic species based on differences in critical DNA sequences (O'Donnell *et al.*, 2000; 2004; 2008; Starkey *et al.*, 2007; Yli-Mattila *et al.*, 2009).

Three different species concepts have been used to define *F. graminearum* (Summerell *et al.*, 2003). The first species concept is the morphological species concept (MSC) which is based on morphological characteristics e.g. colony colour, absence or presence of chlamydospores, macro- and microconidia, shape and size of macro- and microconidia, growth rate, optimal growth temperature, mycotoxin production etc. (Summerell *et al.*, 2003; Leslie and Summerell, 2006). The second, the biological species concept (BSC) is based on sexual cross-fertility with members of the same species capable of producing viable and fertile progeny (Bowden and Leslie 1992; 1999; Leslie *et al.,* 2007). The third concept is the phylogenetic species concept (PSC), which in *F. graminearum* is based on DNA sequence variation in the genes encoding translation elongation factor 1-α (EF 1-α), β-tubulin (β-tub), phosphate permease (*PHO*), UTP-ammonia ligase (*URA*), trichothecene 3-O-acetyltransferase (*TRI101*), reductase (*RED*), mating type (*MAT*), α -tubulin (α-tub), intertranscribed spacer 28S rDNA (ITS 28S rDNA), Histone H3 (*HIS*) and the trichothecene cluster (*TRI*-cluster) (O'Donnell *et al.*, 2000; 2004; 2008; Ward *et al.,* 2002; Starkey *et al.*, 2007; Yli-Mattila *et al.*, 2009).

Knowledge on the population structure of *F. graminearum* is necessary to understand the epidemiology and evolutionary potential of the pathogen as a causal agent of FHB. This knowledge could improve management strategies for disease control, because the genetic diversity of a population indicates the potential such a population has to evolve

as well as information on its past evolutionary steps. Population surveys of *F. graminearum* have been conducted in North America (Walker *et al*., 2001; Zeller *et al*., 2003; 2004; Gale *et al*., 2007; Ward *et al.,* 2008), Canada (Gilbert and Tekauz, 2000; Fernando *et al*., 2006), Europe (Waalwjik *et al.,* 2003; Gagkaeva and Yli-Mattila, 2004; Tóth *et al*., 2004; 2005; Qu *et al.,* 2008; Yli-Mattila *et al*., 2009) and Asia (Carter *et al.,* 2000; Gale *et al*., 2002, Qu *et al.,* 2007; Suga *et al.,* 2008; Yang *et al.,* 2008; Karugia *et al*., 2009). In contrast, no survey data are available on South African *F. graminearum* populations except for a few South African isolates examined by O'Donnell *et al.* (2000) for placement in lineages as well as the isozyme analyses of Boshoff (1996).

The first objective of the current study was to evaluate the tricothecene genotype potential of FHB populations, while the second and main objective of this study focused on the application of AFLP (amplified fragment length polymorphism) analyses to reveal the population genetic structure of *F. graminearum* populations responsible for FHB in South Africa in order to enable the improvement of disease management strategies.

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CHAPTER 2

A review of *Fusarium* as causal organism of Fusarium head blight in wheat

Wheat is a widely grown crop of critical global importance (Curtis, 2002). Improvement of wheat cultivars is therefore of high priority, with disease resistance as one of the most important characters subjected to selection.

The genus *Triticum* was divided into three prime taxonomic groups (einkorn, emmer and dinkel) by Schultz (1913). Later the cytology of these three groups (einkorn = diploid, emmer = tetraploid and dinkel = hexaploid) supported the division made by Schultz (Feldman, 2001; Nesbitt, 2001). The diploid einkorn types, *Triticum monococcum* (Link) Thell. (one-seeded) are one of the earliest, most primitive, cultivated wheats (Nesbitt, 2001). Cultivated einkorn was derived from the wild form of this wheat, a finding confirmed by the close cytogenetic relationship between the two forms (Feldman, 2001). The second class, the tetraploid two-seeded emmer (*T. dicoccum* Schrank ex Schübler) was rediscovered by Aaronsohn (1906) in Lebanon, Syria, Jordan and Israel (Nesbitt, 2001). Today *T. turgidum* var. *durum,* is the only tetraploid species of commercial importance. *Triticum aestivum* L.*,* the hexaploid and third class is commonly known as bread wheat. This species is the most recent step in the evolution of the wheat complex. It is a true breeding allopolyploid, originating from the hybridisation of three wild grass species still found in the Middle East (Feldman, 2001).

The allohexaploid nature of *T.aestivum* has been confirmed by cytogenetic analysis of hybrids between species with different ploidy levels (Feldman, 2001). *Triticum urartu* Tumanian ex Gandilyan (a diploid) is the donor of the A genome, *T. turgidum* L. the donor of the B genome, and *Aegilops taushii* (also known as *Ae. squarrosa*) the donor of the D genome (Nesbitt, 2001; Gill and Friebe, 2002). Wheat was one of the first domesticated crops and also the youngest polyploid species of agricultural importance (Gill and Friebe, 2002). Ninety-five percent of wheat, both hard and soft, grown today is hexaploid (AABBDD) and the other 5% is tetraploid durum wheat, which lacks the D-genome present in bread wheat. Within *T. aestivum*, different bread wheat types can be distinguished, based on growing period, end use and kernel colour (Peña, 2002).

2.1 Economic importance of wheat

Wheat, together with other triticaceae crops, e.g. barley, rye, triticale, rice and maize provide more than 60% of the calories and protein consumed daily by humans, and is the foundation of human nutrition (Sharma *et al.*, 2002). World wheat production is 500 million metric tonnes, and wheat is grown on 17% of cultivated land, i.e. 240 million hectares (Curtis, 2002). Wheat is a basic staple food for more than 40% of the human population (Gill, 2010). Thirty-thousand different varieties of wheat are known and these varieties can be divided into three major groups based on end use (Peña, 2002). Hard wheat is high in protein (10-14% gluten) and is commonly used for bread, while soft wheat is low in protein (6-10% gluten) and is used for biscuits, cakes etc. The third group, durum wheat, is high in protein and usually is used for pasta (Peña, 2002).

Total global grain production increased dramatically between 1950 and 1980 and therefore the area devoted to wheat cultivation also increased (Myers, 1999). Fluctuations in production occurred between 1991 and 1997 and between 2004 and 2009, with a slight decrease from 1998 to 2003 (SAGIS, 2009). World wheat production declined by 1.1% during the 2005/2006 season and by an additional 3.5% during the 2006/2007 season, which was accompanied by a substantial increase in wheat prices. The world population however, has been projected to increase by 25% to 7.5 billion by 2020. A 2% annual increase in grain production is needed to meet human needs in 2050. However, increase in grain production must occur without an increase in available land, due to urban growth and land degradation (Engelman and Le Roy, 1995). Thus, productivity gains are essential for long-term economic growth and for sufficiency in food supplies for an increasing world population (Curtis, 2002).

South Africa contributed 1.1% to the world's total wheat production in the 2004/2005 season. In the 2005/2006 season South African wheat production increased by 14%. However, high South African wheat prices resulted in a relatively low proportion of the crop being exported.

2.2 Diseases of wheat

Wheat diseases contribute to yield loss and quality reduction, which influences fluctuations in production of wheat worldwide. The three major wheat rust diseases (stem, leaf and stripe rust) appear on plants as reddish-brown pustules that form on the

aerial parts of plants and give the appearance of rust (Prescott *et al.*, 1986; Trench *et al.*, 1992). Other important diseases of wheat are powdery mildew characterised as white, pale grey, fuzzy colonies of mycelia on the adaxial surfaces of leaves and leaf sheaths which can cause yield losses (Prescott *et al.*, 1986; Trench *et al.*, 1992; Murray *et al.*, 1998), karnal bunt, which affect wheat flowers (Singh *et al.*, 1992; Trench *et al.*, 1992; Murray *et al.*, 1998), loose smut, a seed borne disease (Trench *et al.*, 1992; Curtis, 2002; Knox *et al.*, 2002), take-all, which attacks seedling roots and causes sudden death of the plant during flowering before the grain matures (Trench *et al.*, 1992) and Fusarium head blight (FHB), which causes grain yield losses and quality reduction (Sutton, 1982; Bai and Shaner, 1994; Parry *et al.*, 1995; McMullen *et al.,* 1997). These diseases cause economic losses due to food and feed restrictions as well as yield and quality reduction of the grain.

2.2.1 Rust diseases

Wheat rusts are amongst the oldest plant diseases known. Their evolution parallels that of their host. These common wheat diseases are distributed worldwide and can cause severe yield losses. The three major rust diseases are caused by different species of *Puccinia* (Singh *et al.*, 1992; 2002). These pathogens are obligate parasites. Rust fungi can produce large numbers of spores and epidemics caused by these pathogens maybe of continental proportions (Kolmer, 2005; Roelfs, 2010). A large number of pathogenic races occur for each of these three diseases. These three rust diseases were named based on the plant location where symptoms occur and their appearance on the plant (Singh *et al.*, 1992; 2002; Kolmer, 2005; Roelfs, 2010).

2.2.1.1 Stem rust (black rust)

Stem rust is caused by *Puccinia graminis* Pers. f. sp. *tritici* Eriks. & E. Henn. This pathogen is a major threat to wheat production and also causes disease on barley, triticale and other related grasses (Trench *et al.,* 1992; Ayliffe *et al.,* 2008). Many commercial wheat cultivars are susceptible to the latest stem rust race, Ug99 or TTKS (Pretorius *et al.,* 2000, Ayliffe *et al.,* 2008). Yield loss and reduction in grain quality and weight can occur under favourable environmental conditions for disease development with availability of a local inoculum source. Stem rust occurs primarily on stems, but may be seen on leaves, sheaths, glumes and sometimes seed (Roelfs, 2010). The disease

initially appears as dark reddish-brown oblong pustules that become black as the disease develops (Prescott *et al.*, 1986; Trench *et al.*, 1992).

2.2.1.2 Leaf rust (brown rust)

Leaf rust, the most common of the three wheat rusts, occurs primarily on the leaves, but may also occur on the glumes (Scott, 1990; Murray *et al.,* 1998). The orange-brown pustules on the upper leaf surface contain a large number of spores that become black as the disease progresses. This disease is caused by *P. triticina* Eriks. This pathogen reproduces by clonal production of urediniospores in most wheat-growing areas (Kolmer, 2010). Leaf rust occurs primarily on wheat, but also has been reported on barley, triticale and other related grasses (Singh *et al.*, 2002; Kolmer, 2005). This disease can reduce yield by reducing the number of kernels (Murray *et al.,* 1998; Singh *et al.,* 2002; Kolmer, 2010).

2.2.1.3 Stripe must (yellow rust)

Stripe rust, also known as yellow rust, occurs in cooler regions than stem and leaf rust (Singh *et al.,* 2002). This disease is caused by *P. striiformis* Westend f. sp. *tritici* Eriks. and appears on wheat heads and leaves as yellow stripes due to its elongated pustules (Murray *et al.*, 1998; Chen, 2010). The tissue around the pustules turns brown as the disease progresses and the colour of the pustules changes from a yellow-orange to black. Many races of this pathogen are known. Economic losses due to stripe rust usually are limited to barley and wheat, but this pathogen also affects rye and at least 18 other perennial grasses. The disease may result in reduction in yield and grain quality (Singh *et al.,* 2002; Kolmer, 2005).

2.2.2 Powdery mildew

Powdery mildew appears as a series of white, pale grey, fuzzy colonies on the upper surfaces of leaves and leaf sheaths (Prescott *et al.*, 1986). *Erysiphe graminis* DC f. sp. *tritici*, the pathogen responsible for this disease on wheat, is a hetrothallic ascomycete that produces barrel-shaped conidia in chains. This pathogen reduces photosynthesis and increases respiration and transpiration rates causing leaves to die as the host tissue becomes chlorotic and then necrotic (Stromberg, 2010). Within the species, some isolates affect only wheat, whereas other isolates may affect other crops, e.g. barley, oats and rye. Yield losses due to powdery mildew may range from 20-25% depending on

the time of infection and environmental conditions (Prescott *et al.*, 1986; Murray *et al.*, 1998).

2.2.3 Karnal bunt (partial bunt)

Karnal bunt, also known as partial bunt, is caused by *Tilletia indica* Mitra. This soil- and/or seed borne disease infects the flowers. This disease may affect wheat, triticale and several other related grasses, but not barley (Prescott *et al.*, 1986; Murray *et al.*, 1998). Symptoms of karnal bunt are difficult to see prior to harvest due to the irregular distribution of the infected kernels. Symptoms of this disease on the plant are easily confused with other wheat diseases. Diseased kernels have a strong diagnostic fishy odour (Carris, 2010). Black spores usually are observed during harvesting, when infected kernels are crushed. Yield losses due to karnal bunt are relatively minor (0.3-0.5%), but the reduction in grain quality is significant (Singh *et al.,* 1992; Carris, 2010). This disease is on the quarantine list of many countries which makes it a high priority in the world's grain trade (Prescott *et al.*, 1986; Singh *et al.,* 1992).

2.2.4 Common bunt

Common bunt, also known as stinking smut, is caused by *Tilletia tritici* (Berk.) and *T. laevis* Kühn (Fuentes-Dávila *et al.*, 2002). Symptoms of common bunt are only visible after the spike has fully emerged and are usually observed during harvesting (Trench *et al.*, 1992; Fuentes-Dávila *et al.*, 2002). Infected spikes may take longer to ripen (Carris, 2010). The disease may result in loss of yield and grain quality reduction. Common bunt can be averted by using certified treated seed (Fuentes-Dávila *et al.*, 2002). The disease can recur in regions where no control strategies are applied (Carris, 2010).

2.2.5 Loose smut

Loose smut occurs primarily on the flowers of a wheat spike just above the rachis. The flowers are replaced by a mass of black spores (Prescott *et al.*, 1986; Carris, 2010). This seed borne disease is caused by the pathogen *Ustilago tritici* (Pers.) Rostr. The pathogen infects developing kernels and remains within the seed embryo (Carris, 2010). Yield loss ranges from 1-30%, depending on the number of spikes infected (Trench *et al.*, 1992; Fuentes-Dávila *et al.*, 2002; Knox *et al.*, 2002). However, this disease has almost no effect on the grain quality (Carris, 2010).

2.2.6 Black chaff

Black chaff is a bacterial disease caused by *Xanthomonas campestris* pv. *translucens* Syn. and may cause sterility if the plant is infected early in the crop's lifecycle (Trench *et al.*, 1992). Leaves, leaf sheaths and glumes can be infected by black chaff. This disease is characterised by narrow chlorotic lesions or stripes on the leaves. Black chaff symptoms have often been confused with the symptoms of melanosis, which is associated with a stem rust resistant gene, *Sr2* (Milus, 2010). The severity of this disease depends on the number of lost leaves and spikes. The disease may be seed borne and may survive on crop residue in the soil (Prescott *et al.*, 1986; Singh *et al.,* 1992). Black chaff occurs worldwide in major cereal growing areas. Although this disease rarely causes significant damage, yield losses of up to 40% and reduction in seed quality may occur (Murray *et al.*, 1998; Milus, 2010).

2.2.7 Take-all

Take-all is an important root disease of wheat caused by the fungal pathogen *Gaeumannomyces graminis* var. *tritici* Walker (*Ggt*) (Prescott *et al.*, 1986; Singh *et al.,* 1992; Trench *et al.*, 1992). Other varieties of this pathogen also infect oats and other grasses (Freeman and Ward, 2004). *Ggt* causes root rot and necrosis of the lower internodes. This disease is widespread in temperate wheat growing areas, especially in areas where soil is poorly drained. In areas with high rainfall or irrigation, the disease usually develops in patches and the infected plants develop white heads and die prematurely (Mathre, 2000; Paulitz, 2010). Crop debris from the previous season is the main source of inoculum for take-all infection especially with continuous cropping of wheat (Prescott *et al.*, 1986; Freeman and Ward, 2004). Yield losses occur through stunting, premature ripening and decreased grain quality and mass. Fungicide treatments for the control of this disease are available, but crop rotation is the most effective control measure (Freeman and Ward, 2004; Paulitz, 2010).

2.2.8 Fusarium crown rot

Crown rot is primarily caused by *F. pseudograminearum*. This pathogen was previously described as *F. graminearum* and was associated with diseases such as head blight of wheat, crown and stalk rot of maize etc. Francis and Burgess (1977) divided *F. graminearum* into two groups based on minor morphological differences and diseases that isolates were associated with. Group I isolates caused crown rot on wheat, while

group II isolates caused FHB on wheat and stalk rot on maize. Group II isolates often formed perithecia homothallically, while group I isolates were not self-fertile. Diagnostic genes from isolates in these two groups were sequenced by Aoki and O'Donnell (1999a) to confirm phenotypic differences and determine genotypic differences between the two groups. Based on this study, these investigations concluded that group I isolates were different from group II isolates and that group I and group II were different species. They gave group I isolates a new name - *F. pseudograminearum*.

Fusarium crown rot occurs primarily on dryland wheat cultivated under moisture stress. This disease was first described in Australia by McKnight in 1951 (McKnight and Hart, 1966), but also has been problematic elsewhere: United States of America (USA) (Smiley *et al.,* 2005), South Africa (Van Wyk *et al.,* 1988) and Canada (Mishra *et al.,* 2006). *Fusarium* crown rot is a soilborne disease that infects the basal parts of the plant just beneath the soil. Symptoms that indicate the presence of this disease include dark brown lesions and pink-purple discolouration on the basal part of infected plants. The most distinctive characteristic of this disease is the premature white heads of grain at the flowering stage, which never fills (Smiley *et al*., 2005; Cook, 2010). Crown rot in Australia is an economically important disease due to the high frequency of yield losses and reduction in grain quality. The disease is difficult to manage; delayed planting dates and crop rotation are only partially effective.

2.3 Fusarium head blight

FHB, also called scab, is a common fungal disease of wheat, barley, oats and maize that results in reduced grain quality and yield (Parry *et al*., 1995). The disease may be caused by any of several species of *Fusarium* including *F. avenaceum* (Fries) Saccardo, *F. graminearum, F. crookwellense, F. culmorum, F. langsethiae* Torp and Nirenberg, *F. poae* (Peck) Wollenweber, *F. sporotrichioides* Sherbakoff, and *Microdochium nivale* (Fr.) Samuels & I. C. Hallett (Dill-Macky, 2010). Scab is a devastating disease of wheat in a number of regions, e.g. North and South America, Europe, Asia and South Africa, especially when moist weather occurs during the growing season (Cook, 1981; Dill-Macky, 2010). FHB has caused catastrophic losses in the north-central wheat growing regions of North America and reached epidemic proportions (McMullen *et al*., 1997). This disease that not only reduces crop yield and seed quality, but also results in grain

being contaminated with mycotoxins that can affect food and feed safety (Desjardins, 2006).

A number of reviews (Atanasoff, 1920; Sutton, 1982; Jenkins *et al.*, 1988; Parry *et al.*, 1995; Boshoff, 1996; Champeil *et al.*, 2004) are available for FHB or scab. They conclude that there is no good control available for scab and that no significant solutions or control strategies for the problem have progressed beyond proof of concept.

This disease affects the flowering parts of the host. Small grain cereals susceptible to scab include barley, rye, oats, rice and maize, but the most important is wheat (Parry *et al.*, 1995). FHB can turn a potentially high yield into a loss (Parry *et al.*, 1995; McMullen *et al.*, 1997). Yield reduction usually results from shrivelled tombstone kernels that blow away with the chaff during harvest (Osborne and Stein, 2007). Some of these damaged kernels, however, remain with the healthy grain, reducing test weight and seed quality. *Fusarium graminearum* can degrade grain proteins and gluten, which reduces the baking quality of the flour (Parry *et al.*, 1995). Indirect effects of FHB include poor seed germination, seedling blight and poor stand establishment.

Fusarium graminearum can also produce mycotoxins that are harmful to humans and domesticated animals (Desjardins, 2006). The three most prominent mycotoxins are deoxynivalenol (DON), nivalenol (NIV) and zearalenone (ZEN) (Parry *et al.*, 1995; Desjardins, 2006).

2.3.1 Naming of the disease

W.G. Smith first described FHB as wheat scab in 1884, when the disease was reported in England. Later in the same century, Chester (1890), Arthur (1891) and Detmers (1892) all reported that scab was an important wheat disease. Chester (1890) gave the first detailed description of FHB and attributed the disease to *Fusisporium culmorum* as reported by Smith in 1884 (Parry *et al.*, 1995). Atanasoff (1920) stated that scab was not a suitable common name and used the term Fusarium blight. Dounin (1926) again changed the common name in 1926 to fusariosis. This disease is currently known as both scab and FHB (Stack, 2003).

2.3.2 History of Fusarium head blight

Research on FHB in the USA can be divided into four eras (Stack, 2003). The first era began in 1880 with the beginning of plant pathology. The second era was characterised by experimental studies and began in 1908. This era includes the largest outbreak of FHB in the USA, which occurred in 1919 (Stack, 2003). The third era, known as the dark ages of FHB research, began in the 1950s and the fourth era began in the 1980s and is characterised by a period of intense research on FHB in North America (Parry *et al.*, 1995; Stack, 2003; Dill-Macky, 2010).

Since the late 1930s, FHB epidemics have been documented in Australia (1978 and 1983), Canada (1939-1943, 1980, 1993 and 1994) (Sutton, 1982; Fernando *et al.*, 1997; Stack, 2003), China, Brazil, Argentina, Central Europe, Kenya, USA and several other countries (Windels, 1999; Muthomi and Mutitu, 2003, Muthomi *et al.*, 2008). Epidemics are characterised by high inoculum levels that build up due to no-till and reduced tillage practices along with wheat-maize rotations, warm temperatures and high humidity (McMullen *et al.*, 1997).

The first report of FHB on wheat in South Africa was in the North-West Province in 1980 (Figure 2.1) (Scott *et al.*, 1988), followed by an outbreak in George in the Southern Cape in 1983. Initially the disease in George was restricted to a single irrigation pivot, but it soon began to spread throughout the district (De Jager, 1987). FHB occurred in northern KwaZulu-Natal (Winterton, Greytown, Dannhauser, Dundee and Newcastle) and the eastern Free State (Reitz and Frankfort) on a susceptible cultivar, Zaragosa, in 1985 and again in 1986 (De Jager, 1987). More outbreaks of FHB occurred near George in 1987 and sporadic reports of this disease were made in the Swellendam district at about the same time (Boshoff, 1996). More recently epidemics were reported in the Northern Cape in the early 1990s and are still continuing (Kriel and Pretorius, 2006; Kriel, personal communication).

2.3.3 Distribution and disease life cycle of Fusarium head blight

FHB is a global disease that affects most of the cereal-growing areas of the world and occurs in a number of regions (North and South America, Europe, Asia, Australia, Canada, Kenya and South Africa) (Cook, 1981; Scott *et al.,* 1988; Boshoff, 1996; Carter *et al.*, 2000; Gale *et al.*, 2002; Waalwjik *et al.*, 2003; Fernando *et al.*, 2006; Guo *et al.*,

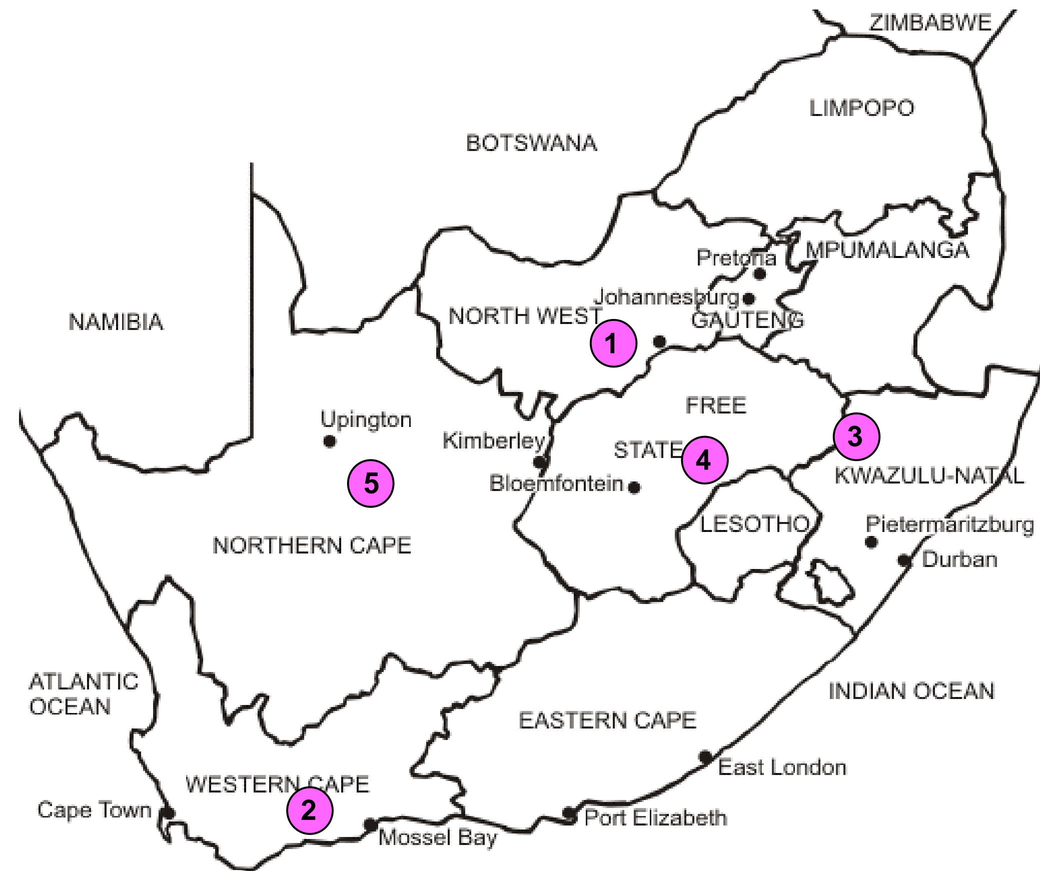

Figure 2.1 South African provinces where FHB epidemics have been reported since 1981 indicated with coloured dots in ascending order of year of epidemic. **1 - 1980** **2 - 1983** **3 - 1985** **4 - 1986** **5 - 1990s**

2008; Karugia *et al*. 2009). This disease is endemic in China where two thirds of the country's provinces produce wheat. This monocyclic disease is primarily dependent on inoculum which survives in the field until the subsequent growing season (Trail, 2009).

Different sources of inoculum for the development of FHB, e.g., crop debris from previous seasons, seeds, other plants, weeds, insects and other fungi are known of which host debris is probably the most important reservoir for the fungus (Parry *et al*., 1995; Champeil *et al*., 2004; Osborne and Stein, 2007). Ascospores from the sexual stage and macroconidia from the anamorph stage are thought to be the principal sources of inoculum for FHB (Figure 2.2) (Sutton, 1982; Bai and Shaner, 1994; Parry *et al*., 1995). The severity of the disease can increase when wheat is sown in plant residue (particularly wheat, barley, rice and maize) from the previous year (Windels, 1999). The pathogen survives as a saprophyte on dead host tissue, especially if susceptible crops are planted in successive years, e.g. wheat on wheat, wheat on maize, wheat on barley and wheat on rice rotations in the same field. Crop rotation, tillage methods, date of anthesis and climatic conditions all can influence disease incidence and severity (Windels, 1999).

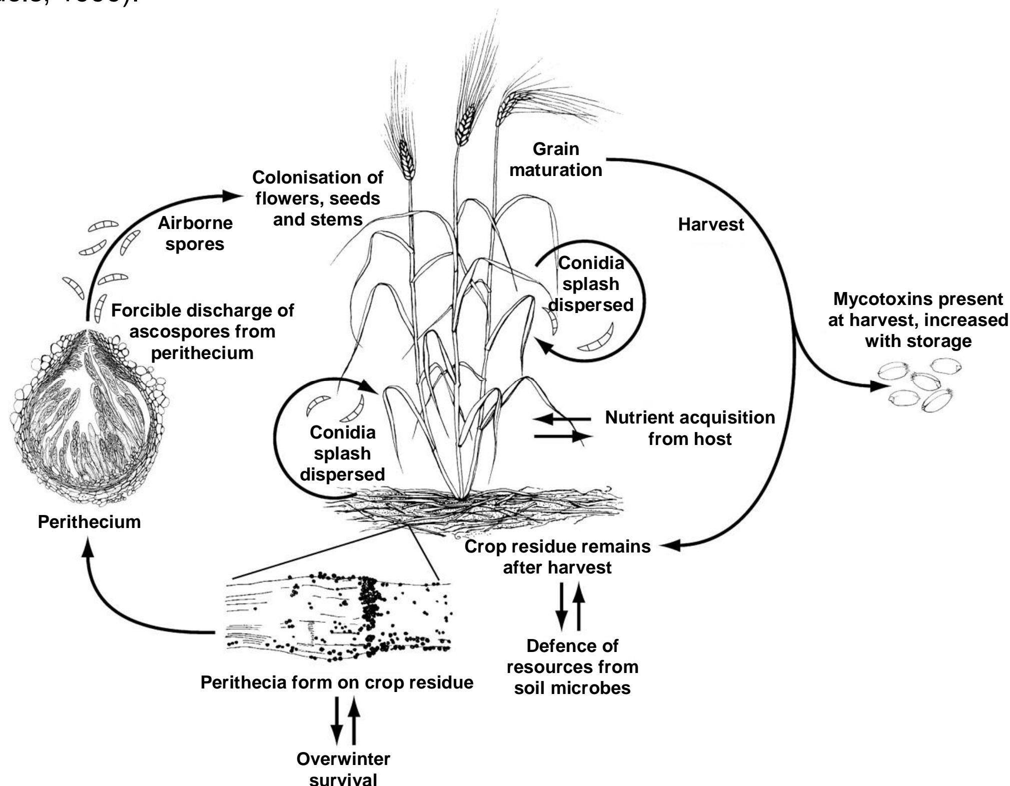

Figure 2.2 Life cycle of Fusarium head blight (with permission: Trail, 2009).

Infections of wheat spikes are initiated by ascospores or macroconidia dispersed by water-splash or air currents onto wheat heads. Water- or rain splash and wind are the most important dispersal factors of ascospores and conidia to wheat heads, but other vectors may include insects and birds. Infections can occur as early as spike emergence, but the most severe infections probably occur during anthesis (Teich and Nelson, 1984; McMullen *et al.*, 1997). Warm moist weather is best for ascospore germination on the wheat heads and initiation of primary infection. Perithecia form at 16℃ and their number increases with temperature up to 25℃. Macroconidia of *F. graminearum* can germinate within three hours of inoculation at an optimal 20-30℃ and by the end of six hours most of these spores will be completely germinated (Shaner, 2003).

The first FHB symptoms observed is a brownish discolouration (necrosis) on the rachis of each spike, and the premature death of the spike (Goswami and Kistler, 2005). The pathogen is readily isolated from infected plant tissues (Boshoff, 1996).

2.3.4 Causal organism

FHB may be caused by several species in the Discolor section of the genus *Fusarium,* namely *F. avenaceum, F. culmorum, F. crookwellense* and *F. graminearum* (Liddell, 2003). *Fusarium graminearum* is common in warmer, more humid parts of the world (Boshoff, 1996; Vigier *et al.*, 1997) whilst *F. culmorum*, *F. avenaceum* and *F. crookwellense* occupy a similar niche in cooler parts of the world (Parry *et al.*, 1995). A relative increase in the frequency of *F. graminearum* relative to *F. culmorum* has occurred in several European countries over the past decade (Waalwjik *et al.*, 2003; Brennan *et al.*, 2005) and may be attributed to an increase in maize production. *Fusarium graminearum, F. culmorum, F. avenaceum* and *F. equiseti* (Corda) Saccardo are commonly recovered from grain samples worldwide (Sutton, 1982; Scott *et al.*, 1988; Parry *et al.*, 1995, Kosiak *et al.*, 2003). *Fusarium graminearum* poses a double threat to cereals since it reduces yield and seed quality and may contaminate the remaining grain with mycotoxins (Desjardins, 2006).

2.3.5 *Fusarium*

Members of the genus *Fusarium* are amongst the most important disease agents of plants. *Fusarium* spp. can cause a wide range of diseases e.g. ear rot, bakanae, wilts

and fusarium patch on many genera of cultivated plants, e.g., maize, rice, turfgrass, tomatoes and bananas, other than small grains. A German mycologist, Link, first described the genus *Fusarium* in 1809 (Stack, 2003). The genus was described as a large, common group of fungi that could grow on many substrates, e.g. soil, water and either living or dead organic substrates. The primary identifying characteristic for members of the genus is the presence of banana-shaped conidia. By the end of the 19th century more than 1000 *Fusarium* species had been described, resulting in great difficulty differentiating species within the genus. This problem was partially resolved with the publication of Wollenweber and Reinking's (1935) monograph, which reduced the 1000 species to about a 100 taxonomic entities. The mycological characters, e.g. spore morphology and pigment, first used by Wollenweber and Reinking (1935) are the basis for most current *Fusarium* classification schemes (Leslie and Summerell, 2006). Booth introduced conidiogeneous cell morphology to distinguish between species in the different *Fusarium* groups. This method of morphological identification is especially important when differentiating some of the species in sections *Liseola* and *Sporotrichiella* (Leslie and Summerell, 2006).

Classification of *Fusarium* species often has not been stable, making it difficult to identify species. Some species of *Fusarium* appear to be ubiquitous while others are limited to specialised habitats as saprophytes or parasites. The number of defined taxa has ranged from the nine species described by Snyder and Hansen (1945), to 44 species and seven varieties described by Booth (1971), 65 species and 55 varieties described by Wollenweber and Reinking (1935); Nelson *et al.* (1983) and more than 70 species and 55 varieties described by Gerlach and Nirenberg (1982) to 70 species, with the number likely to increase (Leslie and Summerell, 2006).

The most common pathogen to cause FHB, *F. graminearum*, has been difficult to identify clearly. Francis and Burgess (1977) divided *F. graminearum* into two groups based on a morphological difference and the effects the members of these groups had on different hosts. Members of group I did not form perithecia in culture and caused diseases associated with wheat crowns, i.e. wheat crown rot. Members of group II formed abundant perithecia homothallicaly in culture and in the field, and were responsible for diseases of aerial parts of plants such as wheat, barley, maize and rice. In 1999, group I was redescribed as *F. pseudograminearum*, with a sexual stage, *Gibberella coronicola*

Aoki and O' Donnell*,* that is known to occur under field conditions (Aoki and O'Donnell, 1999a; 1999b). *Fusarium graminearum* group II is a monophyletic that may be split into at least thirteen distinct lineages/phylogenetic species i.e. *F. austroamericanum, F. meridionale, F. boothii, F. mesoamericanum, F. acaciae-mearnsii, F. asiaticum, F. graminearum, F. cortaderiae, F. brasilicum, F. gerlachii, F. vorosii, F. aethiopicum* and *F. ussurianum* (O'Donnell *et al.*, 2000; 2004; 2008; Starkey *et al.*, 2007; Yli-Mattila *et al.*, 2009). However, not all *Fusarium* researchers accept these phylogenetic species as formal species, since members of these lineages can intercross with one another (Leslie and Bowden, 2008).

2.3.6 Taxonomy

Taxonomy is the science of orderly arrangement, identification, naming and classification of organisms into groups based on similarities of characters. The smallest unit of a taxonomic chain is a species, which Mayr (1996) described as the principal unit of evolution. Accurate description of evolution is possible only if species and their differences are well understood (Mayr, 1996). Charles Darwin (1859) proposed that evolution was responsible for the changes and/or diversity in life that gave rise to new and/or separate species and lineages.

Three different species concepts have been used to define species in *Fusarium* and all three have been used to define one or more species that cause FHB, especially *F. graminearum* (Summerell *et al.*, 2003). These three species concepts are the MSC, the BSC and the PSC, which is secondary to the theoretical concept, the evolutionary species concept. The evolutionary species concept describes a species as a population or a group of populations that shares a common ancestral evolutionary lineage that evolves independently of other such lineages and has its own unique evolutionary role.

2.3.6.1 Morphological species concept

Morphological data have traditionally been the basis of species delimitation, especially in fungi (De Queiroz, 2007). Taylor *et al.* (2000) described the MSC as practical due to its widespread applicability to many fungi. This species concept is regarded as the oldest concept and is based only on phenotypic and physiological characters e.g. colony colour, absence or presence of certain structures (e.g. phialides), shape and size of macro- and microconidia, growth rate, optimal growth temperature etc. (Summerell *et al.*, 2003; Leslie and Summerell, 2006). Morphology is useful for an initial identification of

species and can distinguish some species and species groups. The most common problem with this species concept is that many of the morphological characteristics overlap between species. Thus, morphological data often are inadequate for a definitive identification, even though this concept is useful for asexual organisms (Taylor *et al,* 2000.). Link (1809) used the MSC to initially define *Fusarium* species based on the presence of the banana/canoe-shaped macroconidia. Phenotypic and physiological characters are used to divide the genus *Fusarium* into different sections (Leslie and Summerell, 2006), although these sections are not monophyletic.

2.3.6.2 Biological species concept

The biological species concept is applicable only when the sexual stage can be formed in culture and cannot be applied to asexual populations. Members of a biological species are individuals that can actually or can potentially interbreed with each other (Leslie *et al.*, 2001). Mayr (1996) used three criteria to define and distinguish biological species. These species share characters within a population, rather than individuals. These shared characters delimit and define the extent of variation within a species. Members of the same species also are interfertile. In practice, the biological species concept requires that members of the same species be sexually cross-fertile and that the progeny of these crosses are viable, fertile and relatively numerous (Leslie and Summerell, 2006). The biological species concept helped to distinguish species within the *F. solani* (Mart.) Sacc. species complex (Leslie and Summerell, 2006) as well as the *G. fujikuroi* (Sawada) Ito in Ito & K. Kimura species complex. Reproductive isolation results in different species whose members cannot cross with members of another species (Mayr, 1996). Therefore, according to the BSC, the lineages/phylogenetic species of *F. graminearum* (section 2.3.6.3) proposed by O'Donnell *et al.* (O'Donnell *et al.*, 2000; 2004; 2008; Starkey *et al.*, 2007; Yli-Mattila *et al.*, 2009) can only be described as distinct species if their members are not cross-fertile or if progeny resulting from inter-lineage crosses are either not viable or not fertile.

Bowden and Leslie (1992) used nitrate-nonutilising mutants (*nit* mutants) to show that homothallic, asexual strains of *F. graminearum* could outcross. Female fertility was regarded as a problem. Some of the lineage tester strains of O'Donnell *et al.* do not produce perithecia (Leslie *et al.,* 2007). This problem was overcome by the construction of female-fertile MAT knockout strains (Lee *et al.,* 2003), which can be used to test all *F.*

graminearum strains as males. The three knockout strains were all members of lineage 7. All of the lineage testers were highly fertile when crossed with at least one of the knockout strains, which suggested that all tested lineages, belonged to the same biological species and should not be accorded species rank (Leslie *et al.,* 2007).

2.3.6.3 Phylogenetic species concept

The phylogenetic species concept classifies species based on multiple characters and is usually based on DNA sequences. This species concept usually increases the existing number of species since species previously classified on a non-phylogenetic basis are split into new groups. *Fusarium* spp. can be distinguished based on DNA sequence variation, with the translocation elongation factor 1-α (EF 1-α) gene being the most widely used. β-tubulin (*β-tub*) usually fails in the *F. solani* species complex and sometimes for *F. graminearum* as some isolates have a *β-tub* pseudogene (Leslie *et al.,* 2007). The ribosomal repeat regions ITS 1 and ITS 2, can be used to identify the genus, but cannot be used to resolve all of the species (O'Donnell and Cigelnik, 1997). Identification based on molecular markers is faster if equipment and trained personnel are available and reduces or eliminates variation between laboratories due to environmental variation and diagnostic skill. The PSC can be problematic until it is properly 'calibrated', since the level at which sequences differ at species level is not constant. Validation of a PSC for any group of species requires testing of a population of strains to ensure the reliability, repeatability and frequencies of type I and type II detection errors that result from the protocol. *Fusarium graminearum* is an excellent example of the discord that results when different species concepts result in non-congruent species definitions. There are at least thirteen phylogenetic lineages/species described in the *F. graminearum* clade (O'Donnell *et al.*, 2000; 2004; 2008; Starkey *et al.*, 2007; Yli-Mattila *et al.*, 2009). All are morphologically similar and there is little or no loss in cross-fertility if isolates from different lineages are crossed with the *MAT* knockout strains (Leslie and Bowden, 2008). Members of all of the lineages can cause FHB on wheat grown under greenhouse conditions (Goswami and Kistler, 2005) and there are no pathogenic races known within the species. Lineages may differ in host preference and/or in the mycotoxins produced, but these distinctions are not absolute**.** The division of species in the *F. graminearum* complex based on the PSC is based on strains clustering together due to sequence similarities and the application of a number of genes

(thirteen) that separates these strains into similar groups (Leslie and Bowden, 2008). No single sequence can be used as diagnostic to resolve all thirteen phylogenetic groups.

2.3.7 Mycotoxins

Mycotoxins are low molecular weight toxic metabolites produced by certain fungi (Desjardins and Hohn, 1997; Desjardins, 2006). Research on these secondary metabolites is mostly focussed on the reduction and control thereof in cereal grains. Three major classes of mycotoxins (fumonisons, trichothecenes and zearalenones) produced by *Fusaruim* spp.have been described between 1961 and 1991 (Desjardins, 2006). Most of the fungi that cause FHB also produce mycotoxins that affect both human and animal health. Some of these mycotoxigenic fungi are aggressive pathogens on their plant hosts (Desjardins, 2006). These mycotoxins are chemically and thermally stable and can be found in raw grains and finished products, e.g. flour and bread. *Fusarium* mycotoxins can inhibit enzyme synthesis and yeast growth, which may affect malting (gushing of beer) (Garda-Buffon *et al.*, 2010) and bread quality (low fermentation) (Prange *et al.,* 2005).

Some of the most prominent mycotoxins produced by FHB pathogens belong to the tricothecene class (Desjardins, 2006). Trichothecenes are potent inhibitors of eukaryotic protein synthesis and are toxic to all organisms. These mycotoxins often are produced under cold humid conditions (Champeil *et al.*, 2004). Mycotoxins in this class may have major effects in humans and animals including skin inflammation, digestive disorders, tachycardia, oedema, haemorrhages in internal organs and nervous disorders (Desjardins, 2006). The tricothencene biosynthetic pathway genes in *Fusarium* are closely linked to one another, forming a gene-cluster. This gene-cluster has been identified (Desjardins and Hohn, 1997) and completely sequenced (Ward *et al.,* 2002; Desjardins, 2006) to unravel the influence of these genes on the functioning of the mycotoxins involved. These tricothecenes can be divided into two groups, chemotype A and B (Desjardins, 2006). Chemotype A contains compounds without a keto-group at C-8 and includes the highly toxic T-2 toxin and diacetoxyscripenol. Chemotype B contains compounds with a keto-group at C-8 (Figure 2.3), including DON, 3-DON (3-acetyldeoxynivalenol) and NIV (Desjardins, 2006). Most studies on the production of mycotoxins produced by *F. graminearum* focus on DON.

DON was first isolated from barley in Japan in 1970 (Yoshizawa and Morooka, 1973). It is detected most frequently and in highest concentrations in cereals and can be regarded as an ‘indicator-toxin’ of FHB (Schollenberger *et al.,* 2002). This mycotoxin is the major contaminant of FHB-infected wheat in several countries (Sydenham *et al.,* 1989; Botallico, 1998). DON can affect a wide variety of plants where it inhibits germination and growth of seeds and coleoptile tissues (Snijders and Perkowski, 1990). Cereal grains (wheat, maize and barley) are the most important sources of mycotoxin contamination in human and animal diets (Desjardins, 2006).

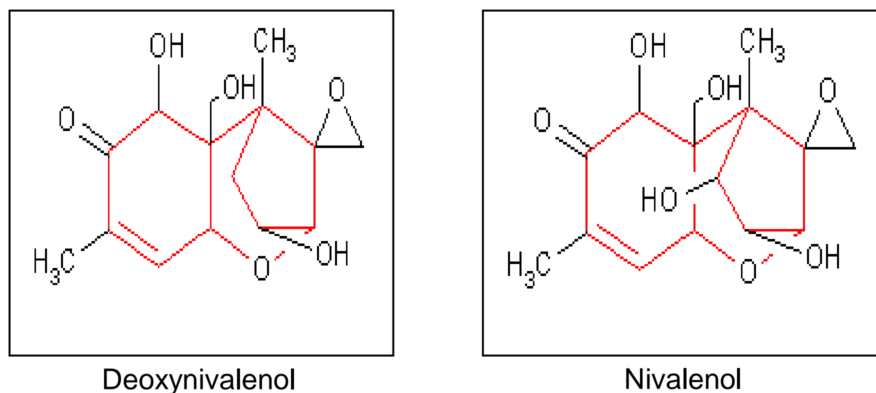

Figure 2.3 Chemical structures of mycotoxins produced by *Fusarium graminearum* (with permission: Desjardins, 2006).

DON contamination in Africa was reported in Zambia in 1974 and South Africa in 1975 (Marasas *et al.,* 1977). Both DON and NIV were detected in infected wheat in the southern Cape region of South Africa. NIV, identified for the first time in 1968, is described as ancestral to DON, even though DON occurs more commonly in contaminated wheat in most wheat-growing countries worldwide. NIV, however, is more frequent in European grains (Desjardins, 2006). The co-occurrence of DON and NIV has been reported in countries other than South Africa, e.g., Korea, Japan, Poland, Taiwan and United Kingdom (UK) (Placinta *et al.,* 1999). DON, however, is up to seven times more active than NIV in inhibiting plant growth. DON producers are more aggressive pathogens than NIV producers in causing FHB (Desjardins *et al.,* 1996) and DON production can be directly correlated with disease incidence (Teich and Nelson, 1984; Champeil *et al.*, 2004).

Increased FHB incidence can be influenced by the previously planted crop and by tillage practices. Therefore high DON levels detected in contaminated grain can be influenced by the same factors as disease incidence. For example, if maize precedes wheat, then the DON concentrations usually are higher than in a field where soybeans precede wheat (Champeil *et al.*, 2004). Beyer *et al.* (2005) suggested that the level of DON contamination caused by *F. graminearum* is dependent on the spore type (macroconidia/ ascospores) in correlation with relative humidity and the interaction between these two factors. An increase in favourable environmental factors can increase the amount of mycotoxin present in the grain. Decreased incidence of fungi in the field usually decreases the mycotoxin content of the grain (Snijders, 2004). DON and NIV accumulation influence the competitiveness of the FHB pathogens, making them more aggressive relative to other fungi (Desjardins *et al.,* 1996; Desjardins, 2006; Desjardins and Proctor, 2007). Thus, correct identification is important for studies of epidemiology, chemical control, resistance breeding and risk assessment of wheat (Nicolaisen *et al.*, 2005).

2.3.8 Pathogenicity

Pathogenicity is the qualitative capacity of a pathogen to infect and cause a disease on a host. Pathogenicity of a pathogen can be expressed in terms of the severity of a disease (Sacristan and Garcia-Arenal, 2008).

Variation in aggresiveness and virulence in the FHB causal species has become a major concern, especially for resistance breeders (Dill-Macky, 2003). Different levels of pathogenicity of different fungal strains on various hosts have been observed for these species (Desjardins and Hohn, 1997). *Fusarium graminearum* and *F. culmorum* are the most pathogenic of the species that cause FHB, with *F. graminearum* being more pathogenic on wheat heads (Desjardins and Hohn, 1997; Desjardins and Proctor, 2007). Only a few pathogenicity factors are known in *F. graminearum*, which does not produce any infection structures, e.g. haustoria or appressoria, during pathogenesis, but colonises the host tissue by producing cell wall degrading enzymes (Bluhm *et al.,* 2007; Dufresne *et al.,* 2008). Trichothecene-producing *Fusarium* spp. are described as destructive pathogens which indicate that the mycotoxin produced by the pathogen play a role in the pathogenesis of *Fusarium* on plants (Desjardins and Hohn, 1997). The thirteen phylogenetic species in the *F. graminearum* species complex as described by

O'Donnell *et al.* may differ in aggressiveness on wheat due to genetic variation in the trichothecenes produced (Ward *et al.*, 2002).

Pathogenicity tests are time-consuming. The identification of pathogenicity genes is important in order to understand the disease process and to develop resistant crops (Idnurm and Howlett, 2001). Two mitogen-activated protein (MAP) kinase genes, *MGV*1 and *GPMK*1, are involved in pathogenesis in *F. graminearum*. Gene disruptions that block toxin biosynthetic pathways are a powerful tool for investigating the role of mycotoxins in plant disease (Desjardins and Proctor, 2007). DON production, spread and virulence of the pathogen are reduced when *MGV*1 gene is disrupted (Hou *et al.,* 2002). Disruption of *GPMK*1 inhibits colonisation of flowering wheat heads and spread of the pathogen from one infected spike to the neighbouring spikes (Urban *et al.,* 2003). The presence of these genes is not necessary for the initial infection of the pathogen, but they are important for the pathogen to spread and colonise additional host tissue. Disruption of the *Tri*5 gene the first step unique to the tricothecene biosynthetic pathway, always reduce virulence of these non-trichothecene producing mutants under laboratory conditions (Proctor *et al.*, 1995). In field trials, the trichothecene non-producing mutants were less virulent than isolates that could produce the toxins (Desjardins *et al.*, 1996). These results provide an explanation for the correlation between the amount of toxin produced and the aggressiveness of the isolate (Desjardins *et al.*, 1996; Desjardins and Proctor, 2007).

2.4 Variation in resistance

Wheat breeders worldwide are trying to breed for resistance to FHB as part of a control strategy to contain this disease. Breeding for resistance, however, is not easy, because each breeder's objective is to create a resistant cultivar adapted to their region with high and stable yield and quality. Genetic variation in resistance to FHB is recognised in most parts of the world (Snijders, 1990; Bai *et al.*, 1999). Wheat varieties are most susceptible to FHB at the flowering stage and grain fill (Dill-Macky, 2010). Resistance is inherited quantitatively (Buerstmayr *et al.*, 2009) and is affected by environmental conditions, which makes it difficult to score disease severity reliably. Resistance components include resistance to penetration and colonisation and the reduction of myctoxin content in the kernels (Snijders, 2004).

The level of resistance to FHB varies from susceptible to tolerant. There are five physiological resistance types to FHB in wheat. Type I and II resistance were described by Schroeder and Christensen (1963), where type I was complete resistance to infection, while type II resistance meant that the pathogen could not spread within the spike following infection. These two resistance types are based on symptom development and can be evaluated under greenhouse conditions following spike and floret inoculation, respectively (Buerstmayr *et al.*, 2009). Type I resistance is measured as both disease incidence and disease severity (Browne, 2009). This dichotomy makes type I resistance difficult to assess. FHB resistance currently is assessed mainly using type II resistance testing methods. Type III resistance is the ability of the host to prevent mycotoxin accumulation in infected kernels (Miller *et al.,* 1985). In experiments to test for type III resistance, seeds from infected spikes are threshed and the damage to the kernels is observed to asses disease severity and the quality of seeds that remain (Rudd *et al.,* 2001; Dill-Macky, 2003). Mesterházy (1995) described type IV and V resistances as yield tolerance and decomposition which implied little or no accumulation of mycotoxins with the fungal infection**,** respectively. Type IV is assessed by measuring yield after artificial inoculation, while DON concentration is measured for type V (Rudd *et al.,* 2001). Environmental factors can influence the expression and/or the effects of these resistance types on FHB severity. All five resistance groups are associated with quantitative trait loci (QTL) (Anderson *et al*., 2001; Browne and Brindle, 2007; Li *et al.,* 2008).

Many sources of partial FHB resistance have been identified, but no sources with complete resistance are known (Ban, 1997; Buerstmayr *et al*., 2009). The most commonly used source of resistance is Sumai 3, a spring wheat variety from China, because of the stable resistance to disease spread in the spike found in this cultivar and its derivatives (Rudd *et al.,* 2001; Dill-Macky, 2003). Major QTL for FHB resistance have been identified on chromosomes 3B, 6B and 5A from Sumai and verified by molecular marker analysis (Waldron *et al.,* 1999; Anderson *et al.,* 2001; Yang *et al.,* 2003; Zwart *et al.,* 2008).

Sumai 3 resistance (Table 2.1) has been used extensively in both winter and spring wheat programmes in the USA. The resistance of this cultivar to FHB is stable and consistent, which enables the analysis of heritability. Other sources (Table 2.1) of resistance that have been used widely include the Brazilian spring wheat cultivar,

Frontana (Steiner *et al.,* 2004; Mardi *et al.,* 2006; Zwart *et al.,* 2008) which has type I resistance and the Japanese line Nobeokabouzu (Rudd *et al.,* 2001). Wheat varieties are not widely adapted and varieties are bred for specific regions or environmental conditions (Singh *et al.,* 1995) making breeding for FHB resistance difficult. A higher level of FHB resistance will lower risks of crop loss due to reduced grain yield, low quality and mycotoxin contamination. Resistance research is directed at the development of genotypes with low disease symptoms and even lower levels of mycotoxins (Buerstmayr *et al*., 2009).

2.5 Molecular identification of *Fusarium* species

Fusarium identification has traditionally been based on morphological studies, which require considerable expertise and are not always accurate. Molecular techniques are now being used to help overcome these problems and to identify fungi within disease complexes.

2.5.1 Ribosomal RNA

The four ribosomal RNAs (rRNAs) known, 5S, 18S, 26S and 28S are highly conserved and universally found in all living cells with the exception of bacteria (Guadet *et al*., 1989). The universal availability of the rRNAs therefore makes it easy to use these molecules as taxonomic indicators. Resolving closely related species were enabled with the use of the short conserved 5S rRNA due to the low information content and uniform rate of nucleotide substitution of this molecule (Qu *et al*., 1988). The development of an easy, rapid sequencing technique of large stretches of rRNA molecules enabled systematic exploitation of these molecules as phylogenetic indicators (Qu *et al*., 1988; Bergeron and Drouin, 2008). Sequencing of rRNA genes has been used as a rapid identification and classification aid of *Fusarium* species associated with human infections (Hennequin *et al.,* 1999; Oechsler *et al.,* 2009). These sequences also have been used to determine the phylogenetic relatedness amongst some related *Fusarium* species (Guadet *et al*., 1989; Logrieco *et al*., 1991).

Table 2.1 Fusarium head blight resistance sources and QTL positions (after Zwart *et al.,* 2008)

Regions	Cultivar	QTL position	References
Asian spring wheat	Sumai 3	**Major QTL**: 3BS, 5AS, 6BS; **minor QTL**: 2AS, 6AS	Waldron *et al.,* 1999; Anderson *et al.,* 2001; Yang *et al.,* 2003; Zwart *et al.,* 2008
	Wangshuibai	**Major QTL**: 3BS; **minor QTL**: 1BS, 2AS, 2AL, 2BL, 2DS, 2DL, 3A, 3D, 4BL, 5A, 5BL, 5D, 6BS, 7AL, 7DS	Zhang *et al.,* 2004; Zhou *et al.,* 2004; Jia *et al.,* 2005; Mardi *et al.,* 2005; Li *et al.,* 2008; Zwart *et al.,* 2008
	Wuhan-1	2DL, 4BS	Somers *et al.,* 2003; Zwart *et al.,* 2008; Tamburic-Ilincic *et al.,* 2009
	Chokwang	**Major QTL**: 5DL; **minor QTL**: 3BS, 4BL	Yang *et al.,* 2005; Zwart *et al.,* 2008
European winter wheat	Arina	**Major QTL**: 4AL, 5BL, 6DL; **minor QTL**: 1BL, 2AL, 2B, 2DL, 3BL, 3DS, 5AL, 6BL, 7AL	Paillard *et al.,* 2004; Draeger *et al.,* 2007; Semagn *et al.,* 2007; Zwart *et al.,* 2008
	Renan	**Major QTL**: 2BS, 5AL; **minor QTL**: 2AL, 3BL, 5AS, 5DL, 6DS	Gervais *et al.,* 2003; Zwart *et al.,* 2008
	Dream	2BL, 6AL, 7BS	Schmolke *et al.,* 2005; Zwart *et al.,* 2008
	Cansas	1BS, 3DL, 5BL, 7BS	Klahr *et al.,* 2007; Zwart *et al.,* 2008
	Fundulea 201R	1B, 3AS, 5AS	Shen *et al.,* 2003; Zwart *et al.,* 2008
	NK93604	1AL, 7AL	Semagn *et al.,* 2007; Zwart *et al.,* 2008
South American spring wheat	Frontana	**Major QTL**: 3AL; **minor QTL**: 5AS, 7AS	Steiner *et al.,* 2004; Mardi *et al.,* 2006; Zwart *et al.,* 2008
North American winter wheat	Ernie	**Major QTL**; 3B, 4BL, 5A, **minor QTL**: 2B	Liu *et al.,* 2007; Zwart *et al.,* 2008
	Freedom	2AS	Zwart *et al.,* 2008

QTL – Quantitative trait loci.

2.5.2 Sequencing

Development of phylogenetic markers to evaluate relationships between fungal groups and to accurately identify fungal species within these groups, which previously had been based on morphology, has become a necessity. Due to the problems with rRNA gene sequences, researchers have turned to sequences of protein coding genes sequences (Roger *et al.*, 1999).

EF 1-α is a short, highly conserved, pervasive protein coding gene, that plays an essential role in protein synthesis (Silar and Picard, 1994; Rösel and Kunze, 1995). This gene has been used to analyse branching order in phylogenetic studies, because those phylogenies are congruent with other molecular phylogenies. Sequences from the EF 1-α gene provide more phylogenetic information than do the rRNA genes (O'Donnell *et al.*, 1998; Knutsen *et al.*, 2004). EF 1-α sequences have been used to identify species, infer phylogenetic relationships between species and to evaluate inter- and intraspecific variation (O'Donnell *et al.*, 1998; Baayen *et al.*, 2000; 2001). However, EF 1-α also can result in low resolution of relationships among and within lineages (Knutsen *et al.*, 2004).

The highly conserved β-tub gene contains more information than the rRNA genes and was proposed by O'Donnell *et al.* (1998) to study phylogenetic relationships within and between groups of *Fusarium* spp. Knutsen *et al.* (2004) however found that β-tubulin and calmodulin provide little resolution among and within lineages which suggested the use of genes which contained even more phylogenetic information for inferring of relationships within and between *Fusarium* groups. Lack of differences in the 5'-end of the nuclear 28S rDNA on phylogenetic level was observed within and between *Fusarium* groups, therefore this marker became unpopular as indicator of phylogenetic relationships (Knutsen *et al.*, 2004).

Gene genealogical studies with sequences of multiple genes, e.g. Figure 2.4 (O'Donnell *et al.*, 2000; 2004; Starkey *et al.*, 2007), have proven highly efficient in identifying phylogenetic species (Taylor *et al.*, 2000) and may be useful in delimiting taxa. The combination of multiple genes also provides better resolution of relationships within and amongst lineages in a fungal group e.g. the *F. graminearum* species complex. The number of lineages in the *F. graminearum* complex have increased from seven to

thirteen over the years as genes have been added to the genealogy (Figure 2.5) (O'Donnell *et al.*, 2000; 2004; 2008; Starkey *et al.*, 2007; Yli-Mattila *et al.*, 2009).

2.5.3 Fingerprinting techniques

The majority of molecular fungal studies conducted today rely on DNA-based genetic markers (McDonald, 1997). Different DNA-based genotyping techniques such as restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), AFLP and simple sequence repeat (SSR) have been used extensively for cultivar and isolate genotyping. These fingerprinting techniques provide information covering the entire genome of an organism, while DNA sequencing focuses on the variation within a single gene. Population genetic structure can be resolved with the application of genetic markers that are selectively neutral, informative, reproducible and easy to analyse (McDonald, 1997). DNA fingerprinting also is popular and robust for population studies of asexually reproducing fungi because it can be used to identify clonal lineages within a population (McDonald and McDermott, 1993).

2.5.3.1 Restriction fragment length polymorphism

Jeffreys *et al.* (1985) first described RFLPs, restriction enzyme-generated fragments of different length, as a molecular technique for assessing differences between and among species. This technique relies on DNA-DNA hybridisation within a genome and can detect variation in both coding and non-coding regions (McDonald and McDermott, 1993). The selectively neutral co-dominant markers produced by RFLPs identify specific genome locations and can be used to create genetic maps of chromosomes (Kohn, 1992). The use of RFLPs in studies of pathogenic fungi, have been limited primarily to mitochondrial DNA, but changed to nuclear DNA for increased polymorphisms to give better resolution and insight in these studies (McDonald, 1997).

RFLPs have been used extensively for identification and characterisation of *Fusaruim* species (Edel *et al.*, 1995; Bentley *et al.*, 1998; Bogale *et al.,* 2007; Leong *et al.,* 2009). This technique assisted in the assessment of intraspecific relationships in *F. oxysporum* Schlechtendahl emend. Snyder & Hansen, especially among pathogenic and non-pathogenic races, as well as the inference of phylogenetic relationships between *Fusarium* groups (Appel and Gordon, 1995; Konstantinova and Yli-Mattila, 2004). RFLPs have been used to analyse genetic divergence within *F. graminearum* populations, to differentiate phylogenetic lineages in this species and to resolve the genetic structure of these populations (Gale *et al.,* 2002; Mishra *et al.,* 2004; Fernando *et al.*, 2006).

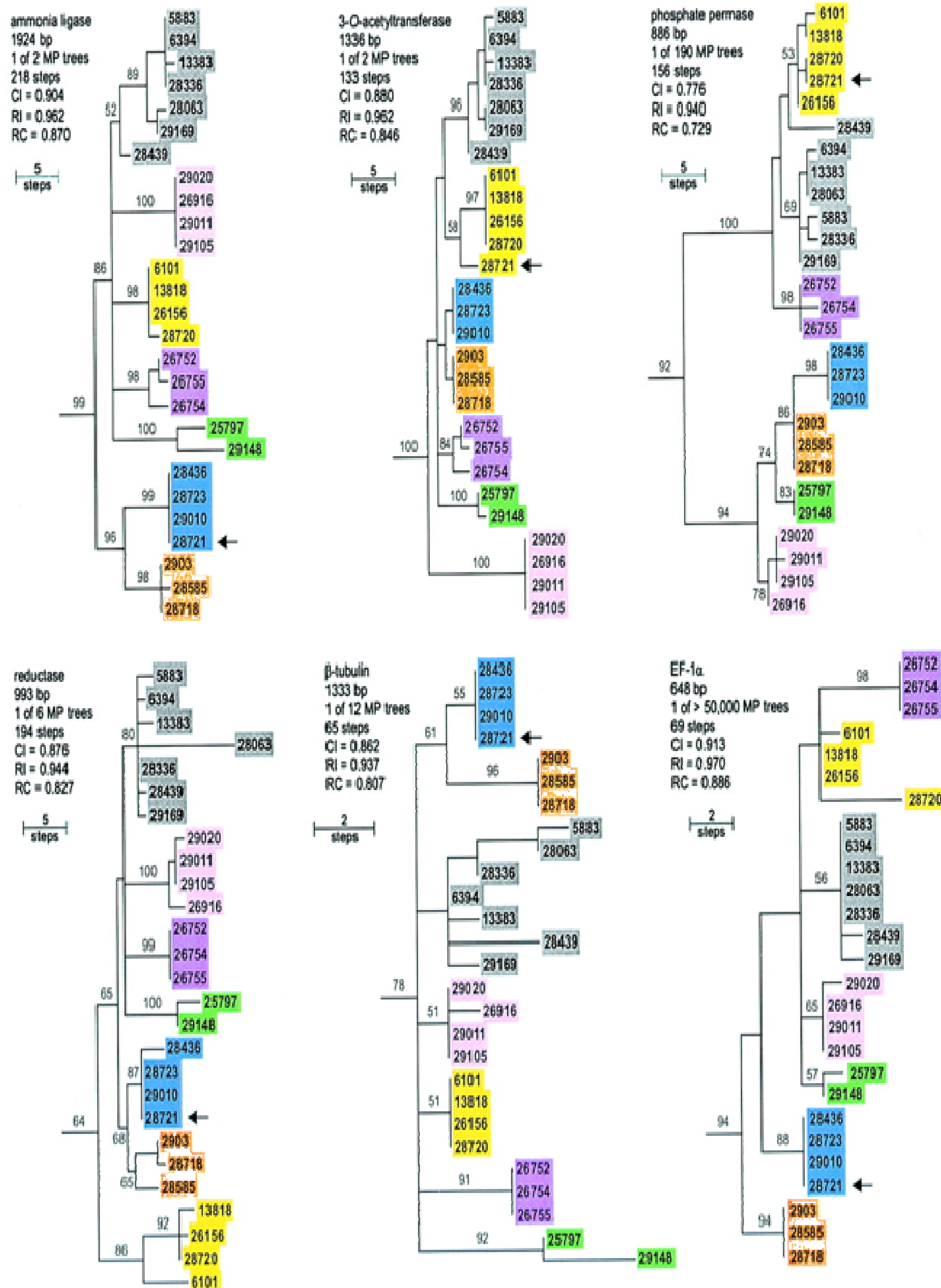

Figure 2.4 Phylogenetic trees representing the six genes which were combined to result in the first seven lineages of the *F. graminearum* clade (with permission: O'Donnell *et al.,* 2000). The coloured sections indicate the different lineages.

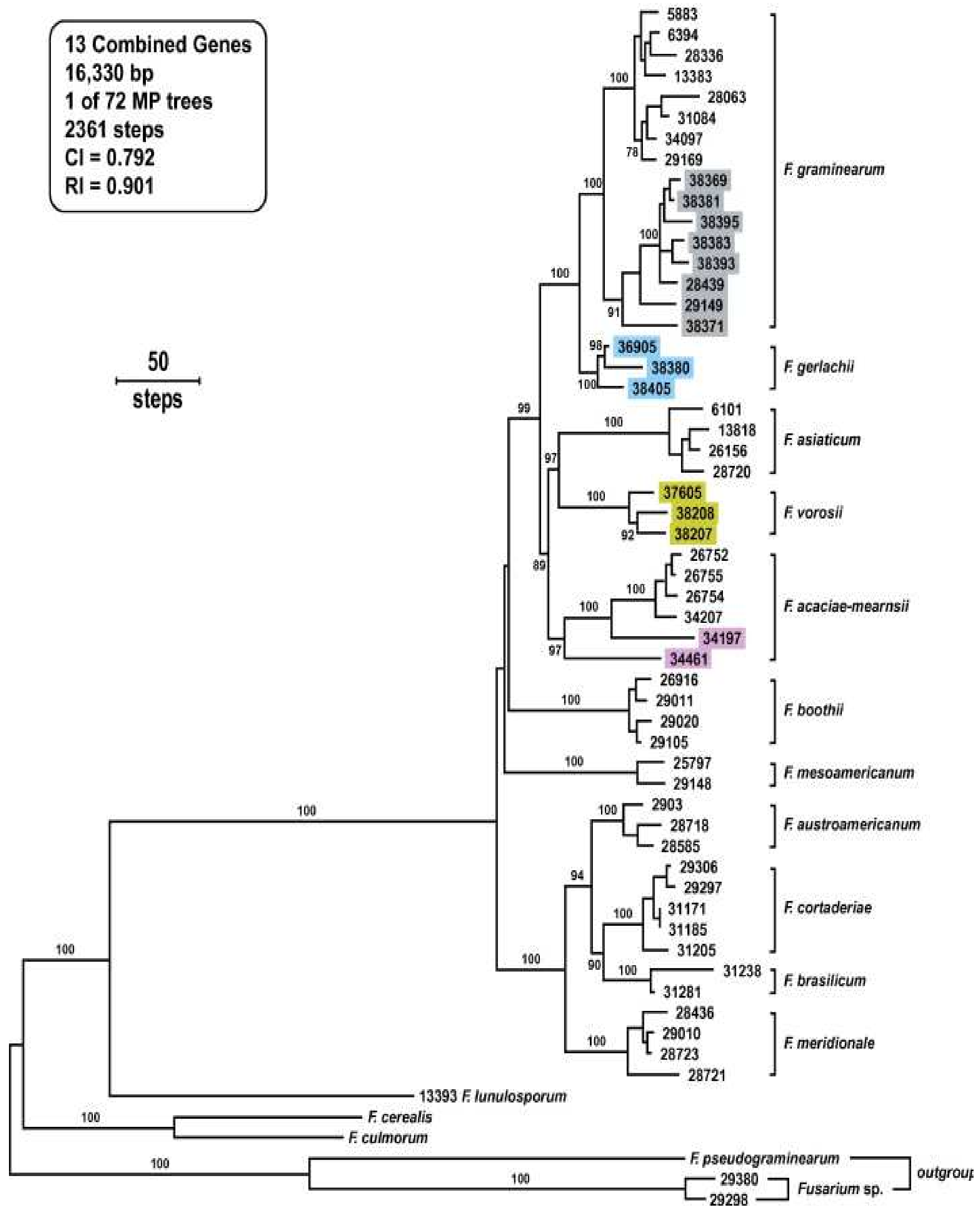

Figure 2.5 Phylogenetic trees representing eleven of the thirteen lineages in *F. graminearum* after combining 13 genes (with permission from O'Donnell: Starkey *et al.,* 2007).

RFLPs are reproducible, but application of this technique is limited due to low levels of polymorphism (McDonald and McDermott, 1993; McDonald, 1997). The requirement for large quantities of pure DNA made RFLPs a demanding, laborious and costly technique for characterising large numbers of samples. At this time most, RFLP markers are converted into polymerase chain reaction (PCR)-based markers prior to population application (Blears *et al.*, 1998; Mueller and Wolfenbarger, 1999).

2.5.3.2 Random amplified polymorphic DNA

Williams *et al.* (1990) described RAPDs as a "random" DNA polymorphism assay based on PCR with single short primers developed to assist in constructing genetic maps and for evaluating genetic diversity. This technique can be applied to any organism from which DNA can be extracted. The relatively low cost and small amount of DNA required for screening purposes increased the popularity of RAPDs being used as genetic markers (Welsh and McClelland, 1990; Williams *et al.* 1990). The number of polymorphisms detected by an individual primer might be limited due to lack of DNA sequences recognised by the primer. The inability to reproduce results between laboratories has reduced the use of RAPDs for the identification of polymorphic fragments for which more robust PCR-based markers can be developed.

RAPDs quickly replaced RFLPs as a DNA-based molecular method for identifying and characterising clones and/or clonal lineages of fungal species, and a number of studies were done on *F. graminearum* (Ouelett and Seifert, 1993; Carter *et al.,* 2000; 2002; Mishra *et al.,* 2004; Cumagun *et al.*, 2007) and other *Fusarium* spp. (Kerényi *et al.*, 1997; Belabid *et al.,* 2004; Nagarajan *et al.,* 2004). Due to prolems with experimental replication, however, the conclusions of these studies are at most confirmatory of conclusions drawn in studies that used more reliable markers.

2.5.3.3 Amplified fragment length polymorphism

AFLP is a PCR-based fingerprinting technique that can detect genomic restriction DNA fragments of any origin and complexity (Zabeau and Vos, 1993; Vos *et al.,* 1995; Blears *et al.*, 1998; Savelkoul *et al.,* 1999). Markers derived from AFLPs are distributed throughout the genome and enable the assessment of the whole genome of an organism (Vos *et al.*, 1995; Blears *et al.*, 1998; Mueller and Wolfenbarger, 1999; Meudt and Clarke, 2007). Polymorphisms detected by AFLPs in different genomic regions are

caused by mutations in the restriction sites, by insertions and deletions in the sequences between the restriction sites, and by mutations in the primer binding sites (Vos *et al.*, 1995; Blears *et al.*, 1998; Savelkoul *et al.,* 1999). AFLPs are highly reproducible, sensitive, provide resolution at the whole genome level and require no prior sequence knowledge. The application of this technique is relatively fast and inexpensive and can be easily adapted to screen a large number of individuals (Meudt and Clarke, 2007). The main disadvantage of AFLP markers is that they are dominant markers since alleles are not easily recognised and the technique does not distinguish homozygous dominant and heterozygous individuals (Majer *et al.,* 1998; Mueller and Wolfenbarger*,* 1999). Fortunately most fungi, including *F. graminearum*, are haploid and this problem is irrelevant to studies of haploid isolates.

Generating a "fingerprint" of DNA fragments can help to resolve species that are morphologically indistinguishable. Although AFLP analyses are not informative at genus or family level, the technique has broad systematic applicability and has been used effectively to determine phylogenetic relationships among individuals: bacterial (Huys *et al.*, 1996) and fungal (Majer *et al.*, 1998, Baayen *et al.,* 2000; Schmidt *et al.,* 2004; Bogale *et al.,* 2009). The potential applications of this DNA fingerprinting technique includes linkage mapping, evaluation of phylogenetic relationships amongst individuals based on assessment of genetic diversity at or below species level and characterisation of fungal plant pathogen populations, including population structure and differentiation (Baayen *et al.,* 2000; Jurgenson *et al.*, 2002; Zeller *et al.*, 2003a; Schmidt *et al.,* 2004; Bentley *et al.,* 2008; Bogale *et al.,* 2009). Intra- and interspecific genetic variation is included in the assessment of the population structure (Savelkoul *et al.*, 1999). Gene flow, dispersal, introgression and some cases of population hybridisation can be evaluated using AFLP analyses (Mueller and Wolfenbarger, 1999; Meudt and Clarke, 2007). AFLPs are regarded as a reliable, reproducible, robust method for the analysis of the genetic structure of a population and had been successfully applied for intraspecific genetic diversity analysis of *Fusarium* species (O'Donnell *et al.* 2000; 2004; 2008; Zeller *et al.*, 2003a; Schmidt *et al.,* 2004; Bentley *et al.,* 2008; Bogale *et al.,* 2009).

AFLPs have assisted in the characterisation of *F. udum* Butler (Kiprop *et al.*, 2002) as well as the identification of a new *Fusarium* species in the *G. fujikuroi* species complex (Zeller *et al.,* 2003b). This technique has also been used to evaluate and characterise

the genetic structure of *F. pseudograminearum* populations from Australia (Scott and Chakraborty, 2006; Bentley *et al.,* 2008). Genomic studies on *F. graminearum* have relied on two genetic maps, one based solely on AFLPs (Jurgenson *et al.*, 2002) and another on sequence tagged sites in addition to AFLP markers (Gale *et al.,* 2005). The most recent AFLP studies in *F. graminearum* were to evaluate the integrity of the phylogenetic lineages (Zeller *et al.*, 2003a; 2004). The investigation of the genetic variability of this FHB pathogen has also become popular to assist in resolving the population structure (Zeller *et al.*, 2003a; 2004; Schmale *et al.,* 2006; Karugia *et al.*, 2009).

2.6 Population genetics

Population genetic studies combine different, morphological and genomic, concepts and technologies in order to understand the evolutionary pathways of the individuals involved. Population structure describes the amount, distribution and patterns of genetic diversity due to geographic location, sexual and asexual reproduction ability, mutations and genetic drift between and within populations (McDonald, 1997). The genetic diversity of a population contains information on the potential of the population to evolve as well as on its past evolutionary steps. Analyses of genetic diversity of pathogen populations are necessary to evaluate control strategies. Knowledge of the population structure of a pathogen population may result in a better understanding of the epidemiology and evolutionary potential of the pathogen and could lead to improved strategies for disease control. The diversity within and between populations can be affected by population size, reproduction and gene flow of the population in question (McDonald and McDermott, 1993; McDonald, 1997). Fungal taxonomy has traditionally been based on morphological characteristics (Leslie and Summerell, 2006). Morphological traits have the disadvantage of being influenced by both genetic and environmental factors and cannot provide the most accurate information on genetic similarity. A combination of simultaneously studied genome regions can provide significant insights as part of a population genetic study (Luikart *et al.*, 2003).

Population studies of *Fusarium* have been conducted with various species at different locations. A meta analysis of the population genetics of *F. graminearum* causing FHB suggested that the relationships between populations are complex, only partially understood, and complicated by mixing, presumably due to agriculture and trade. In

some cases, studies of similar populations with similar techniques have reached contradictory conclusions (Ouelett and Seifert, 1993; Dusabenyagasani *et al*., 1999). Further studies of Canadian populations of *F. graminearum* (Mishra *et al*., 2004; Fernando *et al*., 2006; Guo *et al*., 2008) used different molecular techniques [intersimple sequence repeats (ISSR) and sequence related amplified polymorphisms (SRAPs)] to assess genetic diversity between and amongst Canadian FHB populations. Significant genetic variation was detected in these studies between isolates with evidence of inoculum migration from eastern Canada towards the western prairies (Mishra *et al*., 2004).

Genetic population studies in Asia indicated that isolates from China belonged to a homogeneous population of lineage 6 (Gale *et al*., 2002), while significant variation was observed in populations from Nepal (Carter *et al*., 2000) where it was impossible to distinguish *F. graminearum* isolates based on their host. Karugia *et al.* (2009) conducted a genetic diversity study combining phenotypic characteristics, genotypic data and tricothecene chemotypes, because these factors influence the genetic variation within and between populations. This study by Karugia *et al.* (2009) found that FHB in Japan were caused by a homogeneous population.

Fusarium graminearum populations originating from European countries (Germany, Russia, Hungary and Austria) have been evaluated with several different methods including isozymes, sequencing of significant genes, RAPDs and intergenic spacer region RFLPs (IGS-RFLPs). Gagkaeva and Yli-Mattila (2004) proposed that Asian populations of *F. graminearum* were older than the other populations evaluated in this study, which was confirmed by the related frequency of FHB resistant plant genotypes. Genetic variation within the European population in this study was attributed to pathogen aggressiveness. *F. graminearum* populations in Hungary and Austria lacked enough information to reveal the composition of these populations (Tóth *et al*., 2005). Yli-Mattila *et al.* (2009) evaluated diversity within and amongst European and Asian populations. They identified a new phylogenetic lineage, which serves as a sister lineage to lineage 6. These lineages, i.e. *F. asiaticum* and *F. vorosii,* are indigenous to Asia (Yli-Mattila *et al*., 2009).

Fusarium graminearum populations in North America show high levels of phenotypic and genotypic diversity within populations (Walker *et al.*, 2001; Zeller *et al.*, 2003a; 2004). Assessment of the genetic diversity within the North Dakota, Minnesota and Kansas populations led to the discovery of lineage 7 as being the predominant pathogen in these regions. The genetic variation within and the similarities between populations over long geographic distances indicated that these populations form part of a larger randomly mating population (Zeller *et al.*, 2003a; 2004). Therefore it can be concluded that the possibility of different disease resistant types within North American FHB populations are not due to the genetic variation in these populations (Zeller *et al.*, 2003a).

O'Donnell and co-workers suggested that the population structure of *F. graminearum* is poorly understood and subdivided *F. graminearum* into thirteen lineages based on phylogenetic characterisations, primarily based on nuclear DNA sequences. They also suggested that the oldest phylogenetic species of *F. graminearum* originated in the southern hemisphere (Tóth *et al.*, 2004; 2005). Some of the lineages are restricted to certain areas (e.g. lineage 1 to South America), while *F. graminearum,* lineage 7 occurs across several regions. Different research groups had focussed on studies investigating the genetic diversity of this pathogen in order to resolve disease management and to enhance resistance studies (Gale *et al.*, 2002; Zeller *et al.*, 2003a; Karugia *et al.*, 2009).

Although diversity and population analyses of FHB populations of *F. graminearum* have been done worldwide, no significant information is available on South African populations, although a few South African isolates were examined by O'Donnell *et al.* (2005, personal communication) for placement in lineage groups. Boshoff (1996) used isozymes to characterise isolates and assess the genetic variation of a *F. graminearum* population from South Africa. The use of isozymes to evaluate the population genetic structure of this population resulted in a significant amount of variation found within groups. No further population genetic structure reports on *F. graminearum* populations causing FHB have been made in South Africa. Reports of possible different *Fusarium* chemotype populations have been made for the USA and parts of Europe that might have established the differences in pathogen aggressiveness (Ward *et al.,* 2008; Karugia *et al.*, 2009; Yli-Mattila *et al.*, 2009).

The full extent of pathogen diversity, especially in centers of diversity and the significance of this diversity (species/lineage/chemotype) with respect to aggressiveness to hosts and toxicity to animals or humans, were identified as critical research needs during the International Maize and Wheat Improvement Centre (CIMMYT) Workshop on the Global *Fusarium* Initiative for International Collaboration (Ban *et al.,* 2006).

The objective of the current study was to combine different methods (morphological, molecular and chemotoxicity) to give insight into the population dynamics of Fusarium head blight causing species in South Africa.

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CHAPTER 3
Morphological characterisation and trichothecene genotype analysis of the Fusarium head blight population in South Africa

3.1 Introduction

FHB may be caused by several species of *Fusarium*. It is one of the most severe diseases of wheat reported worldwide, especially where moist weather occurs during the flowering period in the growing season (Bai and Shaner, 1994; McMullen *et al.*, 1997). The disease not only reduces crop yield and seed quality, but is associated with mycotoxins such as NIV, DON and ZEN, that can affect food safety (McMullen *et al.*, 1997; Desjardins, 2006; Leslie and Summerell, 2006).

Many *Fusarium* species are associated with FHB, but *F. graminearum* is regarded as the primary causal agent in most geographic regions where the disease occurs. The fungus is homothallic (Leslie and Summerell, 2006) with sexual development thought to be an important part of the disease cycle (Trail, 2009). Based on morphological characters, i.e. spore and cultural characteristics, and biological characters, i.e. cross-fertility, *F. graminearum* is a single genetically diverse, widely distributed species (Leslie and Bowden, 2008). If phylogenetic species descriptions are used, however, then *F. graminearum* can be subdivided into at least 13 phylogenetic species (O'Donnell *et al.*, 2004; Starkey *et al.*, 2007; Yli-Mattila *et al.*, 2009).

The objectives of this study were to identify the morphological species causing FHB in the irrigated wheat-grown areas of South Africa and to determine the trichothecene production genotype of the recovered isolates. It was hypothesised that 1) *F. graminearum* is the dominant FHB causal species, and 2) that one of the DON genotypes will dominate the population, as seen in wheat fields in other temperate wheat-growing regions. This study places South African agriculture in the international context for this disease and it is the first time that FHB populations from irrigated wheat have been analysed.

3.2 Materials and methods

3.2.1 Field samples

Eight-hundred-and-sixty FHB-infected wheat heads from commercial no-till spring wheat fields were collected from the main irrigated wheat-growing regions in South Africa. Samples were collected from seven different locations over two seasons (2005 and 2006) (Figure 3.1). All samples were obtained within the same pivot area at each location (Figure 3.2). There are three hierarchical levels in the sampling strategy. Level 1 represents pivot location. One-hundred-and-eighty wheat heads were collected at three of the seven locations (Prieska, Barkly West and Orania) by sampling at three different spots (sixty wheat heads per spot) in the same pivot area. Eighty wheat heads were collected at each of the other four locations since these locations do not regularly report FHB. Level 2 in the hierarchy represents the location (city or town) at which one or more of the pivots were located. Level 3 in the hierarchy represents the South African province from which the samples were taken (Figures 3.1 and 3.2). Locations were selected based on past and present histories of FHB epidemics in the main irrigated wheat-growing areas in South Africa. All locations from which FHB infected wheat heads were collected are situated within the Orange River basin, but are irrigated from different rivers; Prieska, Orania and Barkly West directly from the Orange River, Douglas and Potchefstroom district from the Vaal River, Greytown from the Tugela River and Cradock from the Great Fish River. This basin includes >64% of the irrigated cereal production area in South Africa (Lange *et al.,* 2007).

3.2.2 Identification of fungal isolates

One *Fusarium* isolate was obtained per infected wheat head. Heads were surface-sterilised by immersing seeds into a 1.25% (v/v) sodium hypochloride solution for 3 min and then rinsing three times for 30 s with sterilised distilled water. Surface-sterilised seeds were plated onto a semi-selective medium for *Fusarium* (Van Wyk *et al.*, 1986) (Appendix I) and incubated under a 12 h light/dark cycle for 4-7 days at 25℃. After 4-7 days fungal hyphae were transferred to potato dextrose agar (PDA; Appendix I) plates and incubated in a sterile growth chamber with a 12 h light/dark cycle at 25℃ for an additional 4-7 days. Fungal isolates were cultured on Spezieller Nährstoffarmer agar (SNA; Appendix I) (Nirenberg, 1976) plates until conidia formed. Cultures originating from SNA plates were purified by subculturing single spores for identification purposes following dilution plating onto fresh SNA (Leslie and Summerell, 2006).

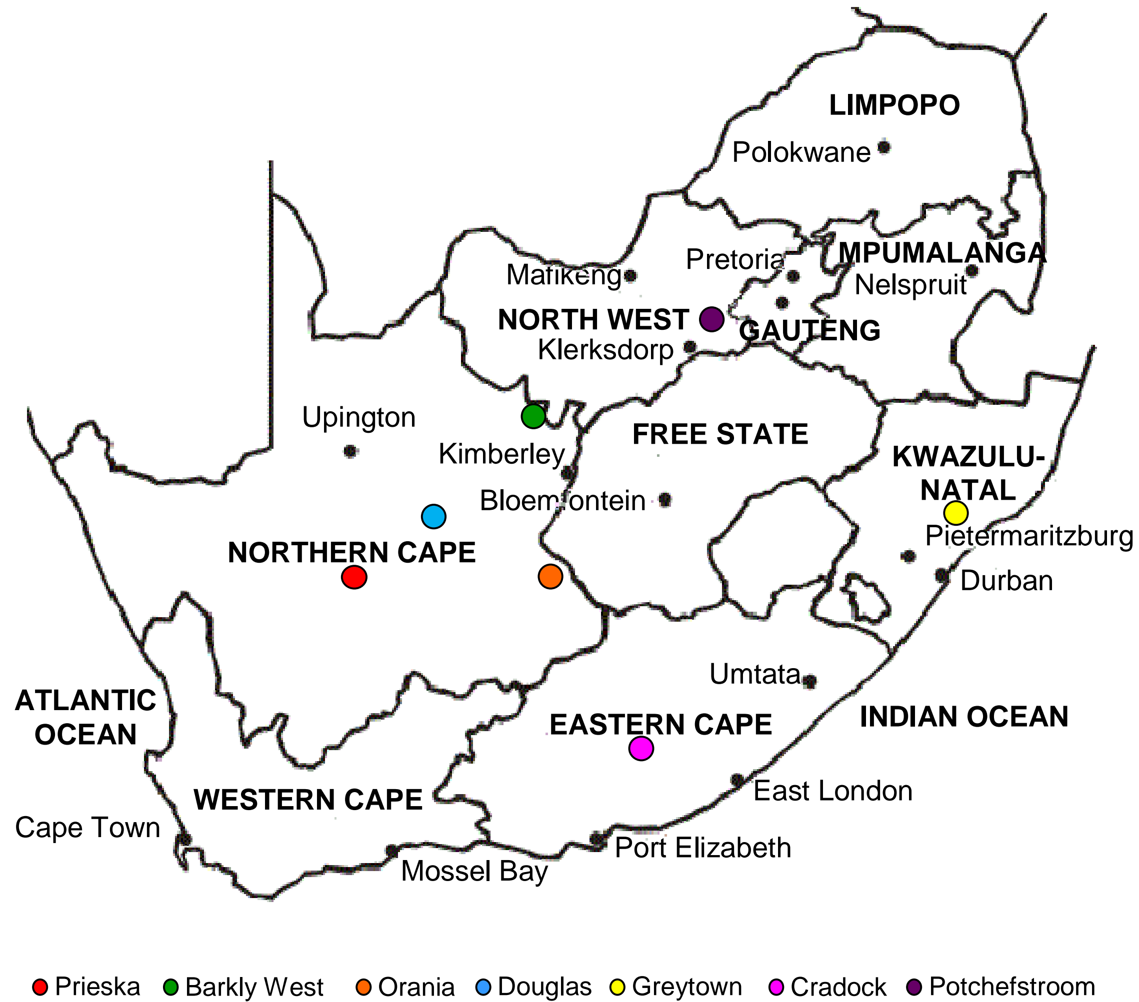
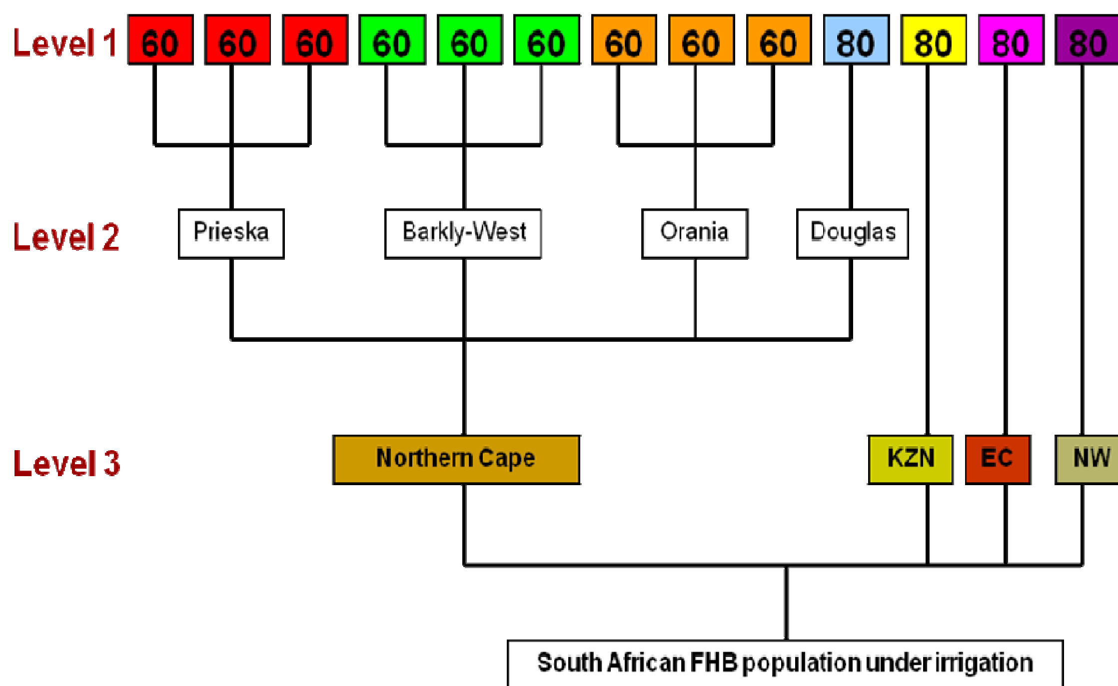


Figure 3.1 Sampling locations within South Africa's main irrigation wheat-growing areas. The map indicates South Africa's nine provinces with some of the main cities within each province.



KZN – KwaZulu Natal EC – Eastern Cape NW – North West GT – Greytown Cr – Cradock Po – Potchefstroom
FHB – Fusarium head blight

Figure 3.2 Sampling strategy for FHB samples collected from seven irrigation wheat-grown areas over four provinces in South Africa.

3.2.2.1 Single spore (purified) cultures

Dilution plating was used to produce single spore colonies. Macroconidia from sporodochia on the SNA plates were suspended in 10 ml sterile distilled water to obtain a spore suspension of 10 spores/ml. One ml of this spore suspension was used to make three successive 1:10 serial dilutions in sterile water. One ml of each of the 10^{-1}, 10^{-2} and 10^{-3} spore suspensions were spread on separate plates of water agar (WA; Appendix I). These plates were incubated overnight in the dark at 25℃. Agar blocks with a single germling were cut from the WA plates with a sterile needle at 45x magnification and transferred to PDA plates. These plates were incubated for 7-10 days with a 12 h light/dark cycle, under ultraviolet light at 25℃ before subculturing on PDA, SNA and carnation leaf agar (CLA; Appendix I) (Fisher *et al*., 1982) for identification. Morphological characterisations followed the protocol of Summerell *et al.* (2003). All macroconidia used for identification purposes were obtained from sporodochia. Morphological characterisation criteria for the identification of *Fusarium* isolates are summarised in Table 3.1.

Table 3.5 Morphological characterisation criteria for identification of Fusarium head blight isolates (Leslie and Summerell, 2006)

Species	Colony colour	Sporodochia	Macroconidia			Microconidia			Chlamydospores
	Pigment	**CLA**	**P**	**S**	**BC**	**P**	**S**	**Ph**	**P**
F. graminearum	Carmine red/ Burgundy	Orange to reddish brown	Yes	3-5 septa, slender, slightly curved	Defined foot shape	No	-	-	Yes, some in macroconidia
F. crookwellense	Carmine red	Orange to brown	Yes	Distinctive, 5 septa	Foot shaped	No	-	-	None
F. culmorum	Carmine red	Orange	Yes	Thick, short, 3-4 septa	Slight notch	No	-	-	None
F. equiseti	Brownish	Orange	Yes	5-7 septa, long, slender	Long distinct Foot shaped	No	-	-	Yes, yellowish brown
F. semitectum	Light brown	Orange	Yes	3-5 septa, slender	Foot shaped	No	Mesoconidia occur		Yes, rare
F. chlamydosporum	Yellow to brownish	Orange	Yes, rare	Typical, 3-5 septa	Notched	Yes	Straight	Poly	Yes, abundantly

CLA – Carnation leaf agar P – Present S – Shape BC – Basal cell Ph - Phialide

3.2.2.2 Deoxyribonucleic acid extraction and trichothecene genotype analysis using the polymerase chain reaction

3.2.2.2.1 DNA isolation

All isolates morphologically identified as *Fusarium* species were subcultured onto ten PDA plates per isolate. Cultures were incubated in a 12 h light/dark cycle for 7-10 days at 25°C for optimal growth. After ten days mycelia were scraped from PDA plates and transferred to a sterile tube. Mycelia were freeze-dried for three days at -60°C using a Viritis Advantage Freeze Mobile II (New York, USA). Freeze-dried samples were stored at -80°C until further use.

Before isolation of total genomic DNA, the freeze-dried fungal material was homogenised using Qiagen's TissueLyser (Haan, Germany). A piece of the material was transferred to a 2 ml microcentrifuge tube with two 5 mm stainless steel ball bearings and homogenised for 30 s at 30 r/s. Isolation of total genomic DNA was done by using a CTAB (hexadecyltrimethylammonium bromide) DNA isolation method (Saghai-Maroof *et al.*, 1984). CTAB extraction buffer with a volume of 750 µl [100 mM Tris-Cl (tris (hydroxymethyl) aminomethane), pH 8.0; 20 mM EDTA (ethylene-diaminetetraacetate), pH 8.0; 1.4 M NaCl; 2% (w/v) CTAB; 0.2% (v/v) β-mercaptho-ethanol] was added to approximately 250 µl of grounded fungal tissue and incubated at 65°C for 1 h. A volume of 500 µl chloroform:isoamylalcohol [24:1 (v/v)] was added and mixed well. Samples were centrifuged at 12000 x *g* for 5 min at 5°C. DNA from the aqueous phase was precipitated with 0.66 volumes isopropanol and incubated at room temperature (20-25°C) for 20 min. After incubation samples were centrifuged at 12000 x *g* for 5 min at 5°C. The supernatant was discarded and tubes drained upside down. The precipitate was washed by adding 500 µl ice-cold 70% (v/v) ethanol and incubated for 20 min at room temperature. Samples were centrifuged at 12000 x *g* for 5 min at 5°C.

The supernatant was discarded and the pellets were air-dried for 1 h at room temperature (20-25°C). Air-dried DNA samples were re-suspended in TE buffer (10 mM Tris-Cl, pH 8.0; 1 mM EDTA, pH 8.0) overnight at 4°C. RNase A (0.1 mg/ml) was added and incubated at 37°C for 1-2 h. DNA was precipitated with 20 µl 7.5 M ammonium acetate and an equal volume chloroform:isoamylalcohol (24:1) and centrifuged at 12000 x *g* for 5 min at 5°C. DNA from the aqueous phase was precipitated with two volumes

ice-cold 100% ethanol overnight. Samples were centrifuged at 12000 x *g* for 15 min at 5ºC and the supernatant discarded. Samples were washed twice with ice-cold 70% ethanol by centrifuging at 12000 x *g* for 10 min at 5ºC each time. The supernatant was discarded and samples air-dried. Air-dried samples were re-suspended in 50 µl TE buffer (pH 8.0) and held overnight at 4ºC. DNA quantity and quality were estimated from a 0.8% (w/v) agarose gel with electrophoresis at 80 V in UNTAN (40 mM Tris-Cl; 2 mM EDTA, pH adjusted to pH 7.4 with acetic acid) buffer. DNA was visualised with ethidium bromide staining under UV light. The concentration of the DNA samples was determined by using a UV spectrophotometer and measuring absorbance at A_{260} and A_{280}. Samples were diluted to a working concentration of 200 ng/µl for subsequent experiments.

3.2.2.2.2 Trichothecene genotypes

Fusarium graminearum, F. culmorum and *F. crookwellense* have all been associated with FHB on wheat in South Africa. *Fusarium graminearum* was indicated as the primary causal agent and can be described as cosmopolitan, while the other two species (*F. culmorum* and *F. crookwellense*) are restricted to the cooler regions of the South African wheat-growing areas. Two of the identified species (*F. graminearum* and *F. culmorum*) produce both DON and NIV, while *F. crookwellense* has been reported to produce only NIV (Desjardins, 2006).

The trichothecene genotype of the morphologically identified *Fusarium* isolates was determined following PCR amplification with PCR primer pairs *Tri7*F and *Tri7*R and *Tri13*F and*Tri13*R (Table 3.2) (Chandler *et al*., 2003). These primer pairs target the *Tri7* and *Tri13* genes in the trichothecene biosynthetic gene cluster. These genes encode proteins that convert DON to NIV (*Tri13*) and NIV to 4-acetyl-NIV (*Tri7*). The selected primer pairs can be used to identify either DON and NIV genotypes (Tri7F + Tri7R and Tri13F + Tri13R) or just one chemotype at a time (DON: *Tri7*F + *Tri7*DON and *Tri13*F + *Tri13*DONR; NIV: *Tri7*F + *Tri7*NIV and *Tri13*NIVF + *Tri13*R). Primer pair combination *Tri13* + *Tri13*R targets the *Tri13* gene and can be used to identify both genotypes in a single reaction by amplifying a DNA fragment of 1075 base pairs (bp) associated with the NIV allele and/or a 799 bp fragment associated with the DON allele. Amplified fragment sizes between 458 and 535 bp resulting from amplification with the *Tri7* + *Tri7*R primer pair amplification are associated with the DON genotype, while an amplified fragment size of 436 bp is associated with the NIV genotype (Table 3.3).

Table 3.6 Sequences of primers used to determine trichothecene genotype profiles of all *Fusarium* isolates (Chandler *et al.,* 2003)

Primer	Sequence (5'-3')
*Tri7*F	TGCGTGGCAATATCTTCTTCTA
*Tri7*R	TGTGGAAGCCGCAGA
*Tri7*DON	GTGCTAATATTGTGCTAATATTGTGC
*Tri7*NIV	GGTTCAAGTAACGTTCGACAATAG
*Tri13*F	CATCATGAGACTTGTKCRAGTTTGGG
*Tri13*R	TTGAAAGCTCCAATGTCGTG
*Tri13*NIVF	CCAAATCCGAAAACCGCAG
*Tri13*DONR	GCTAGATCGATTGTTGCATTGAG

Table 7.3 Primer pairs, PCR conditions and fragment sizes for determining trichothecene genotype profiles of all *Fusarium* isolates (Chandler *et al.,* 2003)

Assay	Forward primer	Reverse primer	PCR conditions (annealing cycle)	Size of amplified PCR fragment (bp)	
				NIV	DON
Generic *Tri7*	*Tri7* F	*Tri7*R	60°C, 30s, 35 cycles	436	458-535
*Tri7*DON	*Tri7* F	*Tri7*DON	60°C, 30s, 35 cycles	-	381-445
*Tri7*NIV	*Tri7* F	*Tri7*NIV	60°C, 30s, 35 cycles	465	-
Generic *Tri13*	*Tri13* F	*Tri13* R	58°C, 45s, 35 cycles	1075	799
*Tri13*DON	*Tri13* F	*Tri13*DONR	58°C, 45s, 35 cycles	-	282
*Tri13*NIV	*Tri13*NIVF	*Tri 13* R	58°C, 45s, 35 cycles	312	-

Each PCR amplification reaction contained 400 ng DNA, 2 mM $MgCl_2$, 1x Promega *Taq* polymerase buffer (10 mM Tris-Cl, pH 9.0; 50 mM KCl; 0.1% (v/v) Triton X-100), 200 µM of each 2'-deoxynucleotide 5'-triphosphates (dNTPs), 30 ng each of the forward and reverse primer (Integrated DNA Technologies Inc, Coralville, IA, USA) (Table 3.2) and

0.25 U GoTaq® Flexi DNA polymerase (Promega, Madison, WI, USA) in a total reaction volume of 20 µl. The optimised cycling conditions for primers used were one cycle at 94℃ for 2 min, 35 cycles of 94℃ for 30 s, 58 or 6 0℃ (depending on primer combination) for 30-45 s, 72℃ for 30 s followed by a final extension of 72℃ for 5 min and a 10℃ hold (Table 3.3) (Chandler *et al.*, 2003).

3.3 Results

3.3.1 Morphological characterisation

Based on morphological characterisations of 860 isolates, six *Fusarium* species, namely *F. chlamydosporum* (0.2%), *F. crookwellense* (1.2%), *F. culmorum* (2.1%), *F. equiseti* (2.6%), *F. graminearum* (93.2%) and *F. semitectum* (0.7%) were recovered. Three of these species, *F. crookwellense, F. culmorum* and *F. graminearum* are associated with FHB. *Fusarium graminearum* was the dominant causal agent of FHB in South Africa and was present at high frequencies at all seven locations sampled. The other two FHB-causal species, *F. crookwellense* and *F. culmorum*, were restricted to the three locations (Cradock, Greytown and Potchefstroom) in the less temperate wheat production regions. Macroconidia are the single most important morphological character in the identification of a *Fusarium* species. The morphology of this spore alone may suffice to identify an unknown *Fusarium* isolate to species level (Leslie and Summerell, 2006). The three FHB-associated species, *F. crookwellense, F. culmorum* and *F. graminearum*, can be separated on the basis of macroconidial morphology (Figure 3.3).

Common features amongst the macroconidia of these three species include: thick-walled spores, orange sporodochia and clear septations. Macroconidia of *F. crookwellense* (Figure 3.3A-C) are of intermediate length with five septa and have a basal cell with a definite foot shape. Spores of *F. culmorum* (Figure 3.3D-F) are relatively short with 3-4 septa. The apical cells of *F. culmorum* macroconidia are rounded and blunt and lack a distinct foot shape. The morphology of *F. graminearum* (Figure 3.3G-I) macroconidia differs from that of the other two FHB species. These slender spores are of medium length with a well-developed foot-shaped basal cell and 5-6 distinct septa.

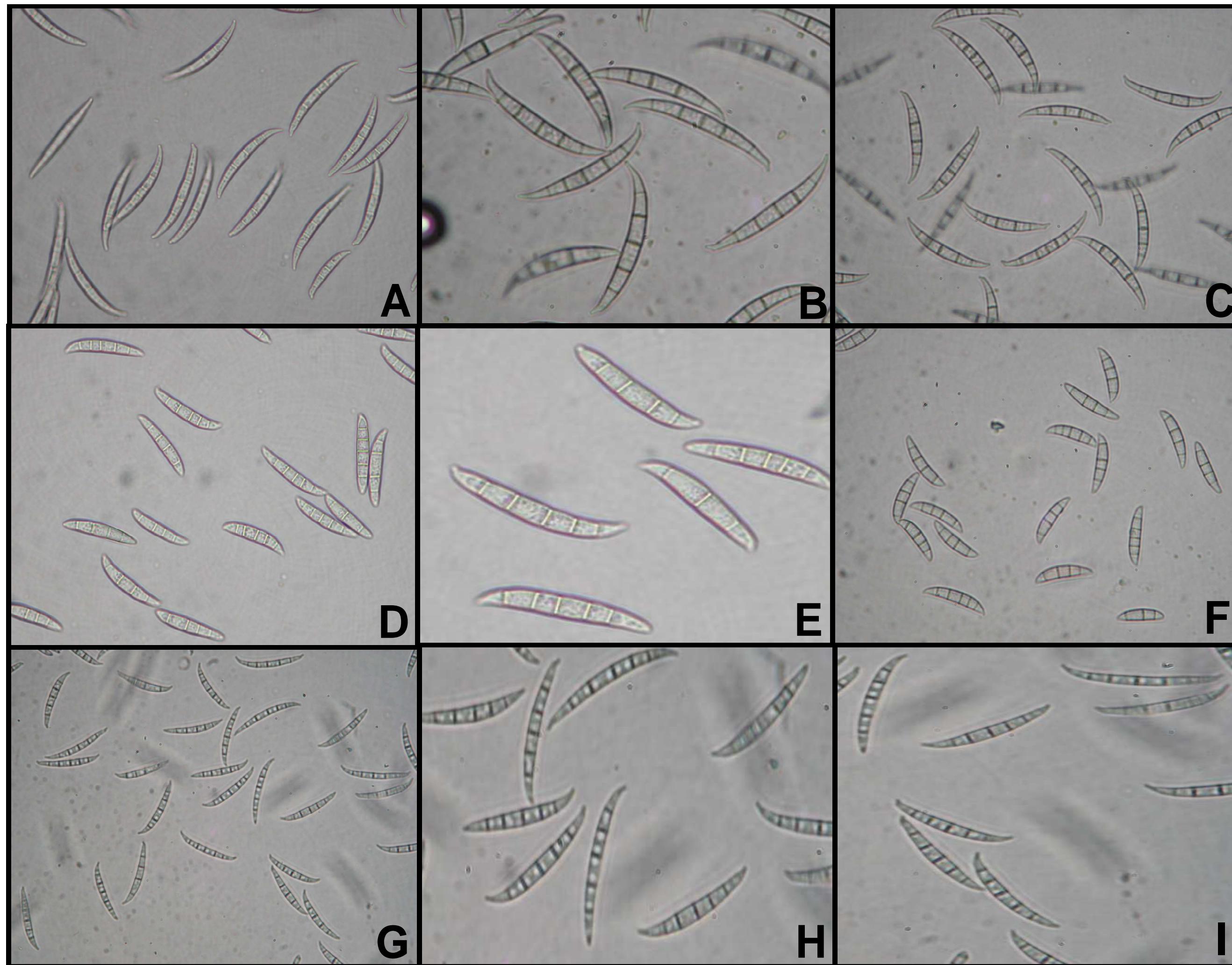


Figure 3.3 Macroconidia of *Fusarium* head blight causal species (A-C: *F. crookwellense* , D-F: *F. culmorum*, G-I: *F. graminearum*) identified in South Africa. A, C, D, F, G, I: Scale = 20 µm; B, E, H: Scale = 40 µm.

Fusarium graminearum was detected in high frequencies (Table 3.4) at all locations sampled. The highest percentage (6%) of *F. equiseti* [often regarded as a saprophyte, but recovered in low frequencies from FHB infected grains in some European countries and Australia (Bottalico and Perrone, 2002; Akinsanmi *et al*., 2004; Ioos *et al*., 2004)], was identified in the Barkly West field population and it occurred at four of the seven locations sampled. Barkly West was the only location at which *F. chlamydosporum* (1%) was recovered. The composition of *Fusarium* species for the field populations at Prieska, Orania and Douglas were similar with only two species detected. The Prieska field population had the highest percentage (99%) of *F. graminearum* isolates.

Four *Fusarium* species, *F. graminearum*, *F. culmorum*, *F. crookwellense* and *F. semitectum* were detected at all the less temperate locations, namely Greytown, Potchefstroom and Cradock. The Greytown field population had the highest number of morphospecies present as well as the highest observed frequencies for *F. crookwellense* and *F. semitectum* (Table 3.4). The Greytown and Potchefstroom field populations had the lowest percentage of *F. graminearum* (both 84%) isolates. The Potchefstroom field population contained the highest frequency of *F. culmorum* (11%) isolates. The lowest frequencies of *F. crookwellense* and *F. semitectum* were observed in the Cradock field population, but this population contained the second highest frequency of *F. culmorum* (8%) (Table 3.4).

Table 3.8 Frequency (%) of *Fusarium* species detected at locations sampled

	F. chlamydosporum	*F. crookwellense*	*F. culmorum*	*F. equiseti*	*F. graminearum*	*F. semitectum*
Prieska	-	-	-	1	99	-
Barkly West	1	-	-	6	93	-
Orania	-	-	-	3	97	-
Douglas	-	-	-	5	95	-
Greytown	-	9	4	-	84	3
Potchefstroom	-	3	11	-	84	2
Cradock	-	1	8	-	90	1

- Indicates the absence of that species at a specific location.

3.3.2 Trichothecene genotype analysis

All isolates identified as *Fusarium* spp. were tested, by means of PCR, for the genotypic potential to produce either DON or NIV, the two mycotoxins associated with FHB contamination of cereal grains. The frequency of isolates with the DON genotype varied by location (Table 3.5) and ranged from 56% in the population at Potchefstroom to 98% in the population at Prieska. All isolates at Prieska, Orania and Douglas had either the DON or the NIV genotype. At the other four locations - Barkly West, Greytown, Cradock and Potchefstroom - between 1% and 4% of the isolates had neither the DON nor the NIV genotype. These were either *F. chlamydosporum* or *F. semitectum* isolates. Most (89%) *F. graminearum* isolates had the DON genotype, while *F. equiseti* isolates were ~ 50:50 DON:NIV. Slightly more than half (56%) of the *F. culmorum* isolates had the NIV genotype, while all of *F. crookwellense* isolates had the NIV genotype.

The trichothecene genotype potential profile of isolates from Prieska indicated that these isolates had a higher DON producing potential (98%) than NIV producing potential (2%) (Table 3.5). Only two *Fusarium* species were identified in this field population and isolates of both species (Table 3.6) mostly had the DON genotype (99% for *F. graminearum* and 100% for *F. equiseti*). The potential for DON production for this location was the highest of the seven locations.

Table 3.5 Frequency of DON and NIV genotypes present in different field populations

Location	%DON genotype	%NIV genotype	No genotype identified
Prieska	98	2	0
Barkly West	86	13	1
Orania	89	11	0
Douglas	90	10	0
Greytown	71	25	4
Cradock	84	15	1
Potchefstroom	56	41	3
Total %	85	14	1

DON – Deoxynivalenol NIV – Nivalenol.

The Barkly West trichothecene genotype potential profile indicated a high DON producing potential (86%) for this field population (Table 3.5). *Fusarium chlamydosporum*, which was detected only in this field population, showed no genetic potential to produce these mycotoxins, while 89% of the *F. graminearum* isolates had the DON genotype. Most members of the third species, *F. equiseti* carried the NIV genotype (60%) (Table 3.6). This was the only location at which the NIV genotype was more common than the DON genotype amongst the *F. equiseti* isolates. The Orania population had the third highest frequency (89%) of the DON genotype (Table 3.5) and both *Fusarium* species identified at this location had a higher DON than NIV producing potential (Table 3.6). The DON genotype at Douglas, the fourth location in the Northern Cape Province, was recorded as the second highest (90%) (Table 3.5). Both *Fusarium* species identified in this field population had higher DON than NIV production potential (Table 3.6).

The Greytown, Potchefstroom and Cradock trichothecene genotype potential profiles indicated high potential (71%, 56% and 84% respectively) (Table 3.5) towards DON production in these field populations. *Fusarium semitectum* isolates from these fields did not carry a detectable trichothecene genotype, while *F. crookwellense* showed 100% NIV production potential at all three locations (Table 3.6). The 100% NIV production potential of *F. crookwellense* confirmed studies that suggested that this pathogen is a potential NIV producer but not a potential DON producer (Desjardins, 2006). *Fusarium graminearum* isolates detected in these populations had a higher DON than NIV potential, while *F. culmorum* only indicated a higher NIV than DON potential in the Greytown and Potchefstroom field populations.

The trichothecene genotype for the entire population indicated that 85% of the isolates had the DON genotype and only 14% of the isolates had the NIV genotype (Table 3.5). There were more isolates with the DON genotype (89%) amongst the *Fusarium graminearum* isolates than there were isolates with the NIV genotype (11%). All *F. crookwellense* isolates had the NIV genotype, while the *F. equiseti* isolates split roughly equally between DON and NIV genotypes. *Fusarium culmorum* isolates tended to have a slightly higher frequency of the NIV genotype (56%) (Figure 3.4).

Table 3.6 Frequency of DON and NIV genotypes present in the different *Fusarium* species present in the different field populations

	Number of isolates	% DON	% NIV
Prieska	**180**	**98**	**2**
F. graminearum	178	99	1
F. equiseti	2	100	0
Barkly West	**180**	**86**	**13**
F. graminearum	168	89	11
F. equiseti	10	40	60
F. chlamydosporum	2	0	0
Orania	**180**	**89**	**11**
F. graminearum	174	90	10
F. equiseti	6	67	33
Douglas	**80**	**90**	**10**
F. graminearum	76	91	9
F. equiseti	4	75	25
Greytown	**80**	**71**	**25**
F. graminearum	67	84	16
F. crookwellense	7	0	100
F. culmorum	3	33	67
F. semitectum	3	0	0
Cradock	**80**	**84**	**15**
F. graminearum	72	88	12
F. crookwellense	1	0	100
F. culmorum	6	67	33
F. semitectum	1	0	0
Potchefstroom	**80**	**56**	**41**
F. graminearum	67	63	37
F. crookwellense	2	0	100
F. culmorum	9	67	33
F. semitectum	2	0	0

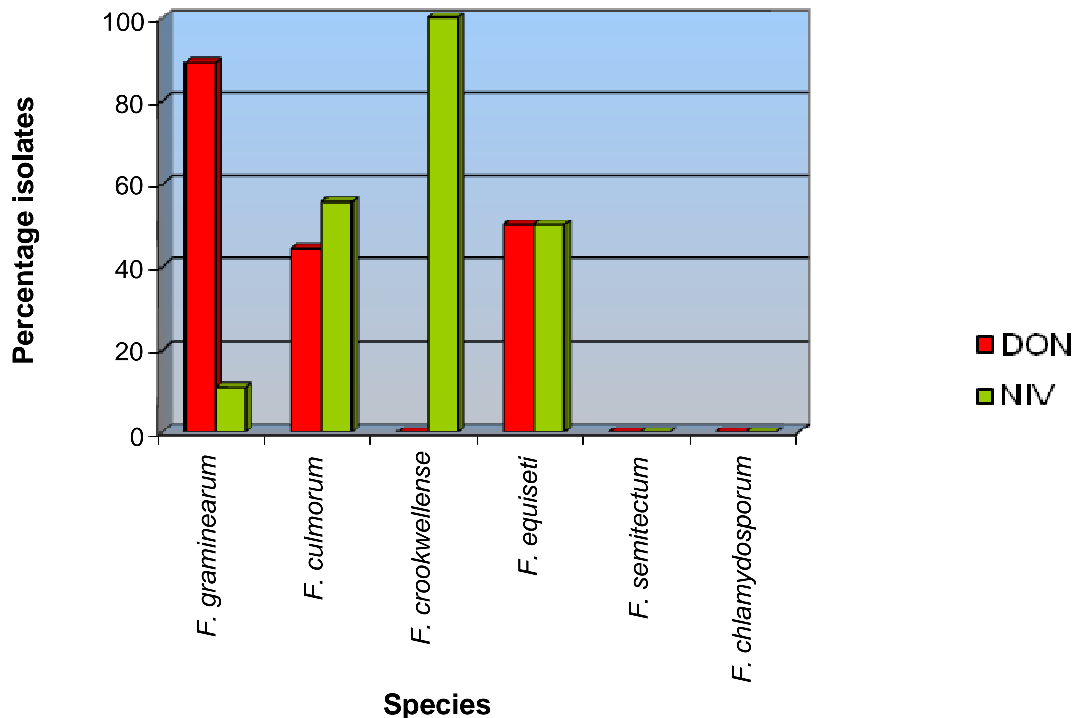


Figure 3.4 Potential trichothecene genotype profiles of each *Fusarium* species by location.

3.4 Discussion

FHB was described as epidemic on farms along the Orange River in the Northern Cape in the early 1990s where wheat was grown under centre-pivot irrigation (Boshoff, 1996). Four of the seven locations (Prieska, Barkly West, Orania and Douglas) where FHB infected wheat heads were collected for this study are situated within the irrigation areas of the Northern Cape. In 1991, Prieska was one of the first locations in the Northern Cape where FHB was detected on wheat under centre-pivot irrigation (Boshoff *et al.*, 1999). After 1991 numerous FHB epidemics were reported in the Northern Cape irrigation scheme on commercial wheat fields along the Orange River, including Barkly West, Orania and Douglas. Factors that contributed to these FHB epidemic outbreaks include tillage practices and crop rotation with maize as the summer crop and wheat as the winter crop.

The first FHB report for South Africa was in 1980 in the North West Province along the Vaal River (Scott *et al.*, 1988). The farm, from which infected wheat heads were sampled in the current study in the Potchefstroom district in the North West Province, also is situated along the Vaal River. However, 2006 was the first time since the report in 1980 that FHB was detected in this area. Epidemic outbreaks of FHB in KwaZulu Natal were first reported in the 1985/86 seasons and included Greytown, one of the locations included in this study. The most severe outbreaks occurred near Winterton, an area usually under centre-pivot irrigation with wheat in rotation with maize in what is traditionally a maize production area. After the first report in the 1985/86 season, follow-up reports on FHB outbreaks in the Greytown area began again in the 2004 season and reached epidemic status during the 2006 season. Cradock, a town in the Eastern Cape province of South Africa, has many irrigated wheat production farms in the district where irrigation is fed from the Great Fish River. In 2006 wheat fields in the Cradock district were plagued for the first time with FHB.

Our data are consistent with previous reports that *F. graminearum* is responsible for most FHB outbreaks in South Africa (Scott *et al.,* 1988; Boshoff, 1996). The present study evaluated more isolates from more locations than previous studies, and, more importantly, covers the bulk of the wheat-growing regions under centre-pivot irrigation in South Africa. Three of the six identified species, *F. crookwellense, F. culmorum* and *F. graminearum,* are associated with FHB in South Africa. *Fusarium graminearum, F. culmorum, F. avenaceum* and *F. equiseti* are all *Fusarium* species associated with FHB (Sutton, 1982; Scott *et al*., 1988; Snijders and Perkowski, 1990) and have been recovered from grain samples worldwide (Parry *et al*., 1995; Kosiak *et al*., 2003; Muthomi *et al.,* 2008). No previous reports of *F. equiseti* being associated with FHB in South Africa have been made, making this study the first official report of *F. equiseti* associated with FHB. This fungus was found only in temperate regions along with the dominant FHB causal pathogen, *F. graminearum*.

The distribution of *Fusarium* species varied by location. Four locations - Prieska, Barkly West, Orania and Douglas - have a history of FHB epidemics since the early 1990s. The wheat-growing areas of this region are all under centre-pivot irrigation and most of the farms, including the ones where samples for this study were collected, maintain a double-cropping rotation system as part of their production practices. The crop rotation

system in these traditional maize fields probably contributes to the observed high disease incidence. These no-till fields are hypothesised to provide the primary inoculum for FHB under centre-pivot irrigation, with warm day temperatures and somewhat cooler night temperatures encouraging sexual reproduction (Boshoff, 1996).

The FHB population from the main irrigated wheat-growing areas in South Africa consisted mostly (93%) of *F. graminearum*. Two other *Fusarium* species, i.e., *F. crookwellense* and *F. culmorum*, were associated with FHB, but were restricted to the less temperate regions of South Africa. These results are consistent with previous reports that *F. graminearum* dominates in temperate, humid areas of the world (Boshoff, 1996; Vigier *et al*., 1997) and that *F. culmorum* and *F. crookwellense* occupy the corresponding niche in cooler regions (Parry *et al*., 1995). In the current study, *F. graminearum* dominated at all seven locations. This suggested a shift in the composition of *Fusarium* spp. causing FHB, especially in the less temperate irrigated regions of South Africa. An increase in the frequency of *F. graminearum* relative to *F. culmorum* also has been reported for several European countries (Waalwjik *et al*., 2003; Brennan *et al*., 2005) and has been attributed to increased maize production.

The high percentage of *F. graminearum* isolates at all seven locations in the present study may be attributed to no-till tillage practices increasing FHB incidence and severity. The removal, destruction or burial of previously infected crop residues might reduce FHB as well as mycotoxin production. Another factor that probably is important in the increased presence of *F. graminearum* in these field populations is rotation with wheat in traditional maize fields. *Fusarium graminearum* is also a major pathogen of maize and can survive on maize stubble. Together, no-till and rotation with maize synergistically creates conditions that contribute to increased levels of *F. graminearum.* Inoculum pressure also contributes to the dominance of *F. graminearum* in these regions. This fungus can produce large numbers of ascospores on crop debris exposed to light and given sufficient moisture. Further complicating potential control, *F. graminearum* spores may be transported for long distances, as aerial masses (Schmale *et al.,* 2006). This massive spore dispersal requires regional changes in tillage and crop rotation practices for significant disease control to occur.

Gilbert and Tekauz (2000) reported that weather conditions have a bigger influence on FHB occurrence than tillage practices. Waalwjik *et al.* (2003) suggested that global warming and associated temperature changes in previously less temperate regions of Europe contribute to the major changes in the distribution of *Fusarium* spp. within the FHB complex. The possibility of temperature changes in the less temperate wheat-growing regions of South Africa, where *F. crookwellense* and *F. culmorum* were initially reported as dominant FHB causing species (Boshoff, 1996), need to be evaluated and determine if the increase of *F. graminearum* in these regions has a similar basis as that proposed for the changes observed in Europe.

FHB epidemics are associated with mycotoxin accumulation, which can influence the pathogenicity of the FHB pathogens and have a detrimental effect on food safety and security. Thus, the trichothecene genotypes present in the FHB population in South Africa was determined. A 19-kb gene-cluster that encodes most of the genes required for trichothecene biosynthesis has been identified and sequenced (Brown *et al.,* 2001; Ward *et al.,* 2002; Desjardins, 2006). This sequence was used to develop allele-specific PCR assays for mycotoxin production (Lee *et al.,* 2001; 2002). The *Tri7* and *Tri13* genes within the cluster have been targeted for assays that distinguish isolates that produce DON from those that produce NIV (Chandler *et al.*, 2003). The DON genotype (85%) dominated in South African populations. Both the DON and NIV genotypes co-occur in Europe and Asia (Desjardins, 2006). The NIV genotype probably is ancestral to the DON genotype (Desjardins, 2006), but the DON producing genotype is more common in isolates from wheat (Edwards, 2004) than the NIV genotype, which had been reported as more pathogenic on maize (Carter *et al.*, 2000). NIV, however, is more frequent in European grains than DON (Bottalico and Perrone, 2002; Desjardins, 2006). NIV is viewed as a more serious threat to human and animal health, whereas DON is important in the host plant/pathogen interaction in cereal grains (Desjardins *et al.,* 1996; Brown *et al.,* 2001; Champeil *et al.,* 2004).

Most *F. graminearum* isolates (89%) at all locations had the DON genotype. Presumably the higher frequency of the DON genotype indicates that *F. graminearum* is the primary cause of FHB and is more aggressive than the other *Fusarium* species associated with FHB (Desjardins *et al.,* 1996; Foroud and Eudes, 2009). The pathogenic aggressiveness of twenty isolates from the Prieska population was tested in a

preliminary study and isolates were all highly aggressive (De Villiers, 2009). All of these isolates had the DON genotype. These preliminary pathogenicity results along with the dominance of the DON genotype in the population are consistent with a physiological correlation between mycotoxin production and pathogen aggressiveness.

Results obtained in this study confirmed the first hypothesis that *F. graminearum* is the dominant causal species of FHB in South African wheat fields under centre-pivot irrigation. A first report of the presence of *F. equiseti* amongst FHB-associated *Fusarium* spp. in South Africa also was made. The second hypothesis i.e. that one of the toxin genotypes dominates in the South African FHB population, also was confirmed by results in this study. The DON genotype dominated in each individual population and in the population as a whole. DON production has previously been reported as a significant factor contributing to the pathogen's aggressiveness, a hypothesis consistent with the South African FHB population being composed of highly virulent isolates of several *Fusarium* spp. The additional aggressiveness provided by the DON genotype might be one reason why *F. graminearum* dominated at all sampled locations. Most of the *F. graminearum* isolates at all locations had the DON genotype, which is consistent with the hypothesis that isolates with the DON genotype are more aggressive pathogens than those with the NIV genotype. The enhanced pathogen aggressiveness of *F. graminearum* isolates in addition to the potential climate change associated temperature changes together might be responsible for this pathogen now dominating in the temperate wheat-growing regions under centre-pivot irrigation in South Africa.

Obst *et al.* (2002) suggested that inoculum pressure was one of the risk factors for FHB severity. Factors that contribute to inoculum pressure include both inoculum density and inoculum dispersal. Unfavourable conditions for the dispersal and development of inoculum in South Africa might be the reason for the 20 year gap between FHB reports in some regions. Along with the dispersal of *Fusarium* inoculum, favourable environmental conditions are still necessary to enable the development and spread of the disease. This may have contributed towards the high percentages of *F. graminearum* present at all of the locations, along with temperature changes that could have been experienced in the previously less temperate wheat-growing areas of South Africa.

Another factor that contributes towards inoculum pressure is airborne inoculum. Airborne inoculum sources have been evaluated (Schaafsma *et al.,* 2005; Schmale *et al.,* 2005; 2006), primarily with wind as dispersal factor of macroconidia (Gilbert and Tekauz, 2000). Wind-driven gradients in small scale experiments suggested that FHB could be primarily distributed via wind-dispersed spores. Schmale *et al.* (2005; 2006) suggested aerial inoculum and dispersal by means of water splash. Aerial dispersal could account for the spread of *F. graminearum* through the irrigated regions of South Africa sampled in this study. Dispersal through the Orange River Basin water systems also is possible, but the spread of *F. graminearum* through river water over long distances, has neither been documented nor tested.

3.5 Concluding remarks

At present wheat prices are low and production costs are high in South Africa (Genis, 2010) and many farmers are planting less wheat. More wheat was imported into rather than exported from South Africa for the first time in 2011 (SABC News, April 2011). Weather conditions and natural disasters are the main contributing factors to this situation, but diseases, especially FHB, cannot be excluded as contributing factors. FHB reduces both yield and grain quality and provides an additional economic disincentive for planting wheat. Information from the present study could help improve resistance breeding strategies against FHB, and provide an incentive to farmers to plant wheat. Recommendations of disease management strategies will help reduce FHB risk factors, and could include changes in tillage practices and control strategies.

Altered tillage practices might include removal or destruction of previously infected crop debris and the inversion of top soil deep enough to minimise the possibility of inoculum build-up. Finally, crop rotation and agronomic practices for each potential wheat field need to be considered in many of the irrigated wheat-growing regions. Graminaceous crops, e.g. maize and barley, are excellent hosts for the development of inoculum. Rotation with a broad leaf crop e.g. soybeans, should reduce the amount of *F. graminearum* inoculum in the wheat fields. Decreasing of inoculum potential in wheat fields also should reduce mycotoxin contamination. Other FHB control strategies include chemical, biological, weed and insect control. Unfortunately South Africa does not have any registered chemical or biological control measures in place.

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CHAPTER 4
Genetic variation in Fusarium head blight causin g species in South Africa

4.1 Introduction

A population is a group of individuals (isolates) that share a common gene pool and originate from a limited geographic area. As these individuals adapt to their local conditions, their gene pool changes. The goal of population genetic studies is to describe the changes, determine their causes, and understand their consequences for the individuals, population and community they are part of (McDonald and McDermott, 1993; McDonald, 1997).

The genetic structure of a population depends on the level and distribution of genetic variation within and amongst populations of the same organism. The structure reflects the evolutionary history and the evolutionary potential of the population. Factors that might influence genetic structure include mutation, mating system, gene flow (migration), population size and the ability to adapt to changes in the environment. Environmental changes that can affect the genetic structure of fungal populations include host availability e.g. resistant varieties, temperature, geographic site, timing, frequency and size of fungicide and fertiliser applications, irrigation, and crop rotation. Due to these influential factors the genetic structure of a population is not always reflected in the geographical distribution of individuals involved (Evanno *et al.,* 2005; Xu, 2006). Understanding of the genetic structure of a fungal pathogen's population assists in the evaluation and improvement of disease control strategies (McDonald and McDermott, 1993; McDonald, 1997).

Fusarium graminearum is the primary causal agent of FHB on wheat worldwide. This pathogen has been described by some researchers as a species complex with at least thirteen phylogenetic lineages/species (O'Donnell *et al.*, 2000; 2004; 2008; Starkey *et al.*, 2007; Yli-Mattila *et al.*, 2009), or by other research groups as a single species based on the biological species concept (Bowden and Leslie, 1992; Leslie and Bowden, 2008; Desjardins and Proctor, 2011). These contrasting views indicate that the population genetic structure of *F. graminearum* is important and that local isolation and adaptation

have occurred. Different research groups have focussed on studies investigating the genetic diversity of this pathogen in order to resolve disease management and enhance resistance studies (Gale *et al*., 2002; Zeller *et al*., 2003; Guo *et al.,* 2008; Karugia *et al*., 2009; Lee *et al.,* 2009; Desjardins and Proctor, 2011).

Population studies have been conducted to give insight into the genetic structure of FHB populations in Asia (Carter *et al*., 2000; Gale *et al*., 2002; Gagkaeva and Yli-Mattila, 2004; Karugia *et al*., 2009; Yli-Mattila *et al*., 2009) and Europe (Tóth *et al*., 2005; Yli-Mattila *et al*., 2009). The Asian FHB population indicated high genetic variation, where almost every isolate had a different genotype. Previously with the examination of one province in China, the Asian population was indicated to be homogeneous, with only lineage 6 isolates present (Gale *et al*., 2002). Later and with more isolates tested across Asia, the presence of lineage 7 isolates was detected. The lineage 6 isolates present, occurred mainly in the warmer regions, with an average annual temperature above 15℃, while the lineage 7 isolates were more promin ent in the cooler regions with an average annual temperature below 15℃ (Qu *et al.,* 2008). The Asian population structure is described to be related to crop rotation. Chemotypes detected in the Asian population affected the population ecology and was responsible for population subdivision (Karugia *et al*., 2009).

The European FHB population consisted of a variation of FHB causal species. Waalwjik *et al.* (2003) suggested major changes in these species across Europe. *Fusarium graminearum* had become the dominant species and with this increase had replaced other FHB species e.g. *F. culmorum* and *F. crookwellense.* The *F. graminearum* population causing FHB in this region indicated high genetic diversity. Variation in pathogen aggressiveness had also been detected in the population genetic structure studies (Gagkaeva and Yli-Mattila, 2004). The *F. graminearum* isolates detected in these population studies belonged to lineages 3, 4, 6 and 7 (Tóth *et al*., 2005; Miedaner *et al.,* 2008). Most of these populations primarily reproduce clonally. These clones disperse over long distances by ascospores (Tóth *et al*., 2005).

Fusarium graminearum isolates from Canada have been characterised with RAPDs, SRAPs and ISSRs. Studies directed by Ouelett and Seifert (1993) indicated a limited amount of genetic diversity amongst isolates, which could be explained by the limited

number of primers used and the small population sample screened. Further research (Dusabenyagasani *et al*., 1999; Mishra *et al*., 2004; Fernando *et al*., 2006; Guo *et al*., 2008) to determine the genetic diversity between and amongst Canadian FHB populations detected diversity within rather than amongst populations. These investigations indicated that the *F. graminearum* populations from Canada belonged to lineages 4 and 7.

Lineage 7 dominates *F. graminearum* populations in North America. The low levels of genetic variation within these populations were regarded as an indication of a larger homogeneous interbreeding population (Zeller *et al*., 2003; 2004). Although the genetic structure of *F. graminearum* populations causing FHB have been analysed on a global scale, only limited research has been done on FHB causal pathogens in South Africa. The only available data are limited to a set of 73 *F. graminearum* isolates that were evaluated for isozyme polymorphisms (Boshoff, 1996). This study identified a significant level of variation within this set of isolates. However, this study did not assess the characters that have been used more recently to assign isolates to lineages. A few South African isolates have been examined by O'Donnell *et al.* (2005, personal communication) and placed in lineages 3 and 5.

The objective of this study was to determine the genetic structure of *F. graminearum* populations causing FHB in the irrigated wheat regions of South Africa using AFLP analyses. The hypothesis was that South African isolates formed part of lineage 3 and 5 and that lineage 3 and 5 were the only lineages present in South Africa.

4.2 Materials and methods

4.2.1 Field samples

Eight-hundred-and-sixty FHB-infected wheat heads were collected from fields in seven different locations over two seasons (2005 and 2006) in the irrigated wheat regions of South Africa using a hierarchical sampling design described in Chapter 3, section 3.2.1. All samples from a location were obtained within the same pivot area. One-hundred-and-eighty wheat heads were collected at three of the seven locations (Prieska, Barkly West and Orania). Eighty wheat heads were collected at each of the other four locations (Douglas, Cradock, Greytown and Potchefstroom) as FHB reports are not received on a

regular basis in these locations. Locations were selected based on past and present histories of FHB epidemics.

4.2.2 Purification of cultures and DNA isolation

Single spore subcultures and DNA isolation were done as described in Chapter 3 (sections 3.2.2.1 and 3.2.2.2.1).

4.2.3 Isolates used for genetic diversity assessment

Three species, *F. graminearum, F. culmorum* and *F. crookwellense,* were morphologically identified as FHB casual species on wheat in South Africa (Chapter 3). *Fusarium culmorum* and *F. crookwellense* occurred at low percentages and only in the less temperate regions of the South African irrigated wheat-grown areas with the highest average summer temperatures ranging between 25-30℃. *F. graminearum* as the dominant casual agent of FHB in South Africa occurred at high levels across all seven sample sites. The identity of these 793 isolates as *F. graminearum* was confirmed by using a *F. graminearum* specific PCR assay developed by Nicholson *et al.* (1998). The Fg16F and Fg16R primer pair combination (Table 4.1) was used to amplify a 410 bp fragment associated with *F. graminearum*.

Each PCR amplification reaction contained 400 ng DNA, 2 mM $MgCl_2$, 1x Promega *Taq* polymerase buffer, 200 µM of each dNTP, 30 ng each of the forward and reverse primer (Integrated DNA technologies) (Table 4.1), and 0.25 U Go*Taq*® Flexi DNA polymerase (Promega) in a total reaction volume of 20 µl. The optimised cycling conditions for the primers used were: 94℃ for 2 min, 35 cycles of 94° C for 30 s, 62℃ for 45 s and 72℃ for 30 s followed by a final extension of 72℃ for 5 min and a 10℃ hold (Nicholson *et al.,* 1998).

Table 4.1 Sequences of primers used to characterise *F. graminearum* isolates (Nicholson *et al.,* 1998)

Primer	Sequence (5'-3')
Fg16F	CTCCGGATATGTTGCGTCAA
Fg16R	GGTAGGTATCCGACATGGCAA

PCR products were separated by electrophoresis through a 2% (w/v) agarose gel at 80 V in UNTAN buffer. DNA was visualised by using ethidium bromide staining under UV light. Samples were compared to the *F. graminearum* reference isolates (Table 4.2), which were used as standards in this experiment, and fragment sizes were determined by comparison with a Lamba/*Eco*RI/*Hin*dIII DNA marker (Promega).

A total of 793 isolates were identified, based on both morphology and PCR-specific amplification, as *F. graminearum* and included in the population genetic analysis. These isolates included 173 isolates from Prieska, 167 isolates from Barkly West, 174 isolates from Orania, 75 isolates from Douglas, 67 isolates from Cradock, 71 isolates from Greytown, 66 isolates from Potchefstroom and 26 reference isolates provided by Dr. Kerry O'Donnell. The 26 reference isolates included representatives of the first nine lineages as described by O' Donnell *et al.* (2000) as well as eight outgroups (Table 4.2).

4.2.4 AFLP analysis

AFLP analysis was performed using primer pair combinations *Eco*RI-AA/*Mse*I-AT, *EcoR*I-CC/*Mse*I-CG and *Eco*RI-TG/*Mse*I-TT. These primer pair combinations were used by Jurgenson *et al*. (2002) and Zeller *et al. (*2003; 2004) to resolve the genetic variability of *F. graminearum* isolates within and between populations. Primers and adapters (Table 4.3) were synthesised by Integrated DNA Technologies Inc, Coralville, IA, USA. Oligonucleotides used as adapters for AFLP analysis were purified by the manufactures using polyacrylamide gel electrophoresis (PAGE). Adapters were prepared by mixing equimolar amounts of the single-stranded oligonucleotides, heating for 10 min at 65℃ in a water bath and then allowing the mixture to cool down to room temperature (20-25℃). AFLP analysis was performed according to Vos *et al.* (1995) with minor modifications as described by Herselman (2003).

Table 4.2 Reference isolates representing nine of the 13 *F. graminearum* lineages described by O'Donnell *et al.* (2000; 2004) as well as outgroup *Fusarium* species used in this study

Lineage	Study codes	Species names *	Geographic origin
1	KOD2, KOD7	*F. austroamericanum*	South America
2	KOD10, KOD24	*F. meridionale*	Asia/New Caledonia/South and Central America/Australia/ South Africa
3	KOD11, KOD12	*F. boothii*	South Africa /Mexico/Central America/Nepal/Korea
4	KOD4, KOD9, KOD27	*F. mesoamericanum*	USA/Central America
5	KOD15, KOD17	*F. acaciae-mearnsii*	South Africa /Australia
6	KOD19, KOD20	*F. asiaticum*	Asia/South America
7	KOD14, KOD21, KOD22	*F. graminearum*	USA/Hungary/Netherlands/Iran
8	KOD3, KOD28	*F. cortaderiae*	South America/Australia/New Zealand
9	KOD1, KOD18	*F. brasilicum*	South America
Outgroups	KOD13, KOD23	*F. crookwellense*	Columbia/Netherlands
	KOD5, KOD6	*F. culmorum*	Denmark
	KOD8, KOD26	*F. pseudograminearum*	Australia/ South Africa

*As proposed by O'Donnell *et al.* (2000; 2004).

Table 4.3 Sequences for adapters and primers used for ligation reactions, pre-selective and selective amplification of *F. graminearum* isolates (Leslie and Summerell, 2006)

Primer	Type	Sequence (5'-3')
*Mse*I adapter-F	Adapter	GACGATGAGTCCTGAG
*Mse*I adapter-R	Adapter	TACTCAGGACTCAT
*Eco*RI adapter-F	Adapter	CTCGTAGACTGCGTACC
*Eco*RI adapter-R	Adapter	AATTGGTACGCAGTCTAC
*Mse*I primer+0	Pre-selective	GACGATGAGTCCTGAGTAA
*Eco*RI primer+0	Pre-selective	CTCGTAGACTGCGTACCAATTC
*Mse*I primer+2	Selective	GACGATGAGTCCTGAGTAA**NN**
		NN=AT, CG or TT
*Eco*RI primer+2	Selective	CTCGTAGACTGCGTACCAATTC**NN**
		NN=AA, CC or TG

4.2.4.1 Restriction digestion and adapter ligation

Two restriction enzymes, *Eco*RI (six bp recognition site) and *Mse*I (four bp recognition site) were used to digest genomic DNA from the *Fusarium* isolates. The 50 µl reaction mixture consisted of approximately 1 µg DNA, 4 U *Mse*I (New England Biolabs, Ipswich, MA, USA) and 1x *Mse*I buffer [50 mM NaCl, 10 mM Tris-Cl pH 7.9, 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT)]. DNA was digested at 37°C for 5 h. This digestion was followed by a second digestion overnight at 37°C using 5 U *Eco*RI (Roche Diagnostics, Mannheim, Germany) and NaCl to a final concentration of 100 mM. A 10 µl reaction mixture containing 50 pmol *Mse*I-adapter, 5 pmol *Eco*RI-adapter, 1 U T4 DNA Ligase (USB Corporation, Cleveland, Ohio, USA), 0.4 mM adenosine 5'-triphosphate (ATP) and 1x T4 DNA Ligase buffer (66 mM Tris-Cl pH 7.6, 6.6 mM $MgCl_2$,10 mM DTT, 66 µM ATP) was added to the 50 µl restriction digestion mix to ligate the adapters. The ligation reaction mixture was incubated overnight at 16°C.

4.2.4.2 Pre-selective amplification

The digested/ligated DNA was amplified in a 20 µl reaction mixture consisting of 5.0 µl DNA, 1x Promega *Taq* polymerase buffer, 2 mM $MgCl_2$, 200 µM of each dNTP, 30 ng of *Mse*I primer+0 (Table 4.3), 30 ng of *Eco*RI primer+0 (Table 4.3) and 1 U Go*Taq*® Flexi DNA polymerase (Promega). The conditions used for the pre-selective amplifications were: 94℃ for 5 min followed by 30 cycles of 94℃ for 30 s, 56℃ for 60 s and 72℃ for 60 s, a final 10 min elongation 72℃ and then held at 10℃. Amplified fragments were separated in a 1.5% (w/v) agarose gel at 80 V for one hour and visualised using ethidium bromide under UV light, after which DNA dilutions (1:15 to 1:50) were made to prepare samples for selective amplification.

4.2.4.3 Selective amplification

The pre-selective amplified DNA was used as the template for the AFLP analysis. The selective PCR reactions were performed using three primer combinations, namely *Eco*RI-AA/*Mse*I-AT, *EcoR*I-CC/*Mse*I-CG and *Eco*RI-TG/*Mse*I-TT. Reactions contained 5 µl pre-amplified DNA, 1x Promega *Taq* polymerase buffer, 2 mM $MgCl_2$, 200 µM of each dNTP, 100 µg/ml bovine serum albumin, 30 ng of *Mse*I primer+2 (Table 4.3), 30 ng of *Eco*RI primer+2 (Table 4.3) and 0.75 U Go*Taq*® Flexi DNA polymerase (Promega) in a final volume of 20 µl. The selected primers had been used to create a genetic linkage map (Jurgenson *et al.* 2002) for *G. zeae* (teleomorph *F. graminearum*) and to estimate genetic diversity within *F. graminearum* populations in North America (Zeller *et al.* 2003; 2004). The AFLP-PCR amplification programme was: 94℃ for 5 min followed by 94℃ for 30 s, 65℃ for 30 s, decreasing by 1℃ every cy cle and 72℃ for 60 s for 9 cycles followed by 94℃ for 30 s, 56℃ for 30 s and 72℃ f or 60 s for 25 cycles and 1 cycle of 72℃ for 2 min followed by a 10℃ hold.

4.2.4.4 Visualisation of amplified fragments

The AFLP-PCR products were separated on a 5% (w/v) denaturing polyacrylamide gel [19:1 acrylamide:bis-acrylamide, 7 M urea, 1x TBE Buffer (89 mM Tris-Cl, 89 mM boric acid, 2.0 mM EDTA)]. The amplified reactions were mixed with equal volumes formamide loading dye [98% (v/v) de-ionised formamide, 10 mM EDTA (pH 8.0), 0.05% (w/v) bromophenol blue, 0.05% (w/v) xylene cyanol]. These reactions were denatured by incubation for 5 min at 94℃. After denaturation, m ixtures were immediately placed on ice prior to loading. Each PAGE gel contained at least sixty individuals, which

represented one primer pair combination and at least three locations. Samples were compared to the *F. graminearum* reference isolates (Table 4.2), which were used as standards in this experiment, and fragment sizes were determined by comparison with a 100 bp DNA ladder (Promega). Samples were set up and loaded arbitrarily to enable unbiased scoring and to compensate for the large sample size. Electrophoresis was performed at constant power of 80 W for approximately 2 h.

The separated AFLP-PCR products were visualised by silver staining by following the Silver Sequence™ DNA Sequencing System manual (Promega). After the final wash step, stained gels were left overnight in an upright position to air-dry and then photographed. The dried stained PAGE gel was positioned on photographic paper (Ilford Multigrade IV RC) the same size as the gel and exposed to a dim white light for approximately 20 s. The fragment sizes were determined by comparison with a 100 bp DNA ladder (Promega). Scorable polymorphic AFLP bands, ranging from 300 bp to 800 bp were scored manually as 1 when present and 0 when absent in each individual and used to construct a binary data matrix. Scored data were used to construct a data matrix for statistical analysis. Homologous AFLP-PCR fragments in different individuals were assumed to represent the same allele.

4.2.5 Genetic diversity evaluation and population genetic analyses

Six different statistical programmes were used for population genetic analyses. A sample of >800 individuals is required for unbiased estimates of population dynamics.

4.2.5.1 F-statistics (F_{ST}) and analysis of molecular variance

F-statistics are the most widely used descriptive statistics in population and evolutionary genetics. Wright (1951) defined F-statistics as a measurement of genetic variance in a hierarchically structured population that can be subdivided into a series of discreet subpopulations. This population model is commonly used to assess population structure and the rate of gene flow between sub-populations. F-statistics also is used to determine the degree of genetic divergence within and between sub-populations. F_{ST} values range from 0, indicating no genetic variation amongst sub-populations, to 1 where populations are fixed for different alleles (Table 4.4).

Table 4.4 Guidelines to assist with the interpretation of F_{ST} as proposed by Wright (1951)

F_{ST} values	Level of genetic variation
0.0 to 0.05	Low
0.05 to 0.15	Moderate
0.15 to 0.25	High
Above 0.25	Very high

As part of the assessment of the genetic structure of the FHB population an analysis of molecular variance (AMOVA) was done using ARLEQUIN 3.11 (Excoffier *et al.,* 2005). AMOVA is an analysis of haplotype variation. This statistical analysis is based on an analysis of variance framework that uses distance between haplotypes as a measure of their evolutionary distance. The AMOVA indexes estimate population subdivision based on the partitioning of the genetic variance as hierarchical components within and between populations following the inference of DNA haplotypes (Excoffier *et al.,* 1992; Evans, 2009).

Subdivisions of the total variance among assessed genotypes include the genetic variation among and within populations and are based on a locus by locus model that uses conventional F-statistics. The significance of the F-statistics parameters and the division of the genetic variance components were tested using 16000 permutations and the ARLEQUIN 3.11 software package (Excoffier *et al.,* 2005).

4.2.5.2 Analysis of genetic variance

Cluster analysis using the sequential agglomerative hierarchical nested cluster analysis (SAHN) clustering method based on the unweighted pair-group method using arithmetic averages (UPGMA) was conducted with a numerical taxonomy and multivariate analysis system (NTSYSpc) version 2.20e software (Rohlf, 2000) with the data in a binary data matrix to construct a rooted dendrogram. UPGMA is a hierarchical algorithm used for clustering isolates into similar groups. Cluster analysis information is sensitive to closely related individuals; therefore the constructed dendrogram illustrates the relationship of the *F. graminearum* isolates to the 26 reference isolates based on AFLP allele variation.

Genetic similarities of isolates were compared by using the Jaccard similarity coefficient (Jaccard, 1908). Cophenetic correlation coefficients were calculated by using COPH and MXCOMP procedures as implemented in NTSYSpc to measure the goodness of fit of the cluster analysis to the similarity based analysis of the binary data matrix. The goodness of fit (r) value indicates a very good fit if $r \geq 0.9$, a good fit with $0.8 \leq r < 0.9$, a poor fit with $0.7 \leq r < 0.8$ and a very poor fit with $r < 0.7$ (Rohlf, 1997).

Allelic polymorphic information content (PIC) was determined using the formula $PIC=1-\Sigma (P_{ij})^2$, with P_{ij} representing the ith allele in the jth population for each location (Botstein *et al.*, 1980). PIC evaluates polymorphism of a marker by characterising the efficiency of each primer for detecting polymorphic loci (Shete *et al.,* 2000).

Ordination of the structure of the genetic diversity within and amongst the seven field populations was analysed based on principal component analysis (PCA) using DARwin 5.0.155 software. PCA was chosen to balance the cluster analysis information, due to its informative nature regarding distances among larger groups. Data are projected on two or three axes to enable visualisation of differences between isolates and the detection of clusters formed by related isolates. Isolates in the PCA function as the uncorrelated variables (principal components) measured against the original variables (the molecular markers). An unrooted, unweighted neighbour-joining (NJ) dendrogram with 500 bootstrap repetitions was constructed with the DARwin software and used to assess the robustness of the nodes. Genetic similarities between isolates were compared by using the Jaccard similarity index (Jaccard, 1908).

A dendrogram based on Nei's (1973) regular and unbiased genetic distance measures was generated using POPGENE 1.31 to assess the genetic variation among and within populations where the AFLP binary dataset was treated as haploid and dominant (Zeller *et al.,* 2003; 2004). This assessment generates a fixation index for groups (G_{ST}) and a genetic distance between populations based on alleles that are present at a ≥5% frequency.

4.2.5.3 Bayesian-based methods

The multilocus Bayesian-based method can be used to identify genetically homogeneous groups of individuals in a larger group and to infer population structure. The Bayesian clustering method implemented in the STRUCTURE 2.2 software (Pritchard *et al.*, 2000) was used to test the genetic structure of the FHB populations in South Africa. Analysis from the STRUCTURE software was used to estimate the 'true' number of sub-populations (*K*) without prior knowledge of the population (Evanno *et al.,* 2005; Evans, 2009). This 'true' number of populations was determined using the admixture model with inference of alpha from the data together with the allele frequency model, where allele frequencies were correlated. Lambda was set to one.

The suggested number of groups in the South Africa FHB population based on the geographical distribution of 793 individuals over 176 loci was seven as seven locations were sampled. The "true" number of populations was calculated following the procedure of Evanno *et al.* (2005) which determines an *ad hoc* statistic ΔK based on the rate of change in the log probability of data between successive *K* values. Evanno *et al.* (2005) suggested that the real number of groups in a population is best detected by using the modal value of the distribution of ΔK. The basic admixture model with unlinked loci and uncorrelated allele frequencies was used. The assumed number of populations varied from one to seven. The programme was run with a burn-in period length and Monte Carlo Markov Chain (MCMC) iterations of 10000 for K=1 to K=10 and repeated for ten runs per *K* value. After the calculation of ΔK=2, the analysis was repeated with K=2 and both a burn-in period and MCMC replications of 900000 to verify consistency. Since the Prieska population contained many clonal isolates the above-mentioned procedures were repeated on all geographical populations but with only one representative of each of the clonal isolates per population. This "clone censored" analysis was conducted to determine whether the pressure of the clonal isolates influenced ΔK.

4.2.5.4 Network analysis

Network 4.5.0.0 software was used to elucidate a minimal spanning network with median joining (MJ) to assess the genetic structure of the population across all seven locations. The results can be used to infer ancestral, evolutionary occurrences by calculating genetic distances between individuals and identifying population expansion events (Bandelt *et al.,* 1995; 1999).

A minimum-spanning tree was used for network simplification where pairs of sequence types are listed in increasing order of their genetic distances. The MJ network algorithm enables the construction of higher-resolution networks from multi-state data. The ancestral node of a network can be determined by comparing the network nodes with suitable outgroups. This process often is referred to as "rooting the network" (Bandelt *et al.,* 1999).

4.3 Results

4.3.1 Classification of *F. graminearum* isolates using PCR analysis

The 793 isolates identified as *F. graminearum* based on morphology (Chapter 3) also were identified as *F. graminearum* in PCR amplifications with primer pair Fg16F/R. Other FHB associated (*F. culmorum* and *F. crookwellense*) species identified in this study and the reference isolates for lineage 7 were included in the PCR analysis as controls. The frequency of *F. graminearum* isolates in individual field populations ranged from 84% in Greytown and Potchefstroom to 99% in Prieska. The 410 bp fragment was amplified in all *F. graminearum* isolates tested, and absent, as expected, in the non-*F. graminearum* controls (data not shown). These results are consistent with the identity of all of these isolates as *F. graminearum*. Only these *F. graminearum* isolates were included in the AFLP assays.

4.3.2 Evaluation of the *F. graminearum* population with AFLPs

AFLP analysis was used to assess the genetic variation in the populations as it is highly informative and reproducible. We identified 173 polymorphic and three monomorphic AFLP fragments across all isolates. These fragments were between 300 and 800 bp in length. Each primer pair generated an informative fingerprint across all isolates and produced between 47 and 63 distinguishable polymorphic AFLP fragments. An example of an AFLP fingerprint generated using primer pair combination *Eco*RI-AA/*Mse*I-AT is given in Figure 4.1

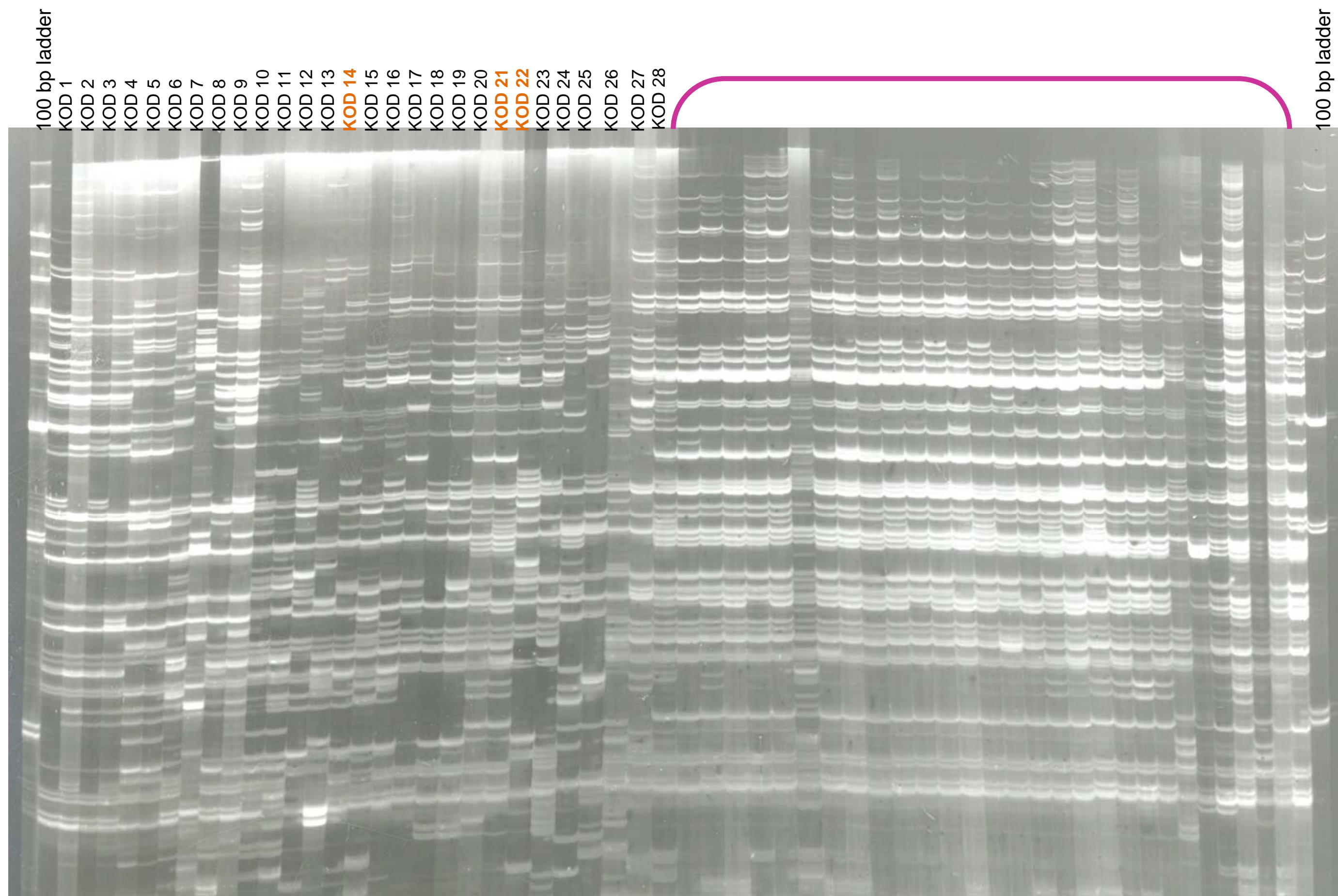

Figure 4.1 **AFLP fingerprint generated using primer pair combination *Eco*RI-AA/*Mse*I-AT. KOD codes indicate reference isolates representing nine of the 13 *F. graminearum* lineages as indicated in Table 4.2. Reference isolates indicated in red represent lineage 7. Isolate profiles marked with pink bracket represent isolates from the seven sampled locations.**

The AFLP primer combinations *Eco*RI-AA/*Mse*I-AT and *Eco*RI-TT/*Mse*I-TG yielded the largest (63 fragments) number of polymorphic fragments, and the smallest number of polymorphic fragments (47) was generated by primer combination *Eco*RI-CC/*Mse*I-CG (Table 4.5). The PIC values for each of the three primer combinations were similar. The similar PIC values in spite of a relatively large range in the number of polymorphic fragments indicate that a primer pair with large number of polymorphic fragments is not necessarily the most informative primer combination.

Table 4.5 Selective primer combinations, scored polymorphic fragments and polymorphic information content values for primers used in this study

Primer combination	Scored polymorphic fragments	Polymorphic information content
*Eco*RI-AA/*Mse*I-AT	63	0.37
*Eco*RI-CC/*Mse*I-CG	47	0.33
*Eco*RI-TT/*Mse*I-TG	63	0.34

4.3.2.1 AMOVA and F-statistics

AMOVA was performed to test the genetic structure of the *F. graminearum* population. Isolates from each of the seven locations were treated as a separate population. Based on the AMOVA results, most of the genetic variation (84%) (Table 4.6) was due to variation within populations and only a small portion (16%) of the variation was due to genetic diversity between different field populations. According to Wright's (1951) guidelines (Table 4.4) the F_{ST} value of 0.16 observed in this study, is consistent with a high level of genetic differentiation within populations. This pattern is consistent with the hypothesis that the overall population can be divided into two or more subpopulations.

The low level of genetic variation between field populations suggests gene flow may occur across large geographical distances. This hypothesis was tested by correlation between genetic and geographical distances (Figure 4.2). No correlation between genetic and geographic distance was detected, even though the locations were separated from one another by 100-700 km.

Table 4.6 Genetic variation of *F. graminearum* field populations in South Africa

Source of variation	df	Sum of squares	Variance components	% variation	P-value	F_{ST}
Between populations	6	4080	5.5	**16.0**	<0.001	**0.16**
Within populations	845	24400	28.8	**84.0**	<0.001	
Total	851	28480	34.3			

4.3.2.2 Analysis of genetic variance

Rooted cluster analysis

The cophenetic correlation, using the goodness of fit (*r*), which measures the correlation between the similarity represented on the dendrogram as well as the actual degree of similarity, resulted in r=0.79 for the UPGMA clustering method based on Jaccard's similarity coefficient. Thus, the dendrogram can be used as an indicator of genetic diversity in the *F. graminearum* population. The entire rooted dendrogram of all 793 South African isolates and 26 reference isolates is given in Appendix II and sections thereof are reproduced in Figures 4.3a and 4.3b.

All of the South African isolates clustered with the representative *F. graminearum* lineage 7 (Figure 4.3a) isolates. There were sporadic clusters of isolates from different locations, but there was no relationship between AFLP clusters and geographic origin, except that isolates from Prieska tended to cluster together. Prieska had the highest number of clonal genotypes (56%) present, while the highest level of genotype diversity (>75%) was found in the Orania population. Other than for Prieska, clonal genotypes were detected in Barkly West (1.2%), Orania (15%), Douglas (11%) and Greytown (15%). Clonal genotypes were also shared between different populations (Douglas and Prieska - 7.1% and Barkly West and Orania - 2.9%).

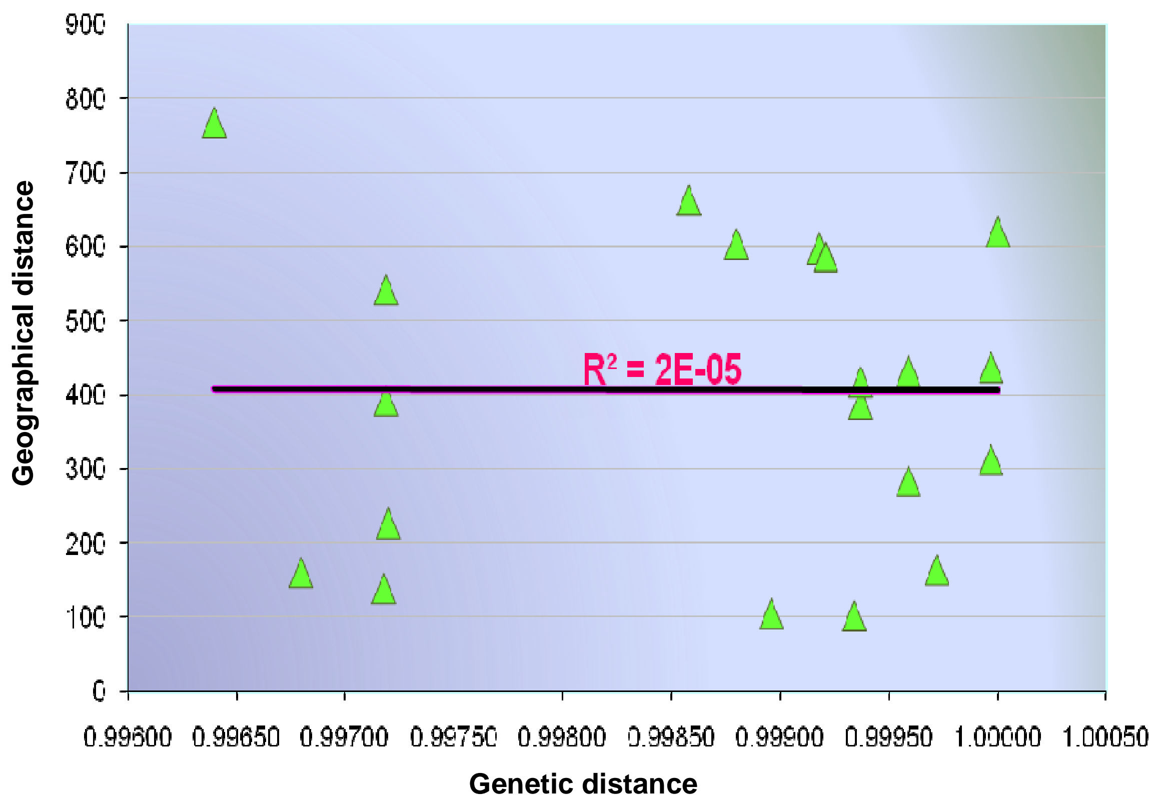

Figure 4.2 Correlation between genetic and geographical distance.

Principal component analysis

PCA using DARwin software (Figure 4.4) is based on the classification of the structure of the genetic diversity in the FHB population and can be divided into four quadrants. Isolates were distributed in all four quadrants, with most of the isolates in quadrants 2 and 3. Prieska was the only location for which the isolates formed a distinct separate group around PC1 of quadrants 1 and 4 and were somewhat isolated from the rest of the isolates in these two quadrants. Only a few isolates from Prieska were observed in quadrants 2 and 3. Most of the clonal isolates from Prieska occurred in the distinct group at the far left end of PC1. Only a few of these Prieska-Prieska (both clones were from Prieska) clones could be observed in quadrants 2 and 3. Most of the clonal isolates from Prieska-Douglas (one of the two isolates of the clonal pair was from Prieska and the other one from Douglas) occurred in quadrant 2 as part of the Douglas isolates with only one such clone observed in quadrant 3.

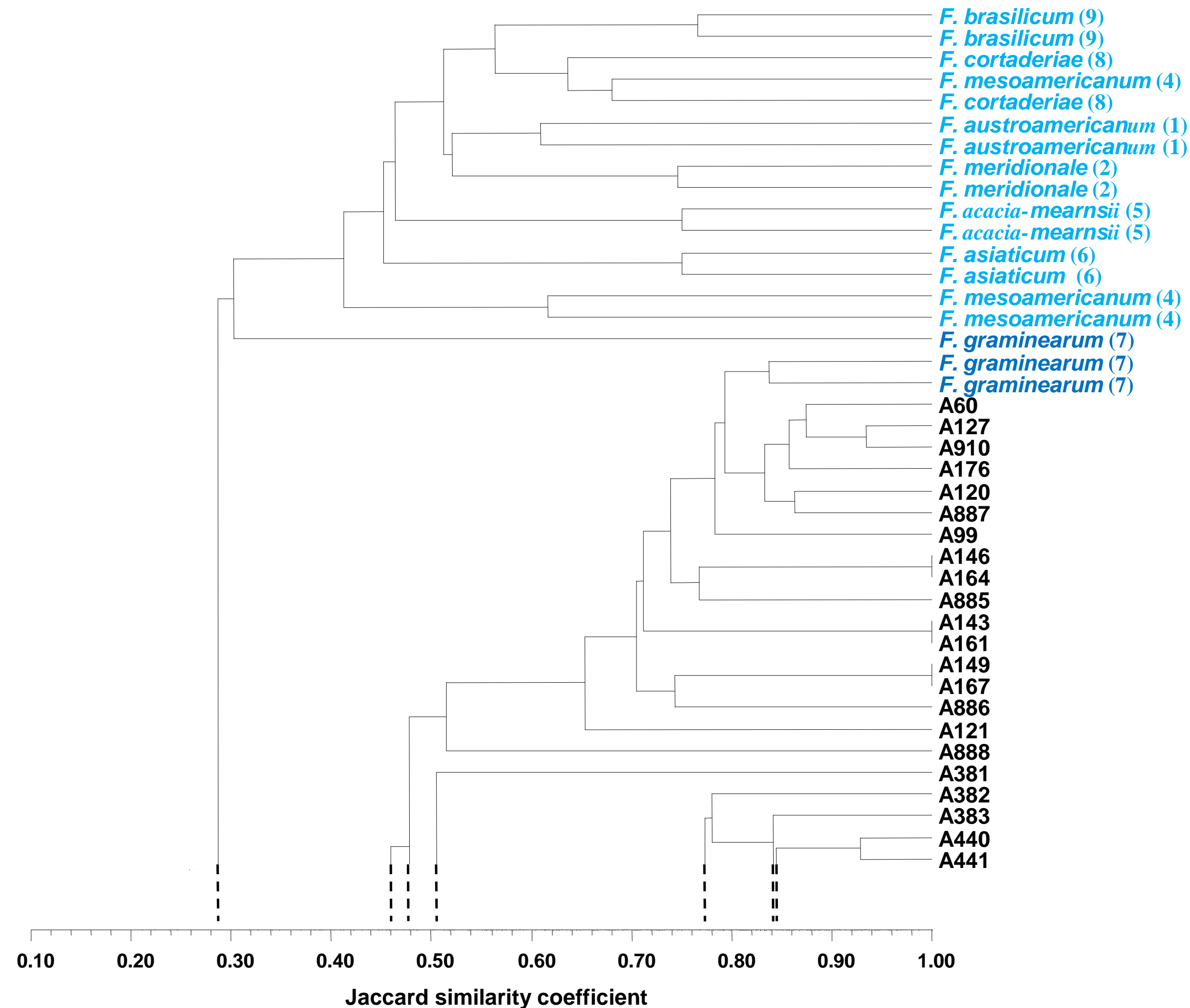

Figure 4.3a First part of the dendrogram generated using NTSYSpc and UPMGA clustering using Jaccard's similarity coefficient, illustrating clustering of South African *F. graminearum* isolates with lineage 7 reference isolates. The different isolates from the nine lineages are indicated in light blue, lineage 7 reference isolates in dark blue and outgroups in red. Isolates from the South African FHB population are indicated in black and were numbered A1-A960. Numbers in brackets indicate known lineage groups. The complete dendrogram is given in Appendix II.

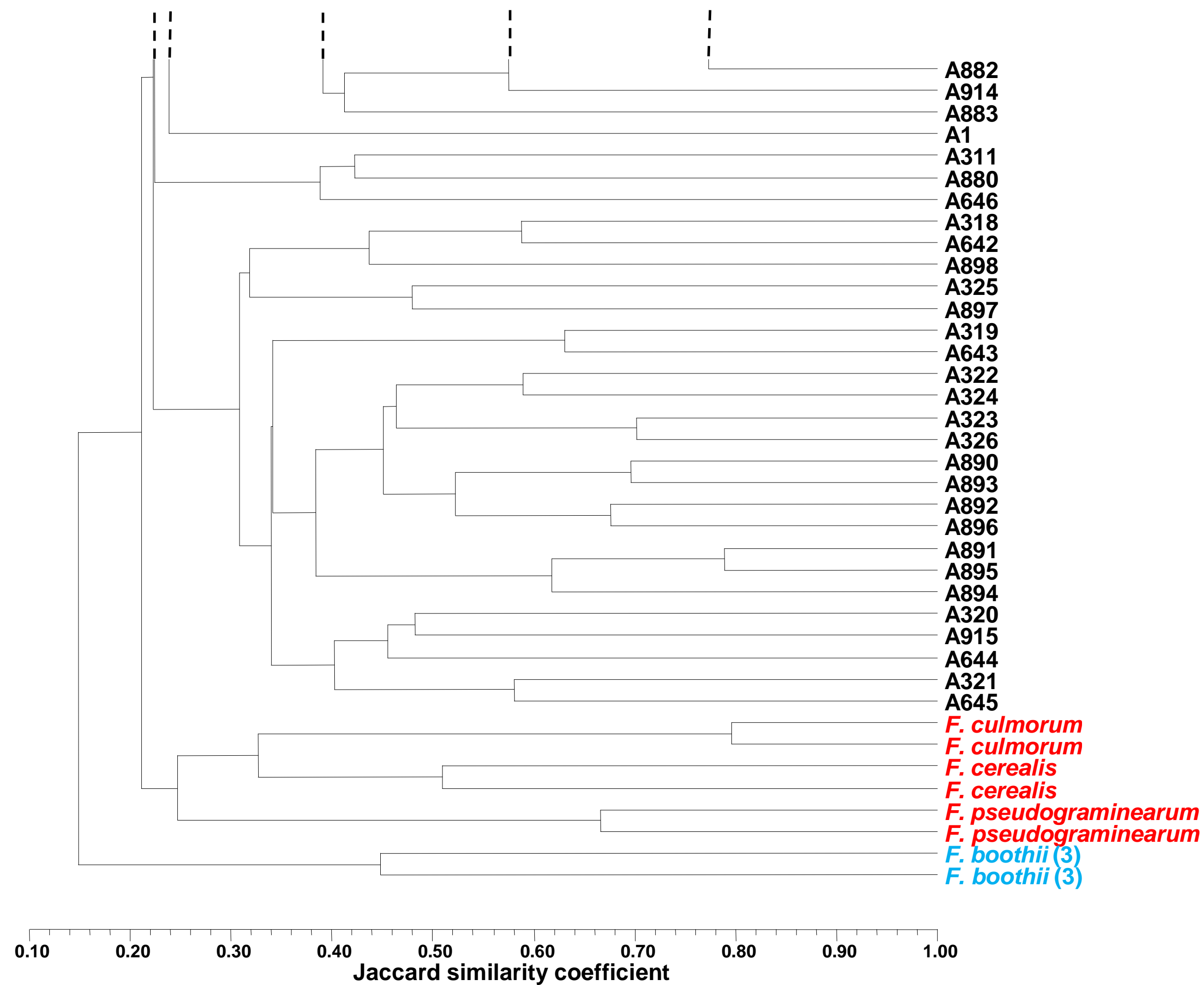

Figure 4.3b **Bottom part of the dendrogram generated using NTSYSpc and UPMGA clustering using Jaccard's similarity coefficient, illustrating clustering of South African *F. graminearum* isolates with lineage 7 reference isolates. The different isolates from the nine lineages are indicated in light blue, lineage 7 reference isolates in dark blue and outgroups in red. Isolates from the South African FHB population are indicated in black and were numbered A1-A960. Numbers in brackets indicate known lineage groups. The complete dendrogram is given in Appendix II.**

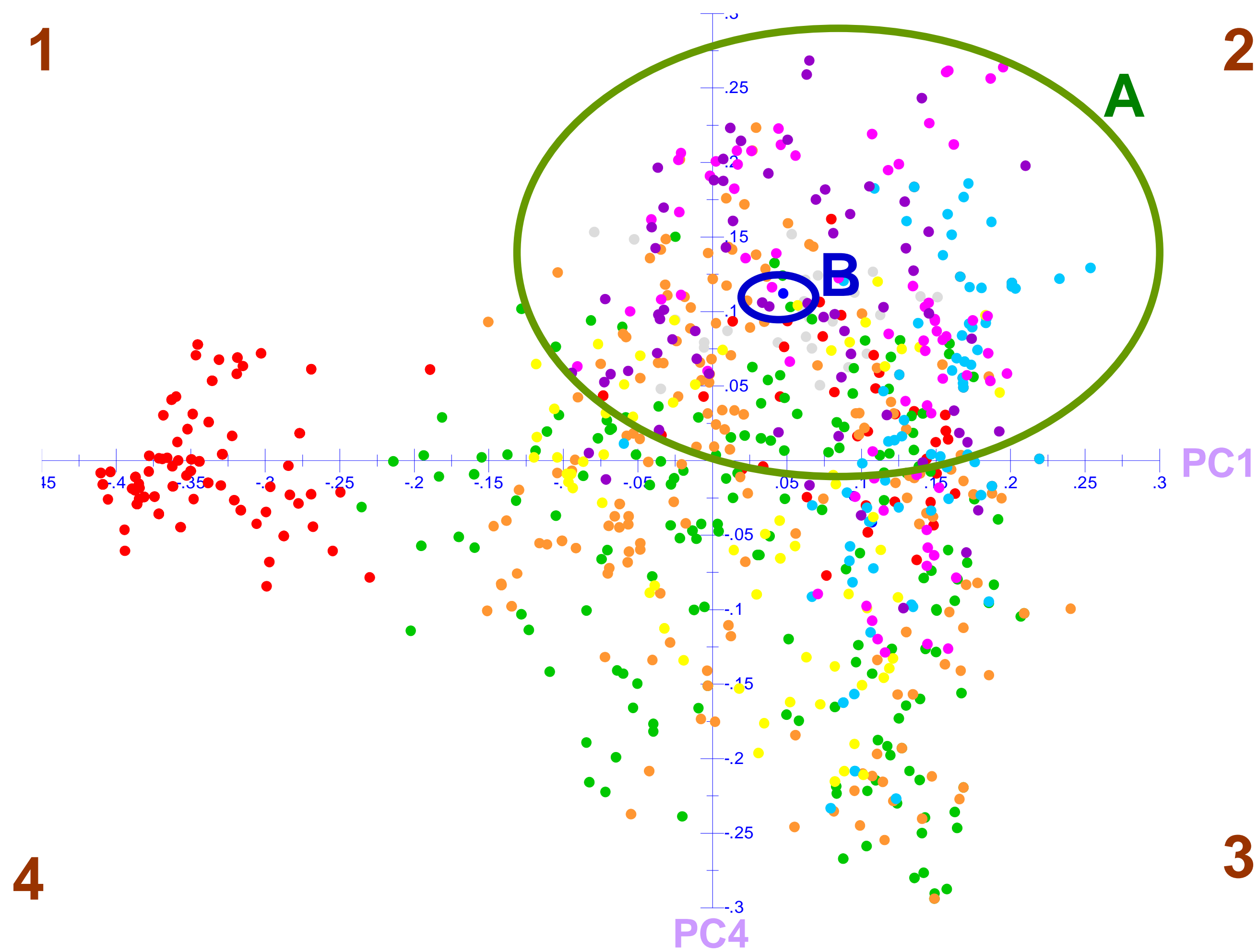

Figure 4.4 Principal component analysis biplot (axes 1 and 4) of 793 *F. graminearum* and 26 reference isolates.

● Prieska ● Barkly West ● Orania ● Douglas ● Greytown ● Cradock ● Potchefstroom ● Lineages ● Outgroups

1-4 = Principal component quadrants A-Isolates from less temperate locations B-Lineage 7 reference isolates.

Isolates from Barkly West and Orania were distributed equally in all four quadrants. Most of the isolates from these two locations clustered together, but the clusters were not always close to each other. A large number of the Barkly West isolates clustered in the central parts of PC1, other than isolates from Orania that were mainly concentrated around PC4, especially in quadrant 1 and 2. Most of the clonal isolates from Orania were found in quadrant 4, close to the intersection of PC1 and 4. A few Barkly West isolates clustered around PC1 in quadrants 1 and 4 towards the distinct group of Prieska isolates.

Isolates from Douglas were found in quadrants 2 and 3 along PC4, with most observed in quadrant 2. Douglas is one of the less temperate locations in the Northern Cape region. Some isolates from this location were somewhat isolated from the others in quadrant 2. Douglas-Prieska clonal isolates were found in quadrants 2 and 3, with most in quadrant 2 and only one in quadrant 3 close to PC1. Some of the Douglas isolates overlapped with isolates in clusters from other locations. Greytown isolates was distributed across all four quadrants. Most of these isolates occurred in quadrants 2 and 3 with only a few in quadrant 4. Most isolates from Cradock were found in quadrants 2 and 3, with quite a few in quadrant 1 and none in quadrant 4. Potchefstroom isolates were present in all four quadrants, but were mainly concentrated with isolates from Cradock in quadrants 1 and 2, especially around PC4 (y-axis). Only one isolate from Potchefstroom was found in quadrant 4, while the largest cluster of these isolates was split between quadrants 1 and 2.

Clustering of isolates from all seven locations with isolates from lineage 7 could be observed in quadrant 2 (indicated by B in Figure 4.4). Isolates from outgroups were observed in quadrants 1 and 2 with most isolates located in quadrant 2.

Unrooted cluster analysis

Associations amongst the field population isolates were analysed by using the NJ method (Figure 4.5) in DARwin. The unrooted NJ tree had the same overall structure as the PCA, with the Prieska field population tending to be separated as indicated with the red bracket in Figure 4.5. The remaining Prieska isolates grouped into two smaller groups (A and B, Figure 4.5) together with isolates from other locations. Most of the

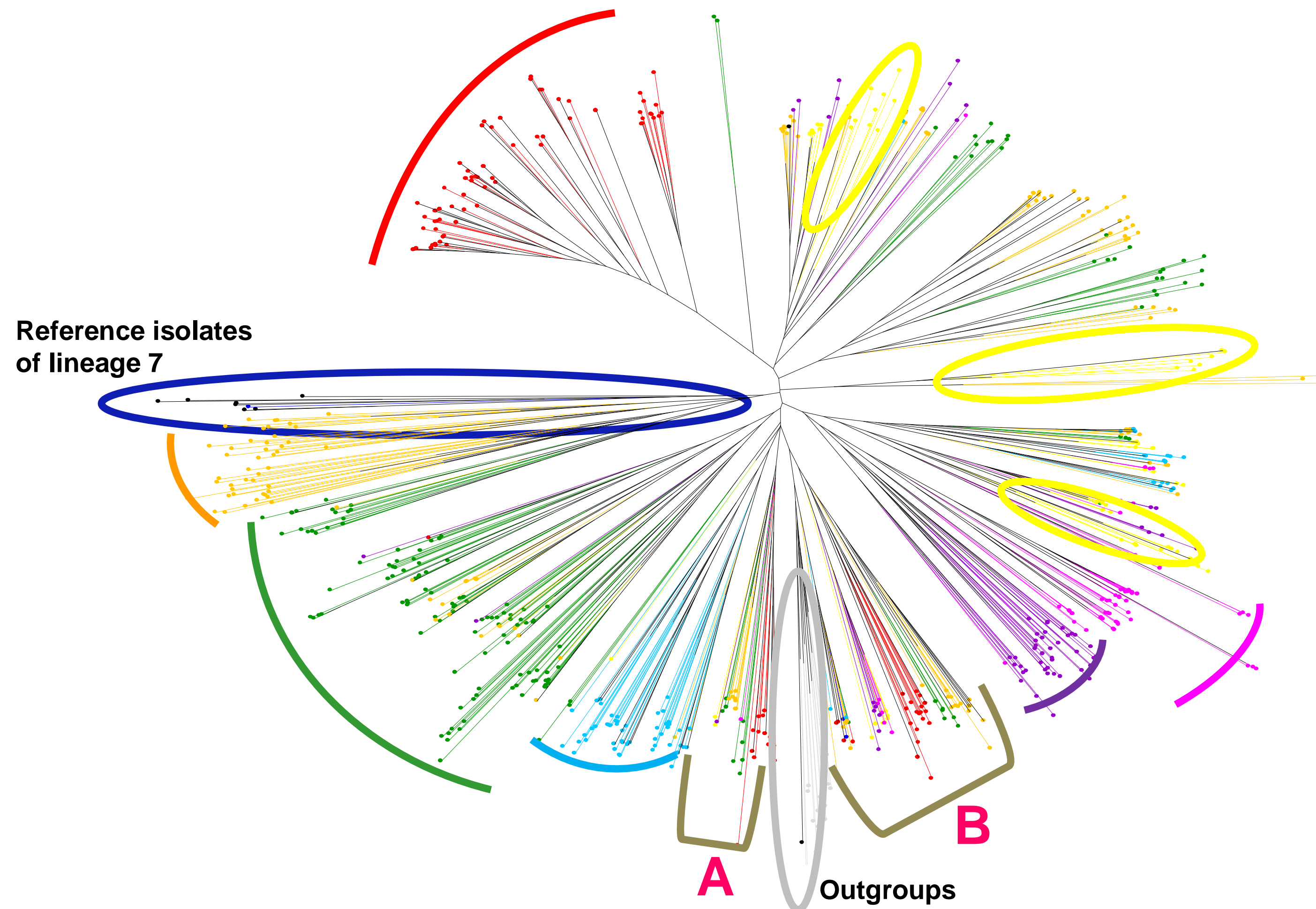

Figure 4.5 Unrooted neighbour-joining (NJ) tree constructed with DARwin5 version 5.0.155 software, illustrating clusters from different locations for the 793 *F. graminearum* isolates and 26 reference isolates.

● Prieska ● Barkly West ● Orania ● Douglas ● Greytown ● Cradock ● Potchefstroom ● Lineages ● Outgroups

A-Contains only isolates from Prieska, Barkly West and Orania B-Contains isolates from six of the seven locations.

isolates from Barkly West, indicated in the green bracket (Figure 4.5), were observed as one large group consisting mainly of isolates from this location and a few isolates from Orania. The rest of the Barkly West isolates formed two smaller groups that also clustered mainly with isolates from Orania. The Orania field population, indicated in the orange bracket in Figure 4.5, is found in one major group and a few smaller groups that clustered with isolates from other locations.

Douglas isolates formed one major group (light blue bracket in Figure 4.5), with a few scattered isolates clustering with isolates from other groups. This cluster also contained all of the Prieska-Douglas clonal isolates. The Greytown population contained three minor groups indicated with yellow circles in Figure 4.5, which included isolates from all six other locations. Potchefstroom and Cradock isolates usually clustered together if a group contained isolates from both locations, although one main group for each location was observed (pink bracket for Cradock and purple bracket for Potchefstroom in Figure 4.5). A few isolates from these two field populations also clustered with isolates from other locations.

Two clusters in Figure 4.5 (A and B) contained isolates from three or more locations. Cluster A contained isolates from Barkly West, Orania and Prieska. These three locations are all in temperate regions in the Northern Cape. Clonal isolates from Prieska are found in this cluster. Cluster B contained isolates from six of the seven locations, with only isolates of Douglas being absent. Clonal isolates from Prieska also occurred in this cluster. These results are consisted with the clustering patterns in Figure 4.4 obtained following PCA.

Genetic relationship between seven field populations

In terms of percentage of polymorphic loci, Barkly West and Orania (both 99% polymorphic loci) tended to be the most diverse populations while Douglas and Potchefstroom (both 89% polymorphic loci) were the most homogeneous, even though genetic diversity in these populations were still high (Table 4.7). Pairwise genetic analysis using Nei’s unbiased genetic diversity yielded genetic identities, ranging from 0.833 (between Prieska and Cradock) to 0.964 (between Orania and Barkly West). Genetic distances ranged from a dissimilarity of 0.036 between Barkly West and Orania to 0.183 between Cradock and Prieska, revealing low dissimilarity/high similarity (Table 4.8).

Table 4.7 Average correlation of 173 polymorphic AFLP loci as a measure for multilocus linkage equilibrium across seven field populations of *F. graminearum* in South Africa

Population	Nr of isolates	% Polymorphic loci	Average correlation	Probability
Prieska	178	98	0.254	<0.05
Barkly West	168	99	0.190	<0.05
Orania	174	99	0.209	<0.05
Douglas	76	89	0.102	<0.05
Greytown	67	91	0.139	<0.05
Cradock	72	90	0.155	<0.05
Potchefstroom	67	89	0.119	<0.05

Table 4.8 Pairwise calculation of Nei's unbiased genetic identity (above diagonal) and genetic distance (below diagonal) based on 176 AFLP loci

	Prieska	Barkly West	Orania	Douglas	Greytown	Cradock	Potchefstroom
Prieska	*****	0.89	0.90	0.85	0.88	0.83	0.85
Barkly West	0.12	*****	0.96	0.92	0.91	0.88	0.89
Orania	0.10	0.04	*****	0.94	0.94	0.92	0.92
Douglas	0.16	0.08	0.07	*****	0.90	0.91	0.90
Greytown	0.12	0.09	0.06	0.10	*****	0.93	0.93
Cradock	0.18	0.13	0.09	0.09	0.07	*****	0.95
Potchefstroom	0.16	0.11	0.08	0.11	0.07	0.05	*****

The average correlation of polymorphic AFLP loci as a measure for multilocus linkage disequilibrium (LD) in each of the populations tested with the null hypothesis indicated that multilocus LD was present in all seven field populations (Table 4.7). These results confirmed results from previous analyses, concluding that genetic variation within a population was higher than genetic variation between field populations. The low genetic distances indicated close relationships between different field populations. These results reflected the existence of these field populations as part of a larger, somewhat older than expected population. The genetic diversity within such populations, even though *F. graminearum* is homothallic, suggests the occurrence of sexual recombination.

Results from genotypic variation analysis using Popgene suggested that the seven field populations share a common ancester, and that the Prieska population is the most isolated (Figure 4.6). The Barkly West and Orania populations were the most similar (0.04 dissimilarity) of the field populations, followed by Cradock and Potchefstroom populations with a dissimilarity value of 0.05. Three main clusters can be identified with the Prieska cluster (cluster 1) being isolated from isolates from the other six locations. The first FHB reports were made at Prieska in the early 1990s. This location also is known for temperature extremes with the average midday temperatures for Prieska ranging from 17.9℃ in June to 32.7℃ in January. T he region is the coldest of the seven locations during July with an average minimum temperature of 1.3℃.

Cluster two included isolates from the other three Northern Cape locations (Barkly West, Orania and Douglas). The Barkly West and Orania populations were the most similar. These two locations have similar climatic conditions, but are geographically separated approximately 156 km.

Douglas tended to be genetically isolated from the other two locations, although it is geographically closer to Barkly West (approximately 119 km) and Orania (approximately 85 km) than these two locations are to each other. Douglas is one of the least temperate locations in the Northern Cape wheat-growing areas.

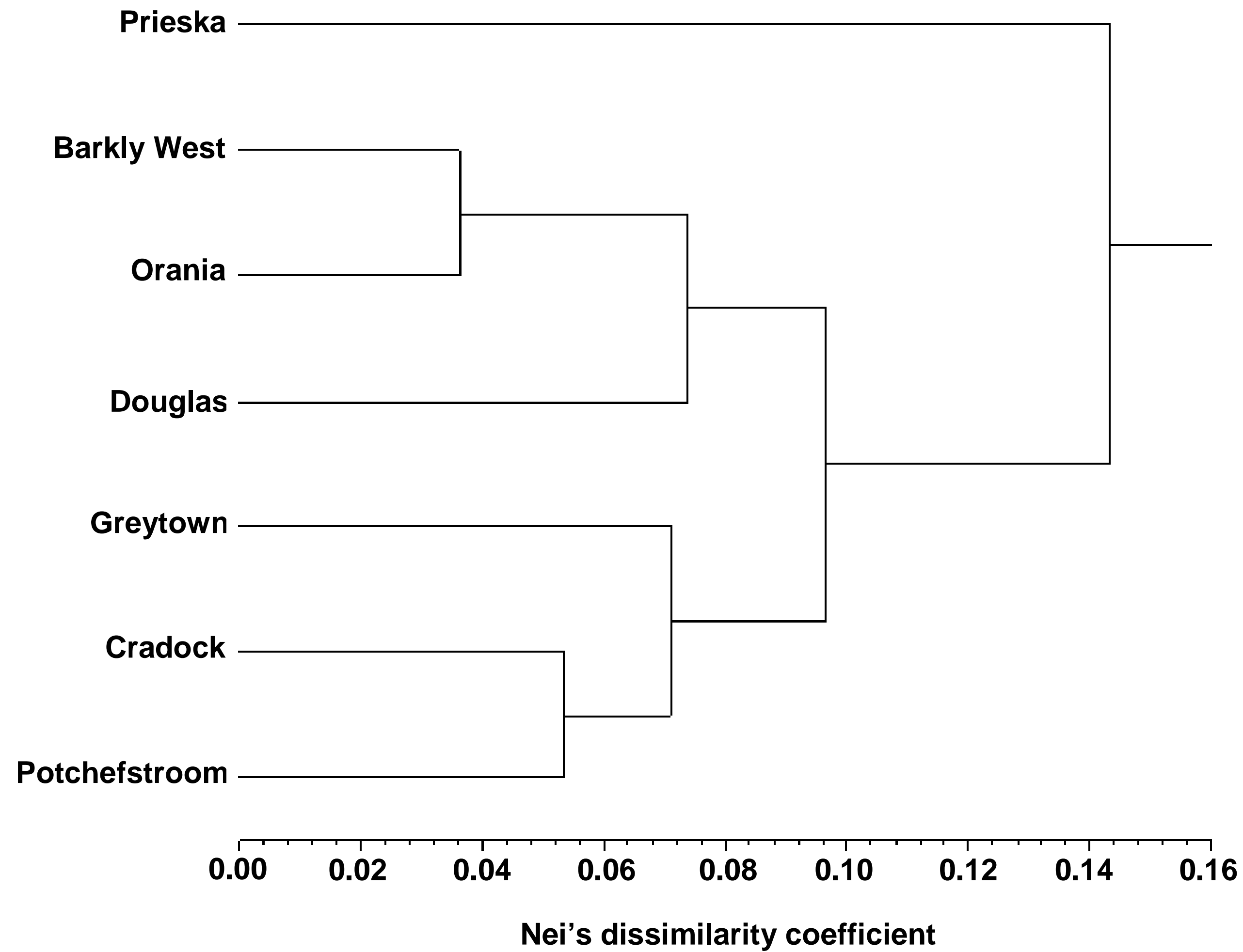

Figure 4.6 Dendrogram illustrating genotypic variation analysis of geographical populations using Popgene software based on Nei's dissimilarity coefficient.

The third cluster from the Popgene dendrogram contained isolates from three locations- Greytown, Cradock and Potchefstroom. These locations are included in the less temperate geographic regions of the wheat-growing areas of South Africa. In this cluster Potchefstroom and Cradock were the most similar field populations. Isolates from these two locations were sampled in the 2006 season when FHB was reported in Potchefstroom for the first time since 1980 and FHB in Cradock was reported for the first time ever in 2006. Greytown, although part of cluster three, clustered separately from the other two locations. Like the other two locations in this cluster, Greytown isolates were sampled during the 2006 FHB epidemic. This location, however, has had multiple FHB reports prior to 2006.

4.3.2.3 Bayesian-based methods (structure analysis)

Structure analysis delineates clusters of individuals based on their genotype by using Bayesian model-based clustering. This analysis is made without prior knowledge of the population at hand. Simulations were run with the South African *F. graminearum* population assuming that the number of clusters was between K=2 and K=10. Figure 4.7 illustrates the most suitable number of K clusters as identified by Evanno *et al.*'s (2005) *ad hoc* ΔK method, which determines the optimum number of genetic clusters in a population. The *F. graminearum* field population in South Africa consists of two populations (K=2), which is the minimum observed number of populations using this protocol. Thus all of the South Africa isolates could have been part of a single population.

Structure analysis with two populations K=2 (Figure 4.8) identified two clusters - one group composed primarily of isolates from the Prieska (red) field population and a second group containing the bulk of the isolates from the other six (green) locations. Although population one (Prieska) was mainly restricted to a single location, genetic components of this population could be seen in isolates from the six other locations. Censoring the clonal isolates in the analysis did not change the results. These results confirmed the genotypic diversity observed within field populations using cluster analysis.

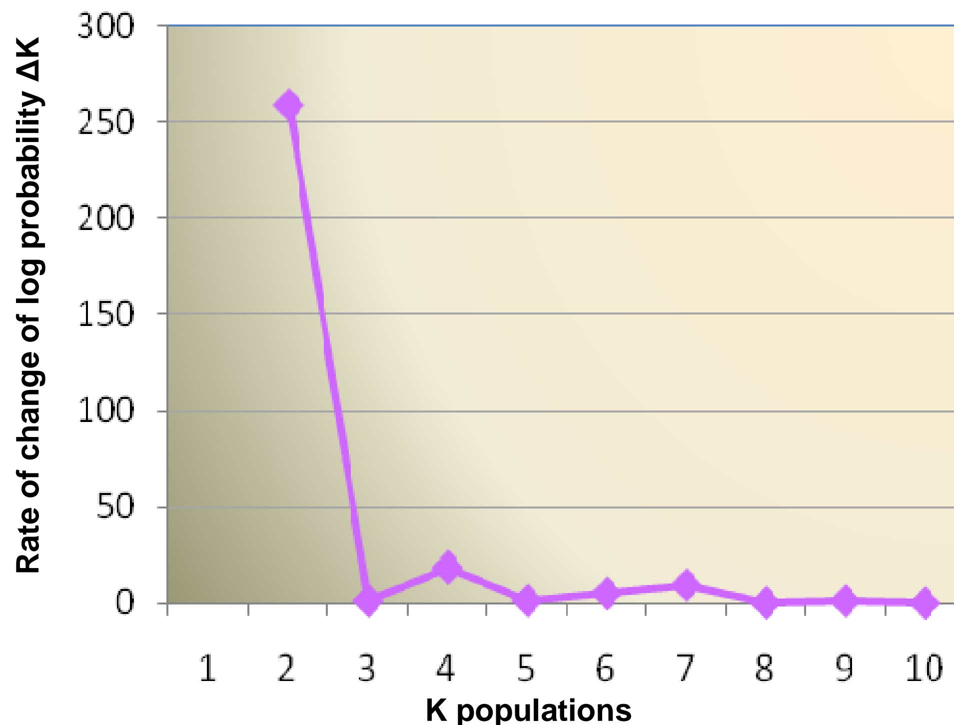

Figure 4.7 A plot of Evanno's *ad hoc ΔK* statistics. Analysis was based on 10000 burn-in and MCMC replications for *K*=2 to 10 and 10 replications per run.

Population 1 consisted mainly of the genetic contribution of Prieska's isolates, with 68% of the genetic contribution of Prieska's isolates assigned to this population (Table 4.9). Only a low percentage of the genetic contribution of isolates from the other six field populations was assigned to this population, ranging from 2% for Douglas to 16% for Orania. Only three of the locations contained isolates with a genetic composition of 50% or higher representative of population 1 (red), with 39% of these isolates from Prieska, 19% from Barkly West and 15% from Orania (Figure 4.8). The genetic contribution of field populations from Barkly West (85%), Orania (84%), Douglas (98%), Cradock (96%), Potchefstroom (91%) and Greytown (84%) mostly contributed to population 2. The genetic contribution of the Prieska field population was only 32% in the latter population (Table 4.9).

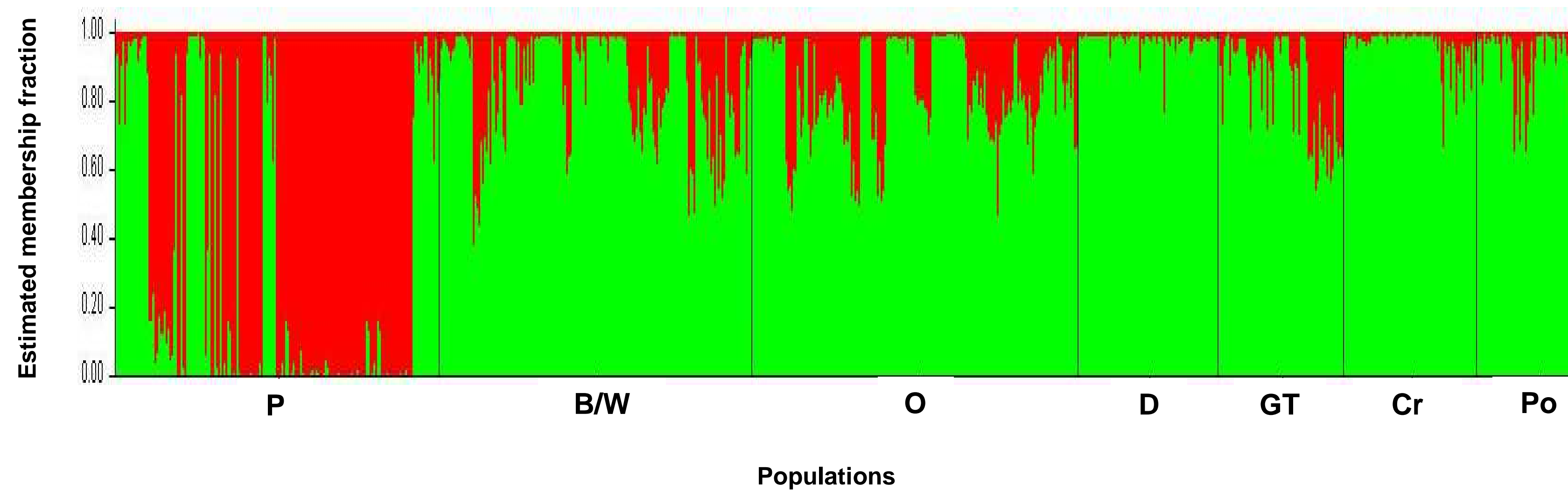

Figure 4.8 Estimated population structure at K=2 for the *F. graminearum* population (793 isolates) of South Africa using the admixture model of population structure. Population 1 is indicated in red and population 2 in green.

P – Prieska B/W – Barkly West O – Orania D – Douglas GT – Greytown Cr – Cradock Po - Potchefstroom.

Table 4.9 Genetic contribution of 793 *F. graminearum* isolates from seven field populations in two populations as detected by structure analysis

Geographic population	Inferred clusters		Number of individuals
	Population 1	Population 2	
Prieska	**0.68**	0.32	173
Barkly West	0.15	**0.85**	167
Orania	0.16	**0.84**	174
Douglas	0.02	**0.98**	75
Greytown	0.16	**0.84**	67
Cradock	0.04	**0.96**	71
Potchefstroom	0.09	**0.91**	66

4.3.2.4 Network analysis

A minimum-spanning network containing the 793 *F. graminearum* isolates from South Africa and 26 reference isolates (Figures 4.9 and 4.10) contained groups similar to those seen in the PCA and NJ cluster analyses. Once again, isolates from the Prieska field population tended to be isolated from the other six locations. More than half (56%) of the Prieska isolates were clonal (representing more than one isolate with the same genetic composition) and are represented as larger red circles in the network. No other field population was as cohesive as or as clearly resolved. All of the isolates from the South African FHB population clustered closer to the *F. graminearum* lineage 7 reference isolates than to representatives of the other lineages, except for three Orania isolates, which clustered with representatives of lineage 3.

Isolates from Orania appeared to be in the centre of the minimum-spanning network (Figure 4.10) with isolates from Cradock at the ends of many clusters. Isolates from Barkly West and Orania often clustered together while isolates from Greytown were scattered throughout the network. Most isolates from Douglas clustered together. Isolates from Potchefstroom and Cradock usually were closely associated with each other. The isolated Prieska population, following closer investigation of the network, required more recombination to generate the observed network structure, as indicated by the five loops illustrated in Figure 4.11. Almost no recombination events were observed in the other field populations.

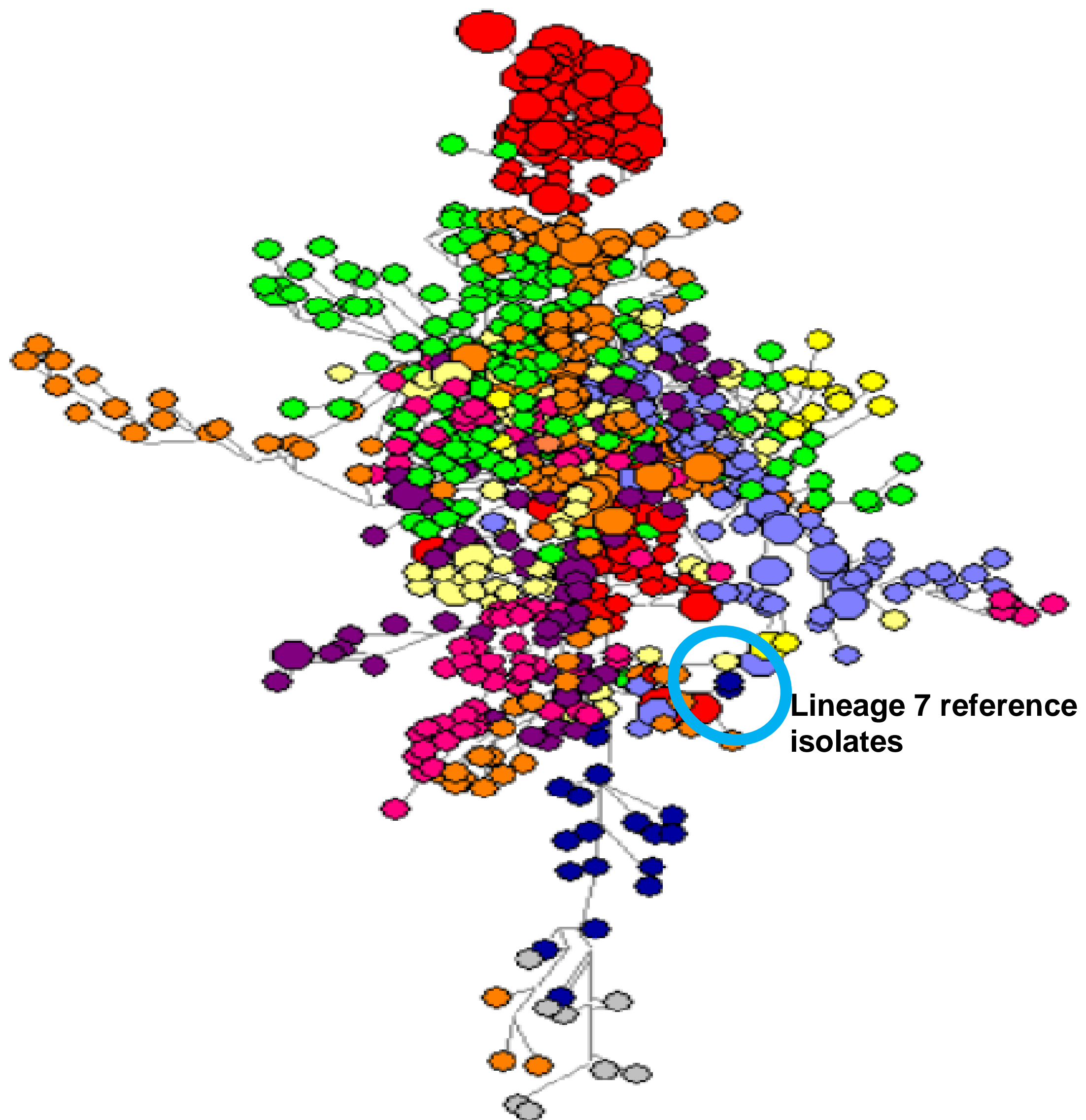

Figure 4.9 Original minimum-spanning network representing the genetic structure of the *F. graminearum* population of South African irrigation areas.

● Prieska ● Barkly West ● Orania ● Douglas ● Greytown ● Cradock ● Potchefstroom ● Lineages ● Outgroups.

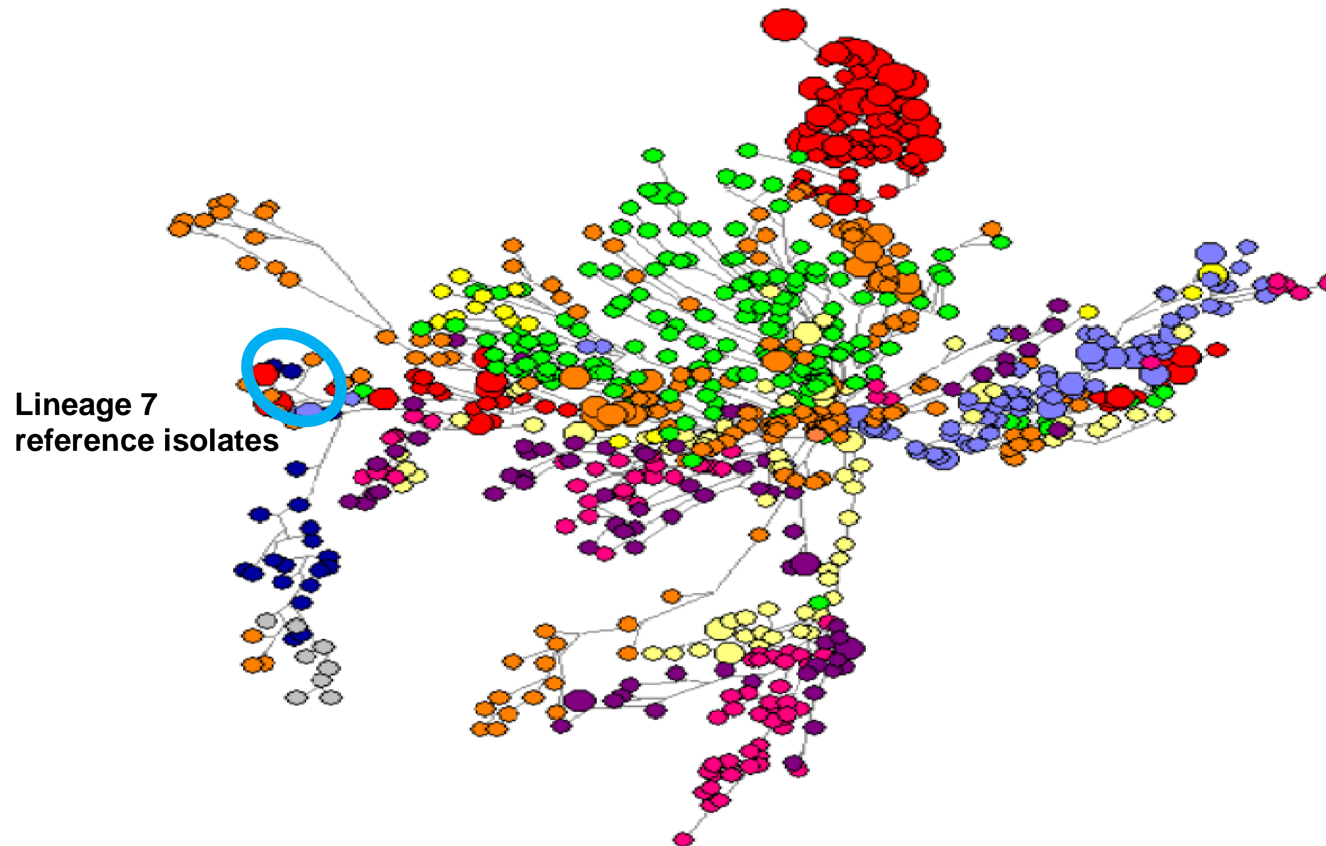

Figure 4.10 Modified minimum-spanning network representing the genetic structure of the *F. graminearum* population of South African irrigation areas.

● Prieska ● Barkly West ● Orania ● Douglas ● Greytown ● Cradock ● Potchefstroom ● Lineages ● Outgroups.

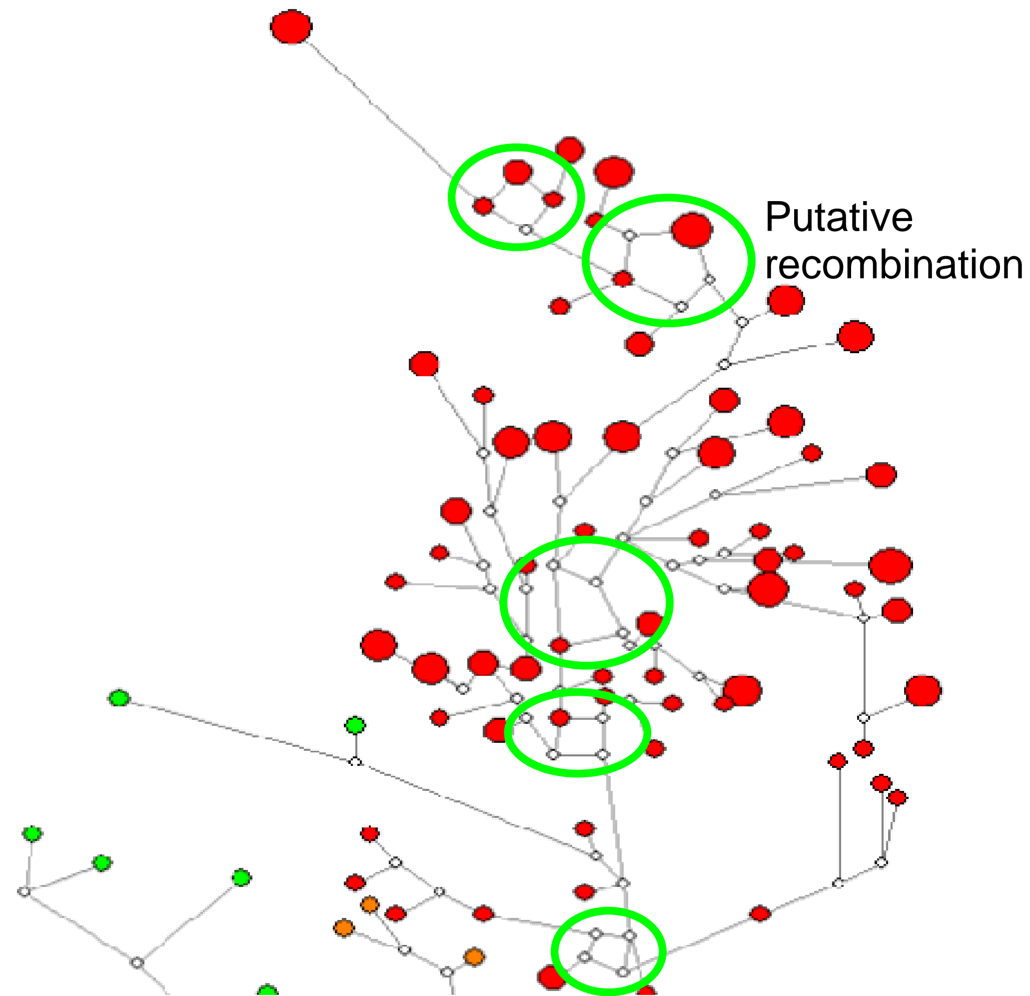

Figure 4.11 Putative recombination as detected in the Prieska field population using minimum-spanning network analysis. Recombination indicated with green circles.

4.4 Discussion

Fusarium graminearum isolates from Canada, North America, Asia and parts of Europe have been characterised to determine the level of genetic diversity within and between populations (Dusabenyagasani *et al.*, 1999; Zeller *et al.*, 2003; 2004; Mishra *et al.*, 2004; Fernando *et al.*, 2006; Guo *et al.*, 2008; Ward *et al.,* 2008). The genetic diversity observed in *F. graminearum* populations depends on the techniques used and the size of the population evaluated. In some cases, e.g. Ouelett and Seifert (1993), both the technique, RFLPs, and the population size limited the genetic diversity observed. AFLP analysis, a highly reproducible and polymorphic fingerprinting technique, was used in numerous population genetic studies to evaluate genetic differences across large parts of the pathogen's genome in a single assay, with no prior sequence information. AFLP analyses of *F. graminearum* populations from different countries and continents indicated that some of these populations contained isolates from more than one lineage, while other *F. graminearum* populations were composed of isolates that belong to a single phylogenetic lineage. Most of these investigations documented high levels of genetic diversity in *F. graminearum* within field populations sampled across large geographic regions (Akinsanmi *et al.*, 2004; Naef and Défago, 2006; Miedaner *et al.,* 2008). Limited studies of the genetic structure of *F. graminearum* populations that cause FHB have been conducted in South Africa (Boshoff, 1996). AFLPs were used in the current study to evaluate and characterise the genetic structure of FHB populations in South Africa and to associate South African isolates with previously described phylogenetic lineages.

To date, South African FHB populations have not been evaluated for genetic structure or association with known lineages. An earlier study by Boshoff (1996) on a group of 73 South Africa *F. graminearum* isolates measured genetic diversity, but did not associate the isolates with a lineage.

O'Donnell *et al.* (2000) placed a few *F. graminearum* isolates from South Africa, which were not associated with FHB, into lineage groups 3 and 5 and referred to group 3 as an African lineage. Two types of dendrograms, rooted and unrooted, were constructed to analyse the genetic diversity amongst and within South African *F. graminearum* populations from centre-pivot irrigation areas based on AFLP data. In both dendrograms, all of the South African *F. graminearum* isolates clustered with the lineage 7 reference

isolates received from O'Donnell (O'Donnell *et al.*, 2000; 2004; Starkey *et al.,* 2007; Yli-Mattila *et al.,* 2009), but none of them were 100% genetically similar to the lineage 7 reference isolates.

These observed clustering patterns are quite different from the results reported by O'Donnell *et al.* (2000). However, the possibility that lineage 3 occurs in South Africa is not discredited by the detection of lineage 7 in the current study. Indeed, the lineage 3 isolates evaluated by O'Donnell *et al.* (2000) were not associated with FHB. Three of the Orania isolates in the minimum-spanning network clustered with reference isolates from lineage 3 and could support the occurrence of lineage 3 in South Africa.

Lineage 7 is regarded to be predominant in the Northern hemisphere (O'Donnell *et al.*, 2000), but has been reported in most of the wheat-grown areas of the world (Miedaner *et al.*, 2008). Most of the other lineage groups were reported to be restricted to certain areas e.g. lineage 6 to Asia (Gale *et al.*, 2002), of which recent reports indicated its presence in USA and Brazil (Gale *et al.*, 2005), lineage 3, which is now present in Europe and the most southern parts of North America as well as lineage 4 (Tóth *et al.*, 2005), which is present in North America and Europe (Miedaner *et al.,* 2008).

The widespread occurrence of lineage 7 suggests that its migration is more rapid and successful than some of the other lineages. South Africa imports large amounts of wheat which could play a role in the transportation and dispersal of FHB from other countries, leading to lineage 7's dominance in wheat in South Africa. Currently South Africa has no legal basis to screen imported wheat for disease infection, which could ease the migration of FHB causing fungi to South Africa from other countries. Insights into the dynamics of the FHB population in South Africa and the possibility of breeding for resistance against FHB would be advantageous for South Africa, and help with the management of the disease in other countries.

Most of the genetic variation in *F. graminearum* (84%) is found common to all of the populations, with a much lower percentage (16%) of genetic variation limited to one or a few populations, suggesting significant gene flow amongst the seven field populations. Some of the earlier studies from outside South Africa found low genetic diversity within groups and a higher diversity amongst groups. However, most of the literature paints a

picture of *F. graminearum* populations' low genetic differentiation between populations and high levels of genetic diversity within groups.

Fusarium graminearum is homothallic and only reproduce sexually without a partner, suggesting a limited amount of gene flow and therefore low levels of genetic diversity. However, Zeller *et al.* (2003; 2004) stated that only a small amount of sexual recombination is necessary to contribute to high genetic diversity. The reproductive barrier of this FHB pathogen is considered important for the delineation of species in the *F. graminearum* complex. Thus, sexual recombination is regarded as a source of the observed genetic diversity within the South African *F. graminearum* field populations, hence the high genetic diversity (84%) within populations. This data illustrated that recombination has an influence on the population dynamics of the FHB pathogen; therefore recombination can be regarded as a mechanism to achieve and maintain high genetic diversity and gene flow within populations. The analyses of the PCA, NJ, population structure and minimum-spanning network all concluded that the seven field FHB populations are a mixture of different isolates, because of the integration of isolates from all locations except Prieska. The Prieska field population was indicated to be isolated and it contained a high percentage of clonal isolates. These results indicated that little sexual reproduction occurred within this population and that individuals in this population reproduced vegetatively.

The fixation index (F_{ST}=0.16) of the South African FHB population was higher than reported in previous studies from the USA (F_{ST}=0.04) (Zeller *et al.*, 2003; 2004) and Asia (F_{ST}=0.016-0.048) (Carter *et al.,* 2000; Gale *et al.*, 2002; Karugia *et al.*, 2009), but was still low enough to indicate that most of the genetic variation present could be found within individual field populations. Even though the genetic variation within these populations from the USA and Asia was indicated as high, the genetic evaluation across the different populations per region indicated low gene flow. Therefore the genetic variation across such populations in different regions was considered as low. The low F_{ST} values suggested that these populations, irrespective of population size and phylogenetic lineage groups (USA with a population size of 253 isolates in 2003 and 523 isolates in 2004, lineage 7; China with a population size of 225 isolates, lineage 6) belonged to larger randomly mating populations in the respective regions. The South African results are consistent with the genetic similarity observed amongst

F. graminearum populations collected from epidemic wheat fields in the USA. Isolates in these populations belonged to lineage 7 (Zeller *et al.*, 2003; 2004). The F_{ST} value as measurement of genetic diversity within and among pathogen populations provides important insights into the evolutionary processes including migration, mutation and genetic drift that influence the structure of the genetic variation of such populations. The genotypic variation within the South African field populations indicated that these populations might have different intraregional structures and/or pathological properties e.g. pathogenicity, aggressiveness or toxin profiles, which needs to be investigated in future studies.

FHB epidemics in South Africa usually are correlated with environmental conditions. The high pairwise comparison values of the genetic identity of populations were consistent with results obtained from the AMOVA. Zeller *et al.* (2003; 2004) and Schmale *et al.* (2006) suggested that low levels of linkage disequilibrium indicate that outcrossing is a common phenomenon between populations. Results from all statistical analysis methods used in this study confirmed the high genotypic diversity within populations at all seven locations. This might confirm the hypothesis of Zeller *et al.* (2003) of the occurrence of sexual recombination within field populations.

FHB has occurred in Prieska since the early 1990s. This population might be the oldest population even though the first FHB report was made in 1980 in North West, where no epidemic outbreaks were reported until 2006. *Fusarium graminearum* populations are regarded as highly flexible in adapting to their environment. This suggests that there might be lower levels of outcrossing in the Prieska population compared to other field populations and this being the reason why this field population tended to be isolated. Higher levels of outcrossing might reduce the clones in this population. Clonal genotypes within a population also suggest the independence of such a population. These clonal genotypes indicated that most individuals within the Prieska population may be reproduced vegetatively, which can be regarded as successful. These results indicated that variation in pathogen aggressiveness might occur within a population. Therefore the presence of the high number of clonal genotypes in Prieska population might be responsible for the isolated behavior of this population.

Based on the minimum-spanning network, Orania is the basal FHB population in South Africa. Individuals from this field population clustered with individuals from all of the other six locations, which suggest that populations at the other six locations might result from the migration of inoculum from Orania. Schmale *et al.* (2006) suggested that FHB inoculum may originate from multiple locations over large geographic areas. Orania also was the only population containing individuals that clustered with the lineage 3 reference isolates. Thus, this population may have been the origin of inoculum dispersal to other locations, including the isolates identified as lineage 3 by O'Donnell *et al.* (2000). It is not known whether members of lineage 5 and lineage 7 differ from one another in ways that might affect their ability to colonise wheat in the portions of South Africa that we sampled.

Individuals from the Cradock field population in the minimum-spanning network appeared to be at the end of most clusters, which indicate that this field population is the youngest FHB population in South Africa. The total South African FHB population can be considered a mixture of different isolates due to the diversity unravelled within the individual field populations. The high genotypic diversity within the South African population suggests that recombination occurs relatively frequently under field conditions. The high gene flow between populations from the different locations suggested that FHB was caused by a randomly mating population of *F. graminearum* in South Africa. These results also indicate the seven individual field populations to be subpopulations of a larger randomly mating population.

4.5 Concluding remarks

Two hypotheses were tested with the current study, where the first hypothesis stated that all South African *F. graminearum* isolates belongs to lineage 3 and 5 as proposed by O'Donnell *et al.* (2000). Results of this study reject this hypothesis, because most *F. graminearum* isolates from this study clustered with lineage 7 reference isolates. The possibility that South African populations contain more than one lineage cannot be excluded since three isolates from the Orania field population clustered with the lineage 3 reference isolates in the minimum-spanning network.

Hypothesis two, i.e. that the South African FHB population can be regarded as a randomly mating population has been confirmed by the low F_{ST} value and the

recombination detected. Even though the proportion of the genetic variation within individual populations was indicated as high, the genetic diversity between the seven field populations from seven different locations indicated similar genetic backgrounds. These results indicated that geographical distance could not be related to increase and/or decrease of genetic diversity among these populations.

Most previous studies found high levels of genetic diversity in individual field populations of *F. graminearum* or populations sampled across a broad geographical scale (Zeller et al., 2003; 2004; Akinsanmi *et al.,* 2004; Naef and Défago, 2006; Schmale *et al.,* 2006). Miedaner *et al.* (2008) reviewed these studies spanning three continents and found that the proportion of the total genetic diversity within individual populations ranged from 30-100%. The high genetic diversity within populations can be explained by the presence of recombination due to ability of *F. graminearum* to reproduce sexually without a partner. Sexual reproduction within the species confirmed previous studies (Leslie and Bowden, 2008) that *F. graminearum* should be regarded as one biological species based on the biological species concept. Low F_{ST} values indicate that the allele frequencies within each population are similar contributing to the FHB population of South Africa being regarded as homogeneous. Therefore it can be concluded that the FHB population of South Africa form part of a worldwide randomly mating population.

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CHAPTER 5
General conclusions

Fusarium head blight, one of the most ruinous wheat diseases worldwide, has also become one of the most intensively researched wheat diseases in the world. FHB had been responsible for substantial (>2.7 billion dollars by 2000) global economic losses in yield and quality. In South Africa, farmers are planting less wheat due to low wheat prices, high production costs and disease risks, of which FHB is a high priority. Consequently more wheat will be imported into than exported from South Africa in 2011. Along with natural disasters and weather conditions, FHB is a contributory factor and economic disincentive for wheat production in South Africa.

FHB can be caused by several *Fusarium* species, e.g., *F. avenaceum, F. culmorum, F. crookwellense, F. langsethiae, F. poae* and *F. graminearum*. *Fusarium graminearum,* however, had been identified and characterised as the most prominent and in some regions the dominant FHB-causing species, worldwide. This pathogen has been responsible for most, if not all of the FHB epidemics in South Africa during at least the past two decades. The pathogen also produces mycotoxins, which can be harmful to both human and animal health.

The main reason to study the South African head blight population, which causes FHB in the centre-pivot irrigated wheat-growing areas, is based on the search for management strategies to minimise disease incidence and severity. Knowledge on the genetic structure of the pathogen population may provide insights into the population dynamics of the pathogen. Genetic differentiation in *F. graminearum* can be based on the toxins produced, AFLP profiles and DNA sequences of thirteen known lineages/species within *F. graminearum*.

Based on morphological identification of *Fusarium* species from seven locations in the centre-pivot irrigated wheat-growing areas of South Africa six *Fusarium* species were identified. Only three of the six species, *F. graminearum, F. culmorum* and *F. crookwellense,* had previously been associated with FHB in South Africa. In the present study, *F. graminearum* was found in high frequencies and was the primary causal pathogen of FHB at all seven locations.

In South Africa, *F. graminearum* usually dominates in the warmer areas, while *F. culmorum* and *F. crookwellense* are more common in the less temperate regions. This characterisation was based on an evaluation of <100 isolates more than ten years ago in a study that was both preliminary and insufficient. *Fusarium culmorum* and *F. crookwellense* were found only in the less temperate wheat-growing locations (Greytown, Potchefstroom and Cradock) included in the present study, but even in these locations, *F. graminearum* dominated. Increased frequency of *F. graminearum* relative to *F. culmorum* in several European countries has been attributed to increased maize production and possible climatic temperature changes. These reasons could explain the current observations in South Africa, since maize production also has increased in all farming regions in South Africa and temperature changes are a phenomenon occurring worldwide.

As part of the evaluation of the genetic structure of the *F. graminearum* population of South Africa, the tricothecene genotype of each *F. graminearum* isolate was determined. These genotypes indicate the potential for mycotoxin production of each pathogen species. DON producers dominated in three of the six *Fusarium* species detected in South Africa. Most of the *F. graminearum* isolates (89%) were DON producers, while isolates of *F. equiseti* had a ~ 50:50 DON:NIV producing potential. Isolates of *F. culmorum* and *F. crookwellense,* associated with FHB in the less temperate locations of South Africa, usually were NIV producers. *Fusarium culmorum* also contained isolates that could potentially produce DON, along with the *F. graminearum* isolates identified in these less temperate locations. The mycotoxin produced by a fungal isolate influences its pathogenicity. DON producers usually are more pathogenic to plants than are NIV producers. The high frequency of DON producers is consistent with the hypothesis that *F. graminearum* is the primary causal agent of FHB in South Africa, as well as providing an explanation for the displacement of other *Fusarium* species in the less temperate regions.

AFLPs were used to unravel the genetic structure of the South African FHB population at DNA level, concentrating only on *F. graminearum* isolates. A total of 793 *F. graminearum* isolates were analysed for placement against 26 reference isolates obtained from Dr. Kerry O'Donnell. The 26 reference isolates included representatives of the nine phylogenetic lineages and eight outgroups.

In these analyses, South African *F. graminearum* isolates clustered with the lineage 7 reference isolates, although none was identical to the lineage 7 reference isolates. Isolates from lineages 3 and 5 have been reported from South Africa, but neither of these lineages is associated with wheat. However, lineage 3 had recently been isolated from infected wheat in Mexico. Lineage 3 isolates from South Africa has been isolated from maize, which indicates that these isolates could increase in a maize/wheat rotation, as maize stubble is thought to be the primary source of inoculum in wheat fields. Lineage 5 isolates were obtained from *Acacia mearnsii*, a plant not always found in the South African wheat-growing regions.

Lineage 7 occurs in most of the wheat-growing regions in the world, making it the most commonly recovered lineage. Thus lineage 7 also may be easily spread from one location to another. This lineage also may have accompanied the large amounts of wheat imported into South Africa from other wheat-growing areas in the world, where lineage 7 is known to occur. Thus breeders will be able to use sources for resistance against FHB in their breeding programmes from most other parts of the world.

Isolates from lineage 7 dominated the South African FHB population, but this population contained significant genotypic variation. The genotypic variation is consistent with outbreeding occurring under field conditions in South Africa.

The Prieska population tended to be isolated from the rest of the populations. Many aspects, e.g., population composition and relatively high frequency of clones, might have contributed towards the isolation of Prieska isolates. Even though FHB is not seed borne in the sense that the pathogen is systemic, seed is a means of spreading the pathogen to new locations. The potential of finding a location in the wheat and maize producing areas of South Africa, without FHB, is relatively slim, but variation within the FHB population could be attributed to the movement of seed.

Prieska, due to its isolation and long distance from most other wheat producing areas, mainly uses seed produced locally which limits the influx of new isolates. This pattern also could explain why the Prieska population contains a higher number of clones than do the other locations. Clonal populations are considered successful if co-adapted gene complexes are transferred directly from the parents to the offspring.

Douglas, the closest site to Prieska, is a known Karnal bunt location, which prevents the movement of seed from this area to any other location without Karnal bunt, although seed may be imported to this area. Since seed production for Douglas also usually occurs locally and this region seldom exports seed to the other wheat-growing regions of South Africa, the exchange of *F. graminearum* isolates with the other regions also is limited. The presence of variation in the Douglas *F. graminearum* population indicates some exchange of isolates, but in low frequencies.

Wheat seed is not produced in Potchefstroom and Cradock so these regions import seed from other locations, which could increase the variation in their populations. Greytown is used as a site by many seed companies for field trials and seed production, which could explain the observed variation in its population. A lot of seed movement occurs between Orania, Barkly West and Douglas, mostly towards Douglas. This exchange enables *F. graminearum* isolates to move easily from one location to another. Therefore the observed variation of isolates in these locations is possible.

Data obtained through AFLP analysis indicated significant gene flow amongst the different field populations with high levels of genetic variation within populations. It also indicated that, although the Prieska population tended to be isolated, all of the populations were a mixture of different isolates and that the seven populations formed part of a larger randomly mating population. These results are consistent with the hypothesis that one of the long distance dispersal methods of FHB inoculum in South Africa might have been the movement of seed from one location to another across the total investigated FHB population.

The low diversity between populations from the seven locations suggests a randomly mating FHB population for South Africa due to similar allele frequencies. Knowledge gained from analyses of the genetic structure of this population might assist with the development of resistance breeding strategies against FHB. This will provide an incentive to farmers to plant wheat, thus improving wheat production in South Africa.

Future research should focus on the new information that became available from this study. One aspect that needs further investigation is the possibility of the change of the dominant FHB causal species in the less temperate regions of South Africa caused by

climate changes and increased maize production. Samples should be collected from a larger number of locations in the less temperate regions of South Africa and the survey should include more seasons and climatic data. Another aspect that needs further investigation is the influence of mycotoxins on the pathogenicity of FHB pathogens. This might also have played a role in the increased frequency of *F. graminearum* in the less temperate regions. Furthermore, the transportation of inoculum by water should be investigated to establish the spread of FHB in South Africa, along with the development and age of the population.

Summary

Wheat, after maize, is globally the most widely cultivated cereal crop and the staple diet of most people around the world. In South Africa, wheat, as the second most important grain crop, is produced in all nine provinces under either irrigation or dryland conditions. Diseases, however, restrain wheat production due to yield and grain quality reduction. Fusarium head blight (FHB) is a globally devastating fungal disease of wheat, primarily caused by *F. graminearum*. This disease is not only responsible for economic losses due to yield and quality reduction, but also contaminates the grain with trichothecene mycotoxins. This study concentrated on the genetic structure of the FHB causing populations present in seven South African wheat-growing areas under centre-pivot irrigation (Prieska, Barkly West, Orania, Douglas, Greytown, Cradock and Potchefstroom). Fusarium graminearum was identified as the dominating causal agent of FHB in South Africa and the tricothecene genotype of these isolates indicated a higher frequency of DON rather than NIV producers. DON producers have been described as more pathogenic to plants than NIV producers. This might explain the high DON frequency as well as the displacement of other *Fusarium* species in the less temperate regions of the South African wheat-growing areas with F. graminearum. AFLP analysis was used to place 793 South African *F. graminearum* isolates into the phylogenetic groups as described by O'Donnell and associates. South African *F. graminearum* isolates clustered with the lineage 7 reference isolates, although previous reports indicated the presence of lineages 3 and 5 in South Africa. Even though the currently studied population was dominated by lineage 7 isolates, significant genetic variation was detected. The observed genetic variation within individual populations was higher than the genetic variation between populations. The genotypic variation was consistent with outbreeding occurring under field conditions in South Africa. Although the Prieska population tended to be isolated, with a relatively high frequency of clones, the low genetic variation between all seven field populations suggested a larger randomly mating FHB population for South Africa due to similar allele frequencies. These results are consistent with previous reports on the genetic structure of FHB populations in other parts of the world. Knowledge gained from analyses of the genetic structure of this population might assist with the development of effective control strategies, i.e. resistance breeding against FHB, using resistance sources from other lineage 7 regions.

This will provide an incentive to farmers to plant wheat, thus improving wheat production in South Africa.

Keywords: Amplified fragment length polymorphism (AFLP), deoxinivalenol (DON), *Fusarium graminearum,* genetic variation, irrigation, mycotoxins, wheat scab

Opsomming

Koring, naas mielies, is die mees verboude graangewas asook stapelvoedsel wêreldwyd. Koring, as tweede belangrikste graangewas, word in al nege Suid-Afrikaanse provinsies, hetsy onder besproeiing of droëlandtoestande, verbou. Koringproduksie word egter nadelig deur die teenwoordigheid van koringsiektes, wat opbrengs en graankwaliteit beïnvloed, geraak. Fusarium aarskroei is wêreldwyd as 'n vernietigende swamsiekte bekend, wat hoofsaaklik deur *F. graminearum* veroorsaak word. Hierdie koringsiekte is nie net vir ekonomiese verlies as gevolg van verlies van opbrengs en graankwaliteit verantwoordelik nie, maar kontamineer die graan met trichothecene mikotoksiene. In hierdie studie is klem op die genetiese struktuur van die fungus populasies verantwoordelik vir aarskroei in sewe Suid-Afrikaanse koringverbouingsgebiede onder besproeiing (Prieska, Barkley Wes, Orania, Douglas, Greytown, Cradock en Potchefstroom) gelê. Fusarium graminearum is as die patogeen wat hoofsaaklik vir aarskroei in Suid-Afrika verantwoordelik is, geïdentifiseer. Mikotoksien toetse van die F. graminearum isolate het daarop gedui dat 'n hoër frekwensie isolate 'n neiging tot DON produksie eerder as NIV produksie toon. Vorige studies het getoon dat individue met die DON genotipe patogenies meer aggressief is as individue met die NIV genotipe. Dit kan dalk die hoë DON frekwensie in die Suid-Afrikaanse aarskroeipopulasie asook die verplasing van ander Fusarium spesies met F. graminearum, veral in die koeler Suid-Afrikaanse koringverbouingsgebiede, verklaar. AFLP (geamplifiseerde fragment lengte polimorfisme) analises is vir die plasing van 793 Suid-Afrikaanse F. graminearum isolate in die filogenetiese groepe, soos beskryf deur O'Donnell en kollegas, gebruik. Die Suid-Afrikaanse *F. graminearum* isolate het saam met die filogenetiese spesie 7 verwysings isolate gegroepeer, alhoewel vorige studies slegs die teenwoordigheid van filogenetiese spesies 3 en 5 in Suid-Afrika aangetoon het. Betekenisvolle genetiese variasie is in die huidige populasie waargeneem, alhoewel filogenetiese spesie 7 isolate die populasie gedomineer het. Die waargenome genetiese variasie binne individuele populasies was hoër as variasie tussen populasies. Die waargenome genotipiese variasie was ooreenstemmend met verbastering onder natuurlike veldtoestande in Suid-Afrika. Alhoewel 'n groot aantal klone in die Prieska populasie waargeneem is en díe populasie geïsoleerd voorgekom het, het die lae genetiese variasie tussen die sewe individuele populasies aangedui dat die Suid-Afrikaanse aarskroei *F. graminearum* isolate weens van die ooreenstemmende alleel

frekwensies deel van ‘n groter willekeurige inteelpopulasie vorm. Hierdie resultate stem met die evaluasie van die genetiese struktuur van aarskroeipopulasies in ander dele van die wêreld ooreen. Inligting ingewin rakende die genetiese struktuur van die Suid-Afrikaanse aarskroeipopulasie kan moontlik gebruik word om effektiewe beheermaatreëls teen aarkroei te ontwikkel. Vir weerstand teen aarskroei kan byvoorbeeld weerstandsbronne uit ander filogenetiese spesie 7 gebiede gebruik word. Hierdie moontlike verbetering asook ontwikkeling van bestaande (fungisiede) en nuwe (weerstandsteling) beheermaatreëls teen aarskroei sal dit dan vir Suid-Afrikaanse koringboere die moeite werd maak om meer koring te plant en sodoende koringproduksie in Suid-Afrika verbeter.

Sleutelwoorde: Aarskroei, besproeiing, deoksinivalenol, *Fusarium graminearum*, geamplifiseerde fragment lengte polimorfisme, genetiese variasie, mikotoksiene

APPENDIX I

Composition of mediums for morphological characterisation of FHB isolates

Van Wyk's Agar (VWA)

1% (w/v) Glycerin
0.05% (w/v) L-Alanine
0.005% (w/v) Rose bengal
0.1% (w/v) Urea
0.1% (w/v) Pentachloronitrobenzene (PCNB)
1.5% (w/v) Agar bacteriological grade (BioLab®, Merck®)

VWA was prepared by suspension and autoclaving at 121℃ of the above-mentioned chemicals in distilled water. This medium is suitable for the selective isolation of *Fusarium* species from plant material, debris and seeds (Van Wyk *et al.*, 1986).

Potato Dextrose Agar (PDA)

3.9% (w/v) PDA bacteriological grade (BioLab®)

PDA was prepared by suspending the PDA powder in distilled water and autoclaving the suspension at 121℃. This carbohydrate-rich medium is suitable for determining colony morphology, pigmentation and growth rates of cultures of most *Fusarium* species under standard conditions.

Spezieller Nährstoffarmer Agar (SNA)

0.1% (w/v) Potassium phosphate (KH_2PO_4)
0.1% (w/v) Potassium nitrate (KNO_3)
0.05% (w/v) Magnesium sulphate ($MgSO_4{\bullet}7H_2O$)
0.05% (w/v) Potassium chloride (KCl)
0.02% (w/v) Glucose
0.02% (w/v) Sucrose
2% (w/v) Agar bacteriological grade (BioLab®, Merck®)

SNA was prepared by suspension and autoclaving of the above-mentioned chemicals in distilled water. This weak nutrient agar can be used for the identification, purification and maintenance of *Fusarium* isolates. It is suitable for sporulation of *Fusarium* isolates and promotes the formation of morphologically uniform macroconidia (Nirenberg, 1976).

Carnation Leaf-Piece Agar (CLA)

2% (w/v) Agar bacteriological grade (BioLab®, Merck®)

CLA is a natural substrate medium that is suitable for the purification and identification of *Fusarium* species. Sporodochia formed on the carnation leaves contain uniform macroconidia, needed for morphological identification. The agar was suspended in distilled water and autoclaved at 121°C. The medium was added to Petri dishes containing carnation leaves under sterile conditions (Fisher *et al.*, 1982).

The carnation leaf pieces were prepared from fresh carnation leaves. After harvest the leaves were dried in an oven until brittle and then sterilised by gamma irradiation. These leaves can be stored at room temperature for up to 12 months before use.

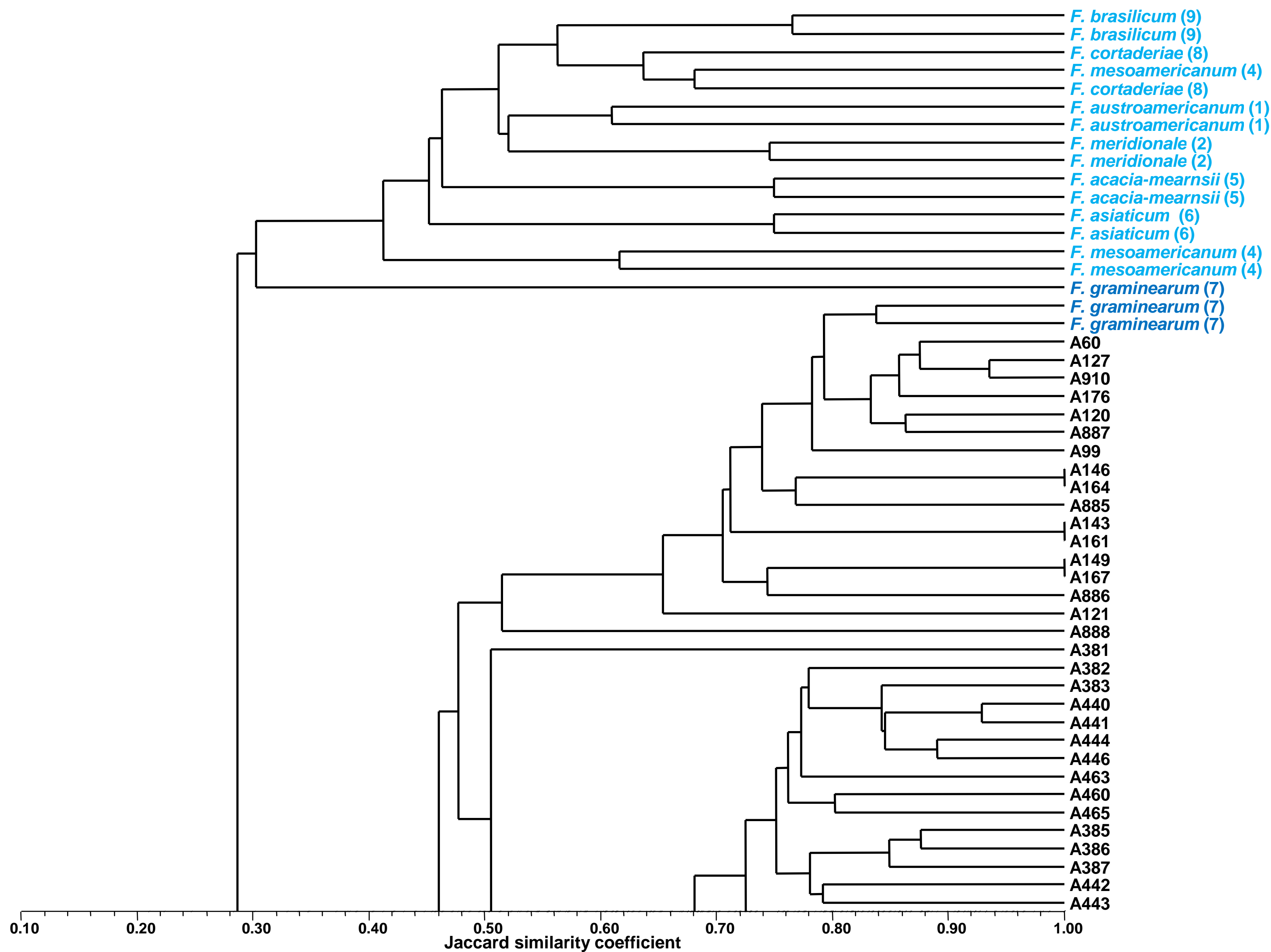
Water Agar (WA)

1.5% (w/v) Agar bacteriological grade (BioLab®, Merck®)

WA was prepared by suspending the agar powder in distilled water and autoclaving the suspension at 121°C. This medium is suitable for germinating *Fusarium* spores under standard sterile conditions.

APPENDIX II

Complete dendrogam of 793 South African isolates generated using NTSYSpc and UPGMA clustering using Jaccard's similarity coefficient, illustrating clustering of South African *F. graminearum* isolates with lineage 7. The different isolates from the nine lineages are indicated in light blue, lineage 7 reference isolates in dark blue and outgroups in red. Isolates from the South African FHB population are indicated in black and were numbered A1-A960. Numbers in brackets indicate known lineage groups.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F. austroamericanum (1)
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F. meridionale (2)
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F. acacia-mearnsii (5)
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F. graminearum (7)
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A910
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A887
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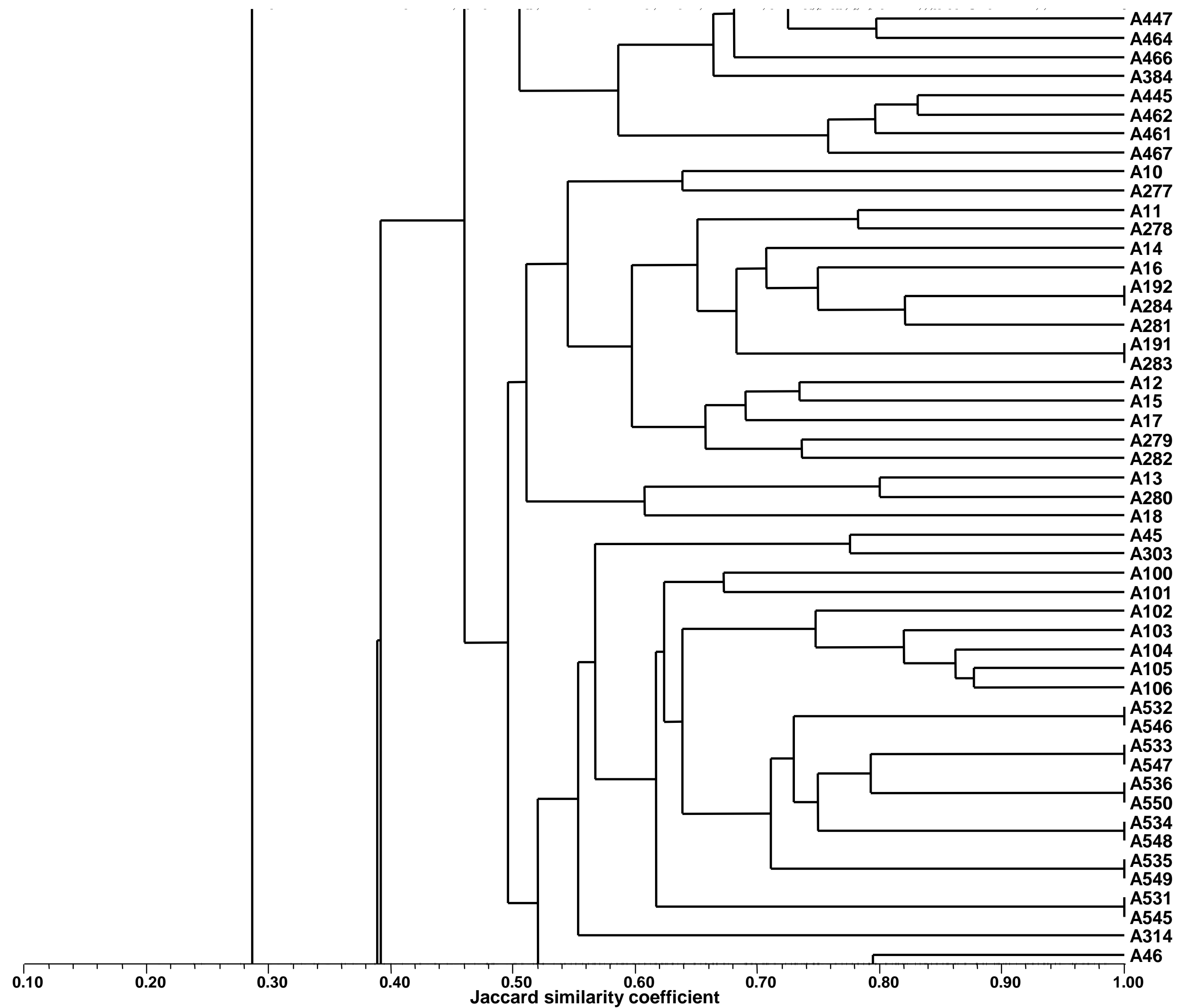
Jaccard similarity coefficient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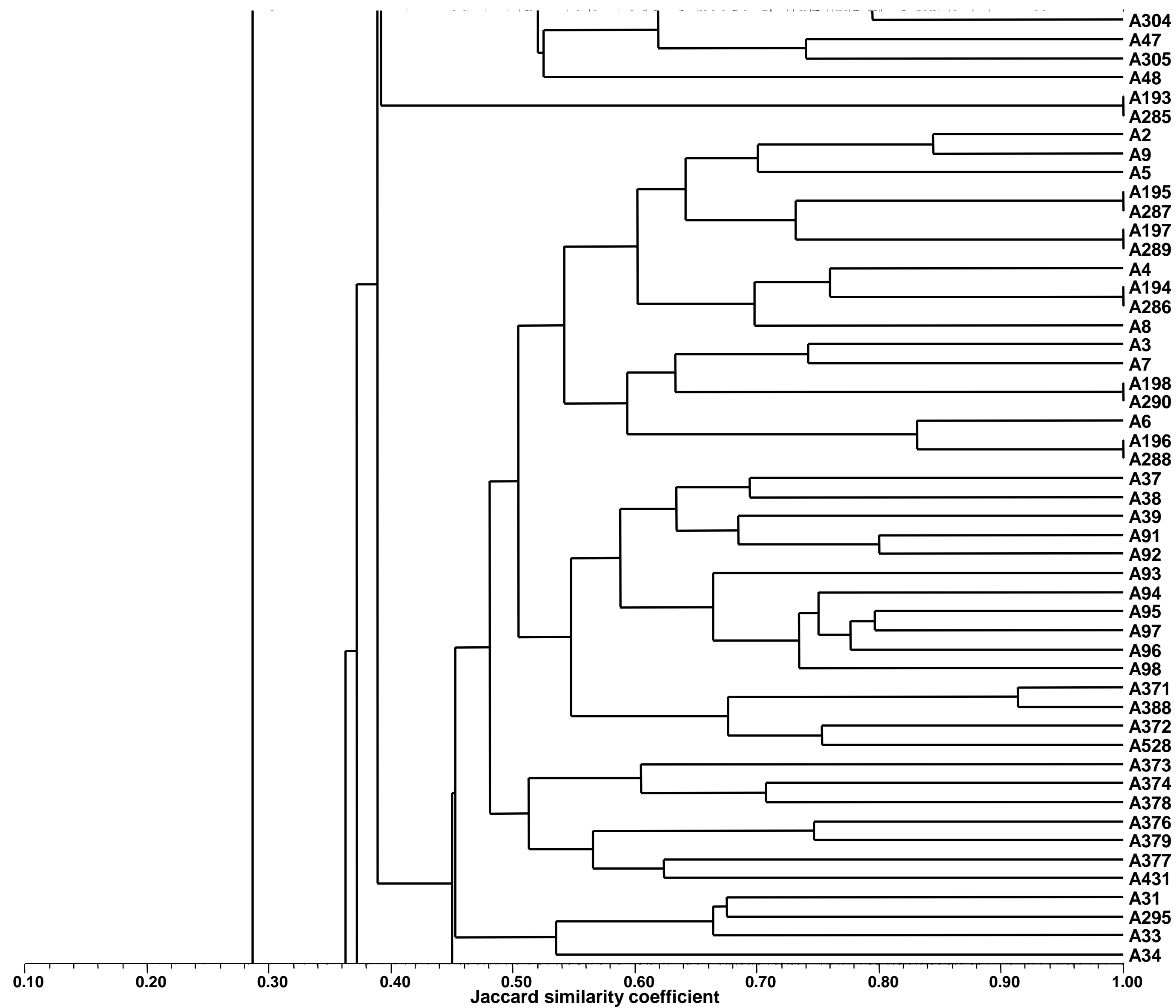
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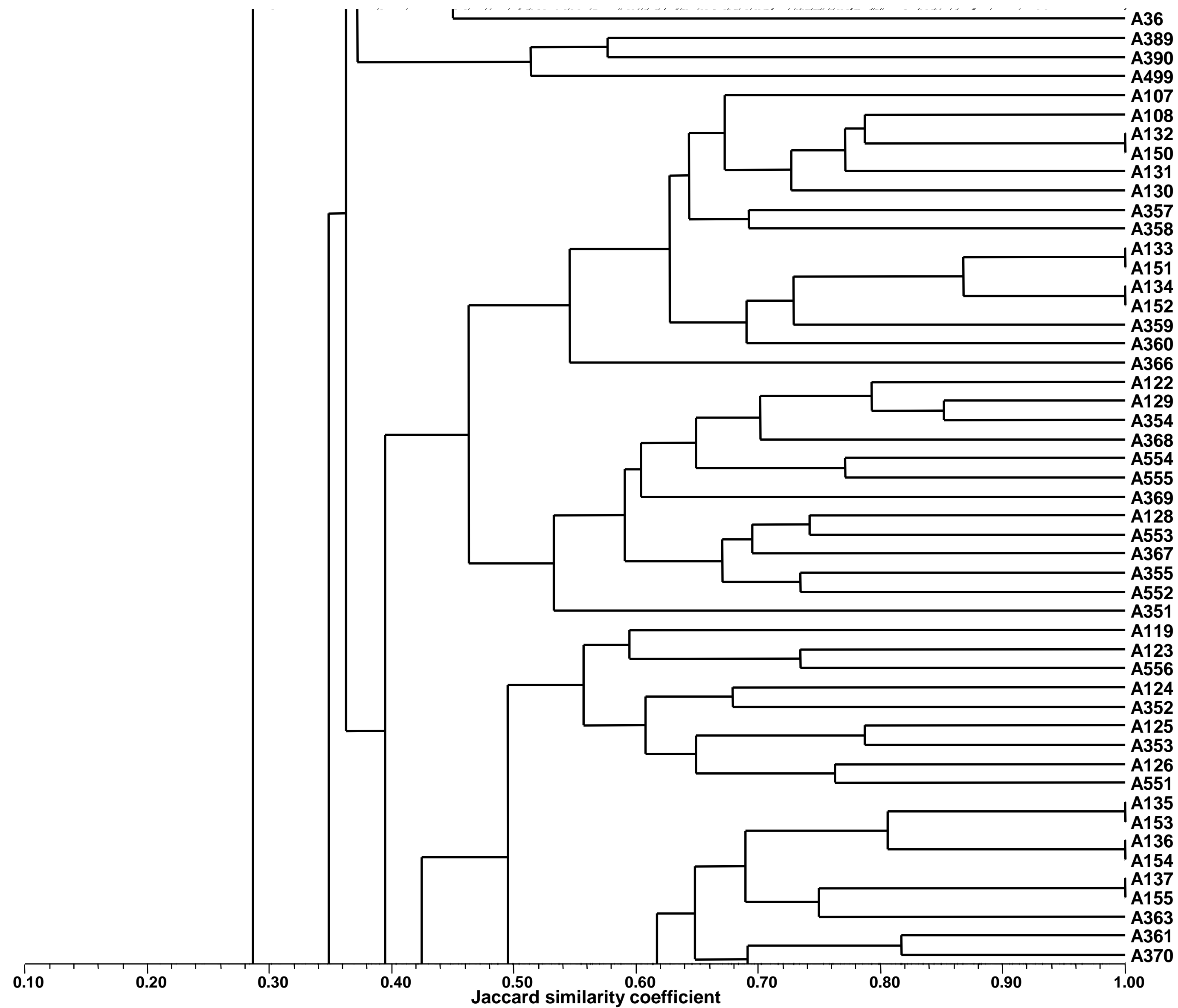
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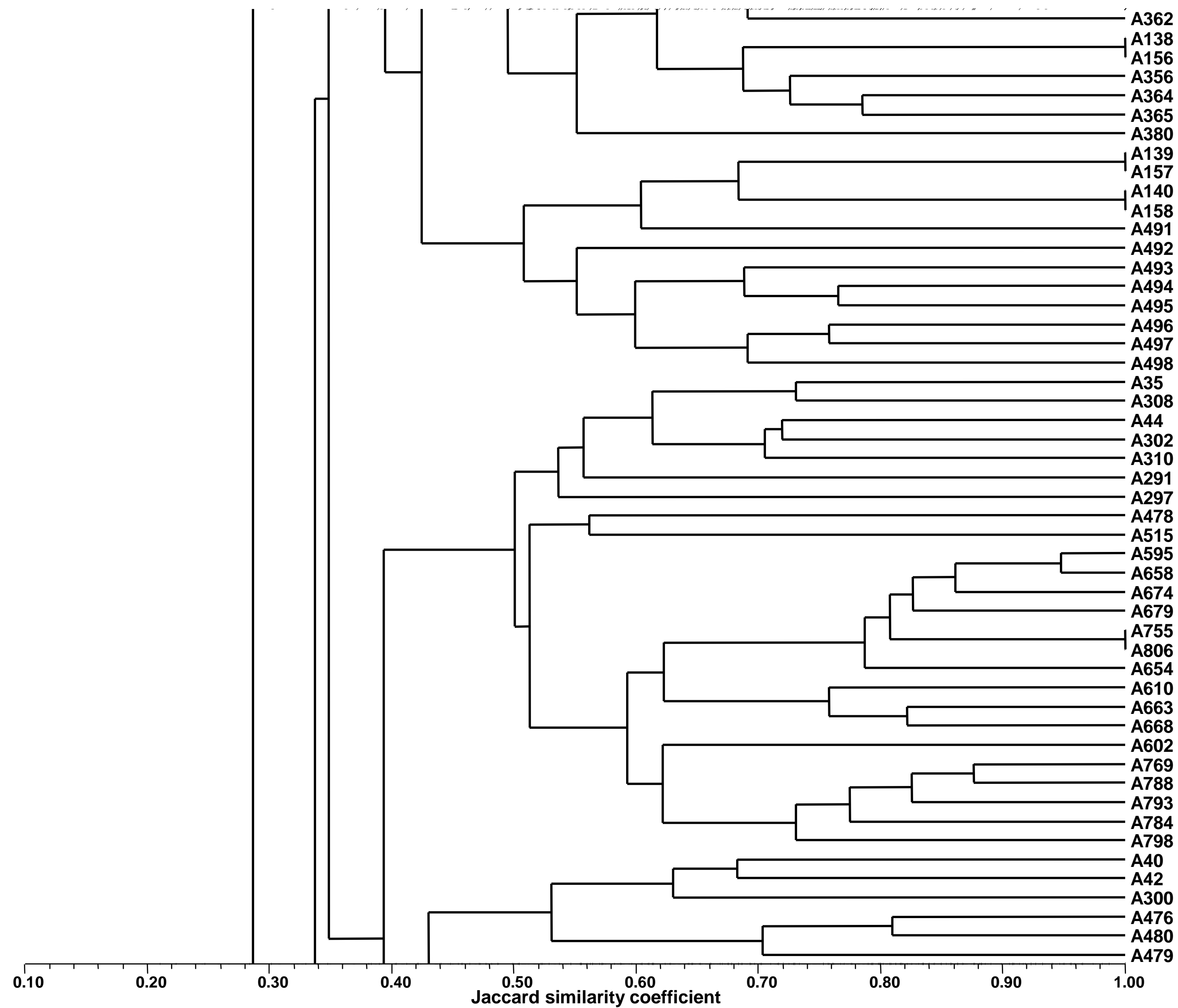
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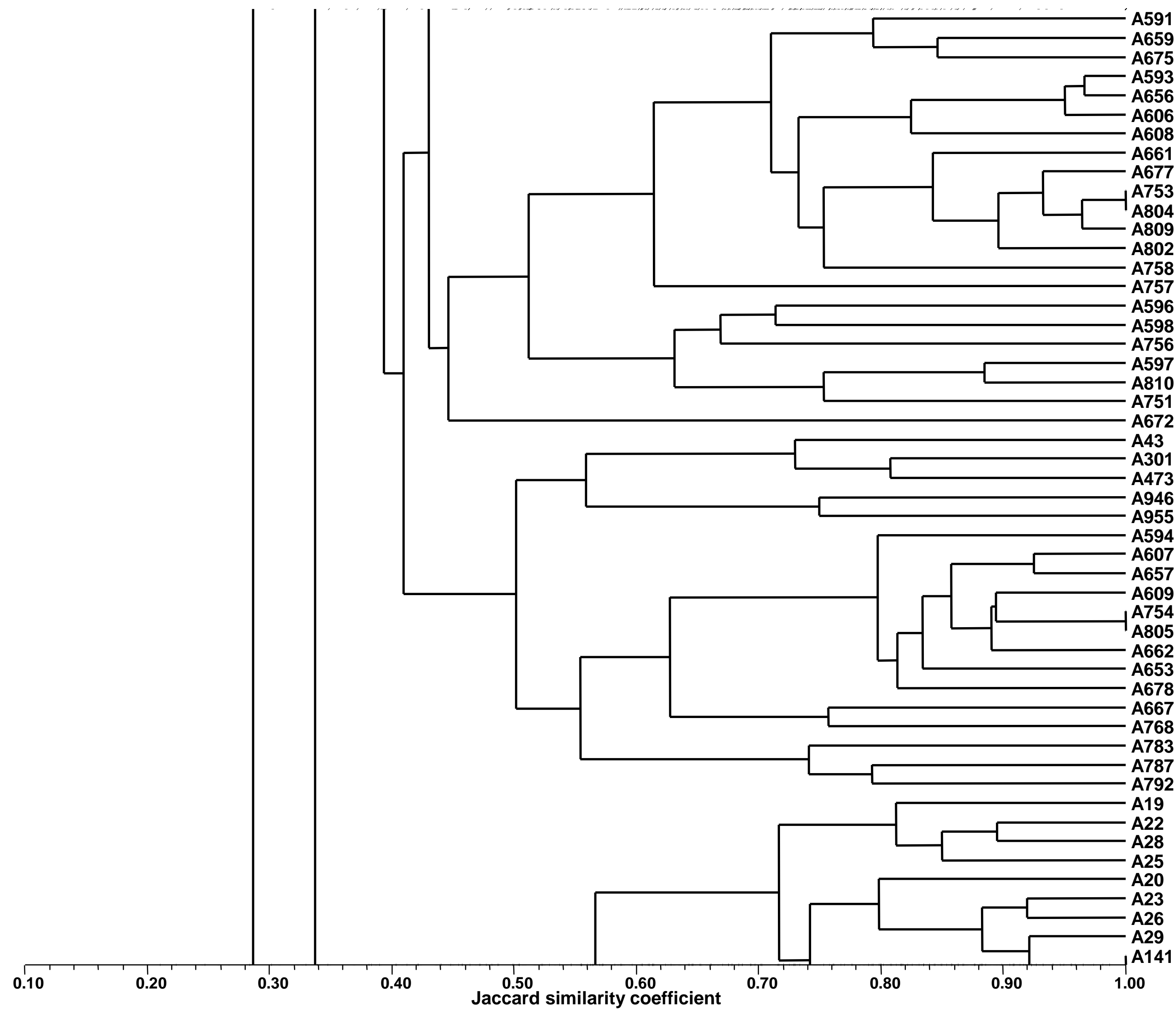
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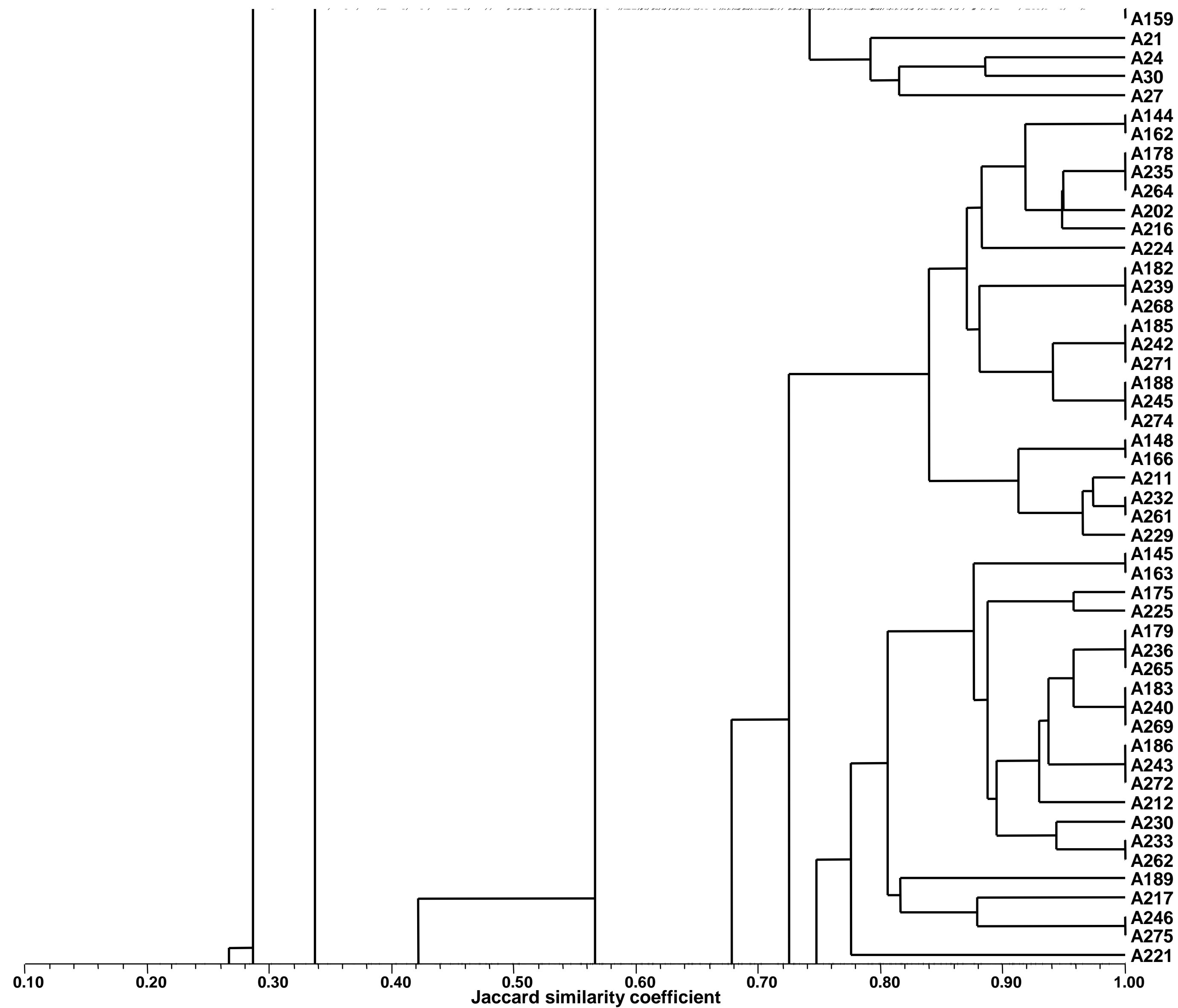
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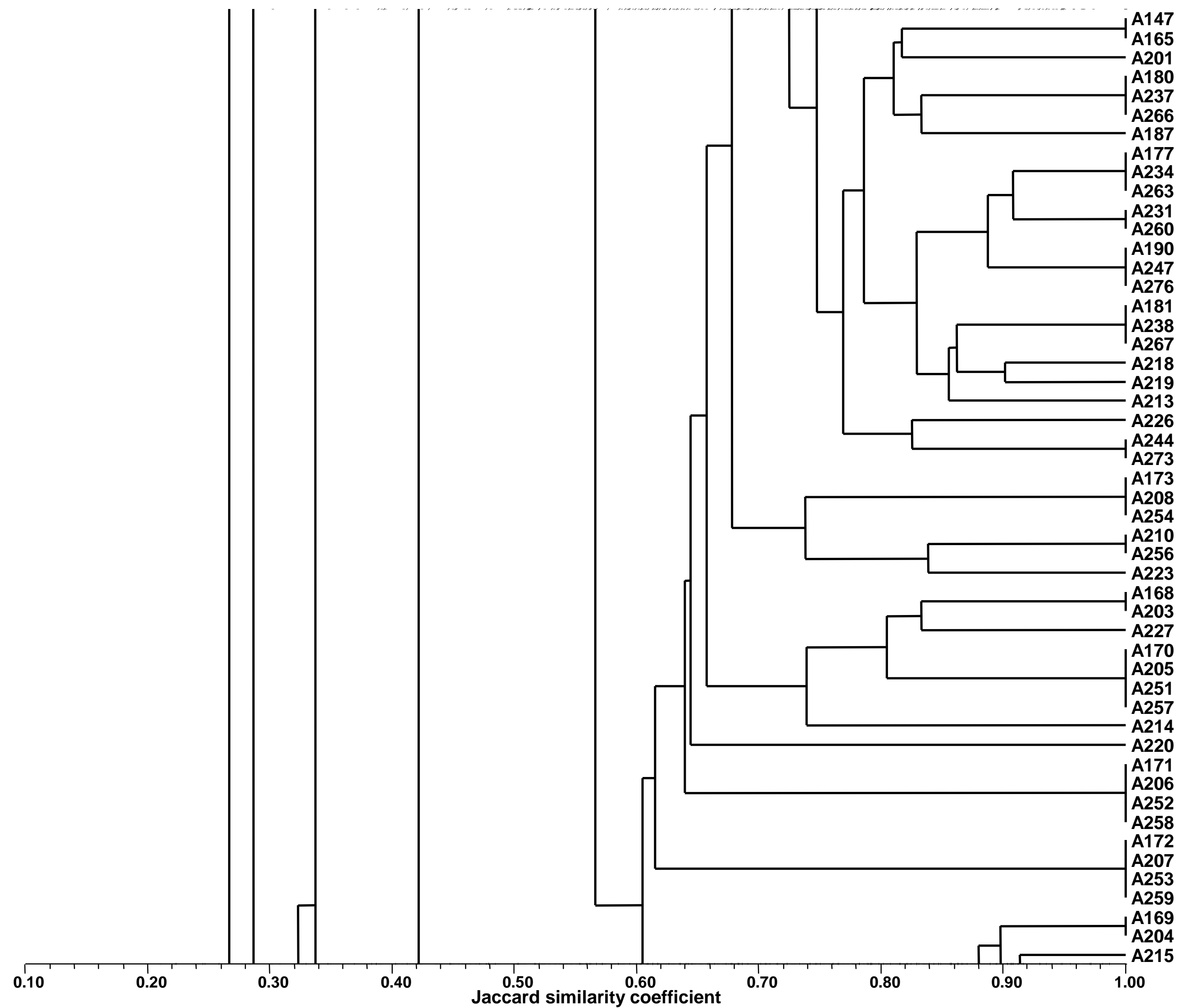
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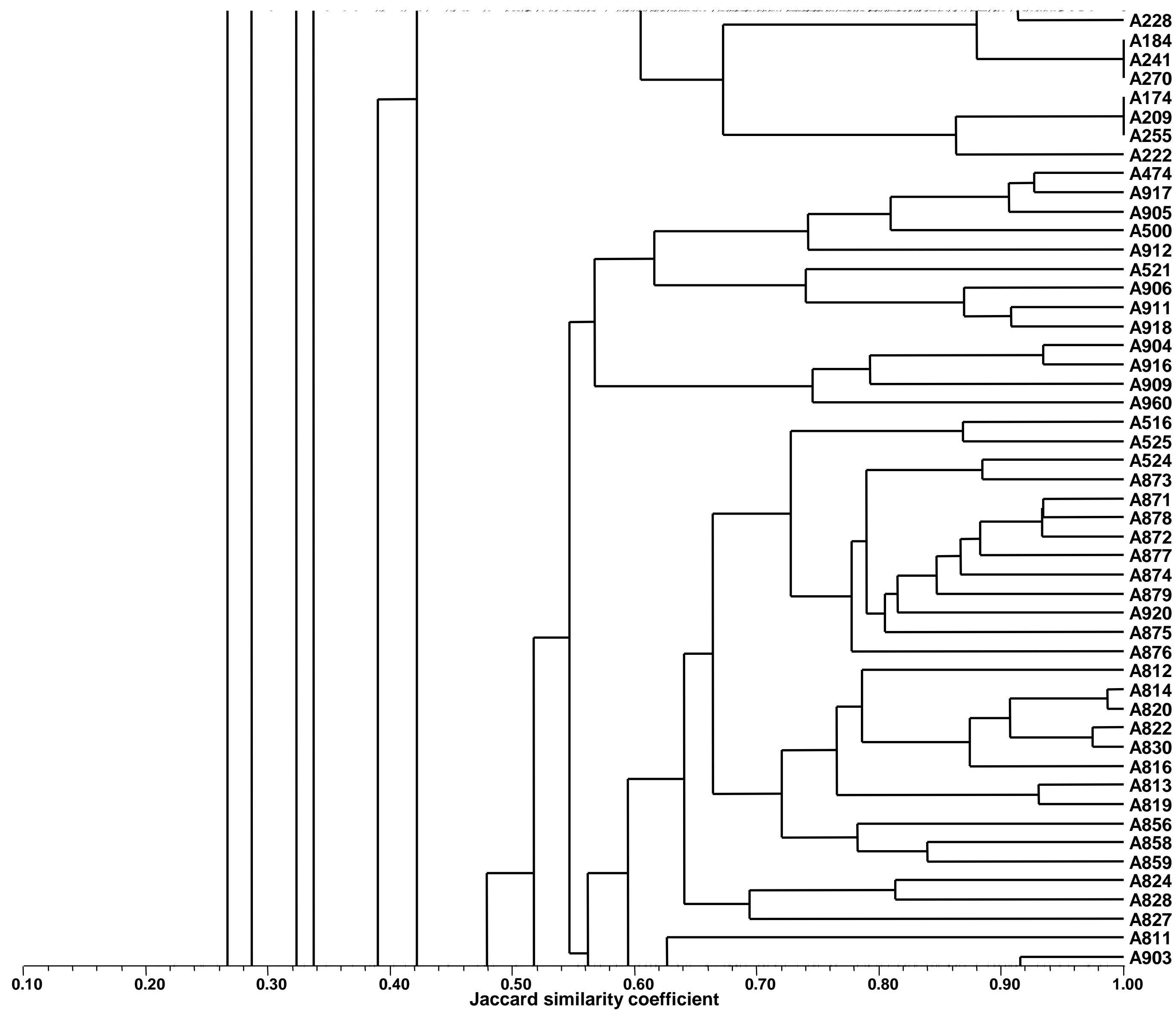
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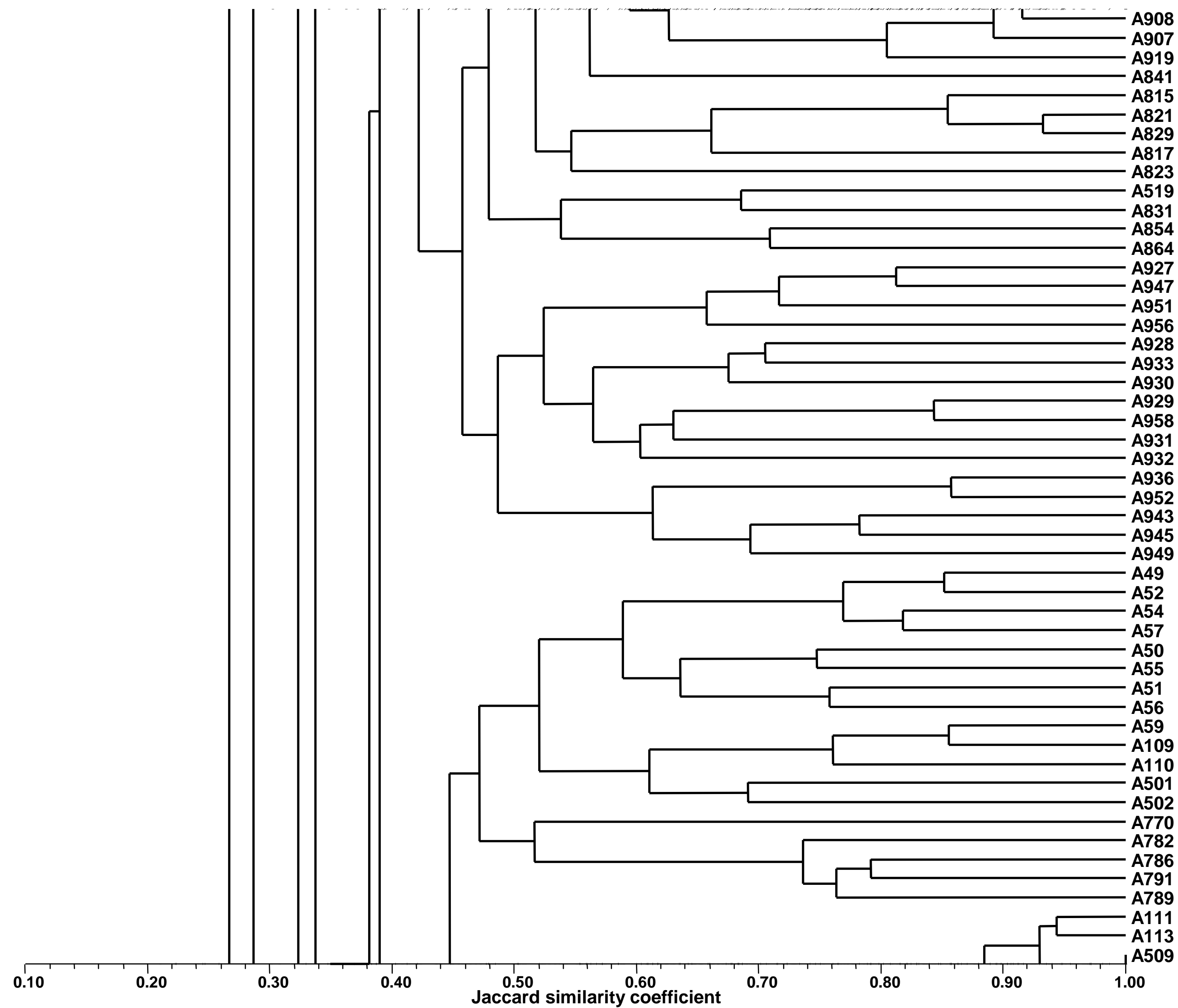
Jaccard similarity coefficient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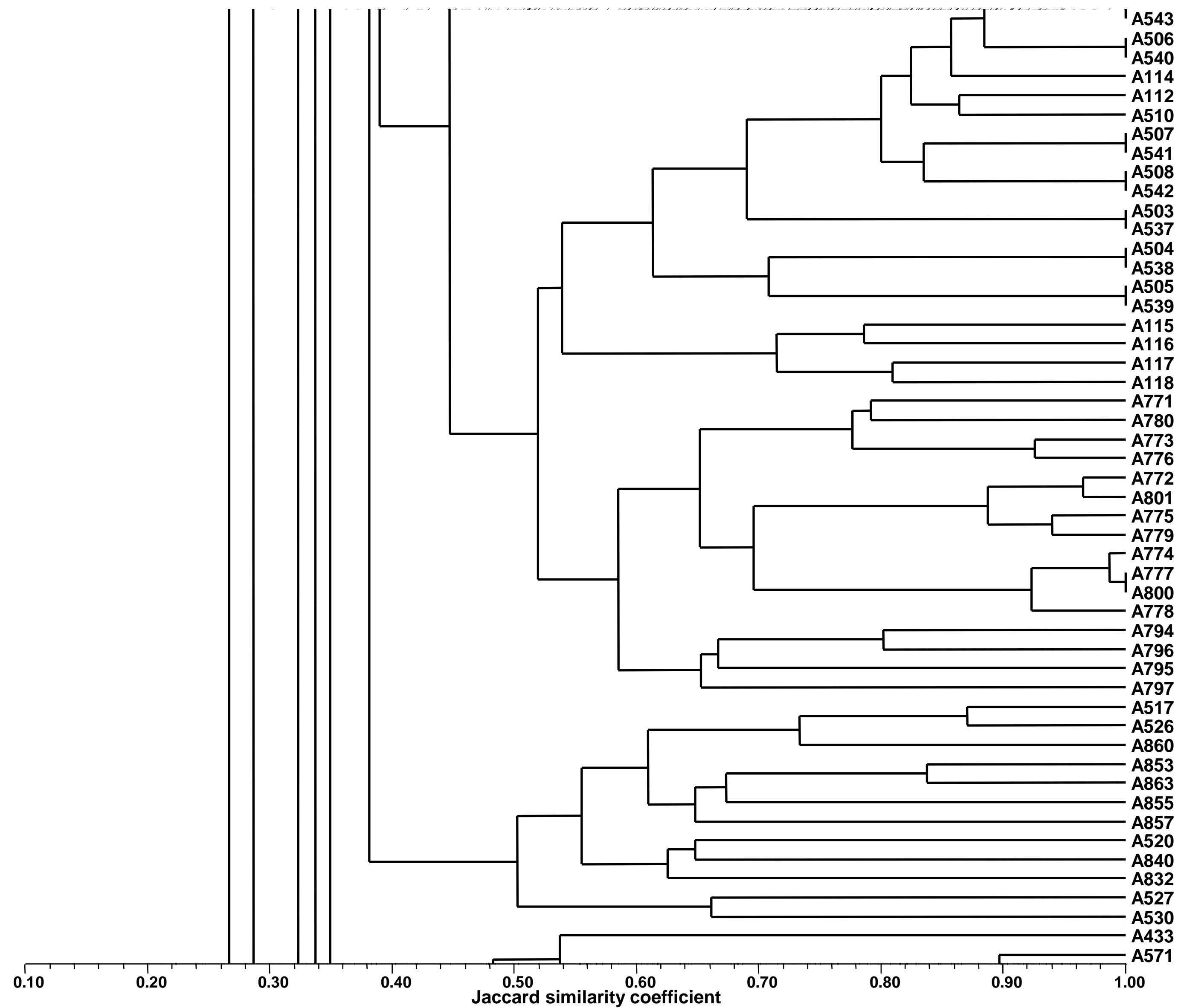
Jaccard similarity coefficient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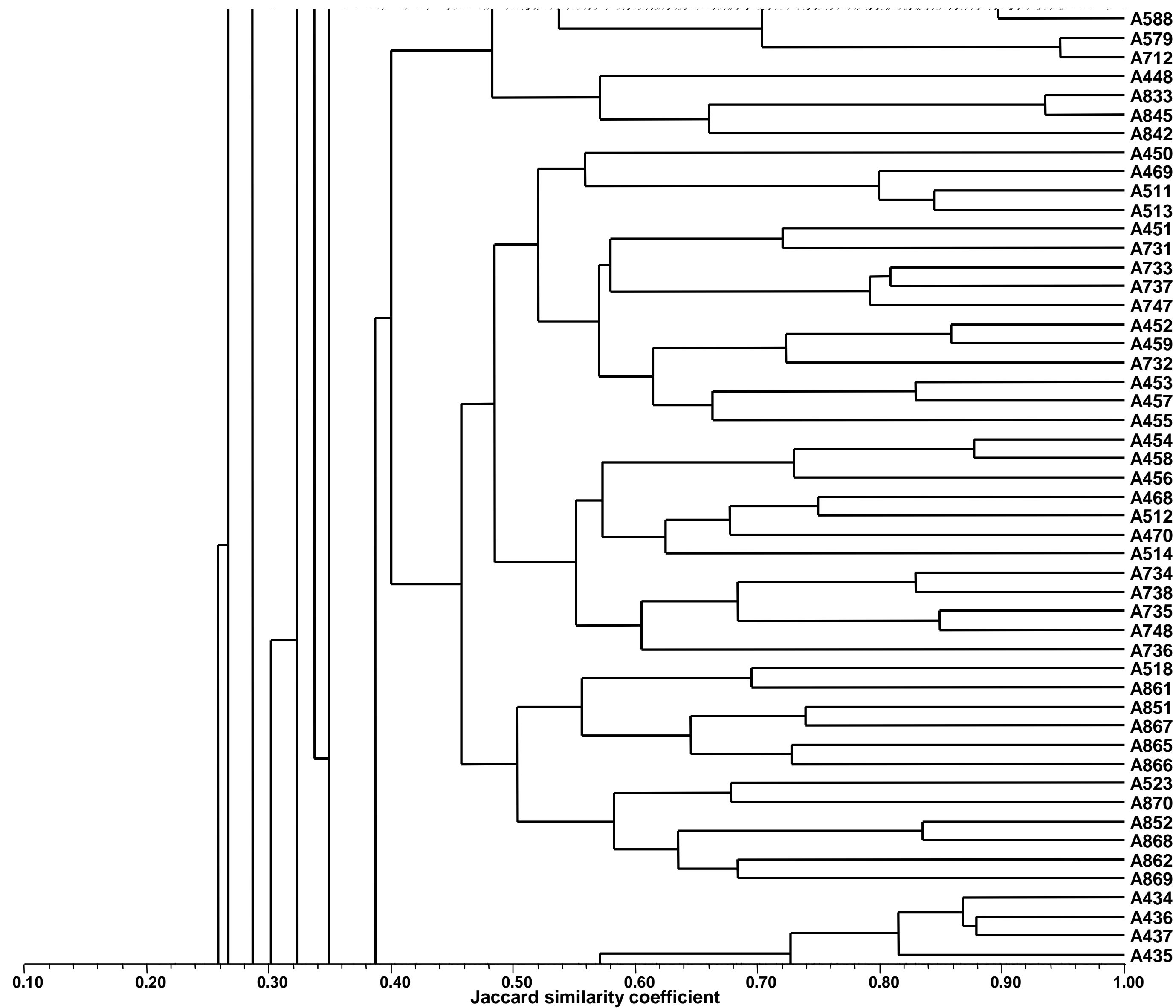
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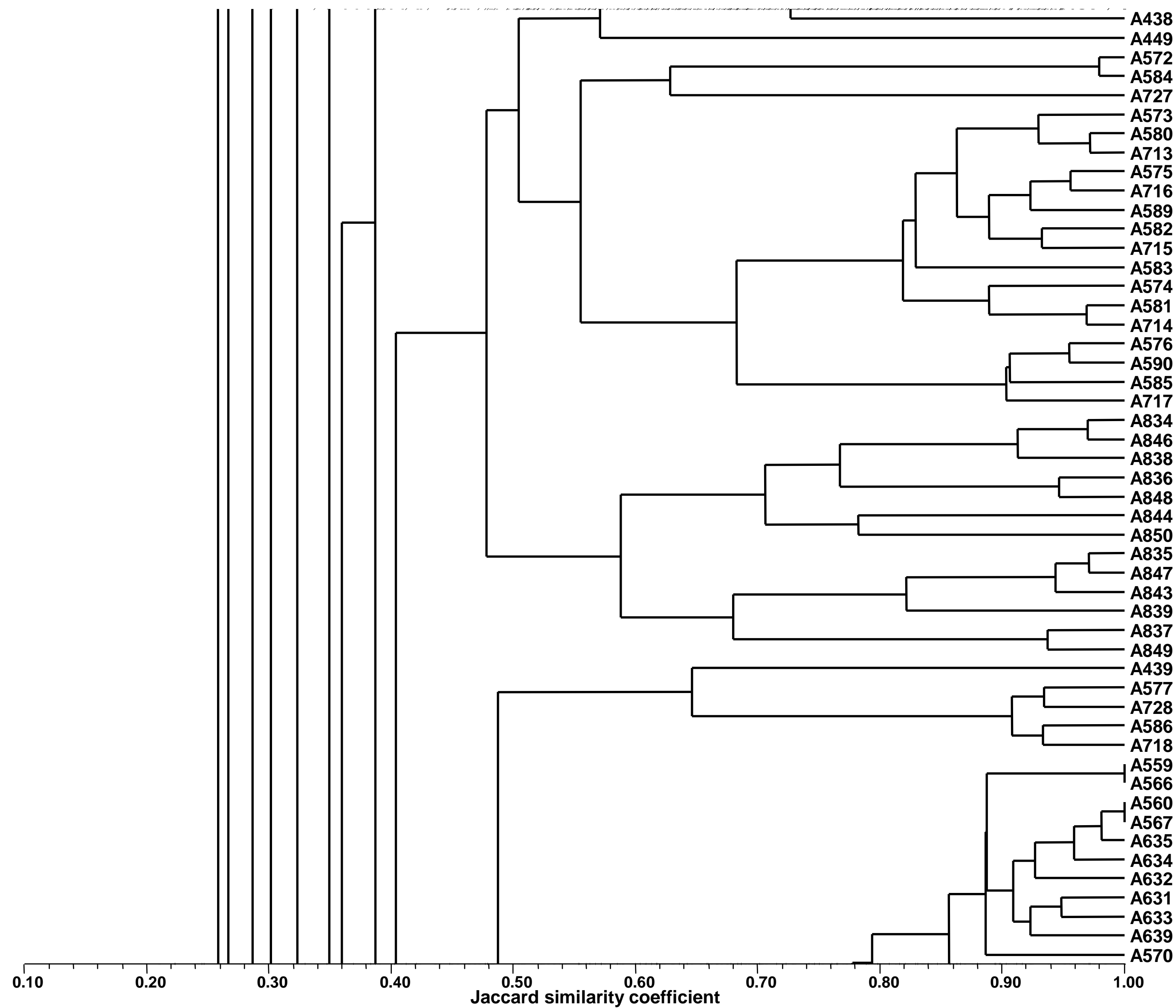
Jaccard similarity coefficient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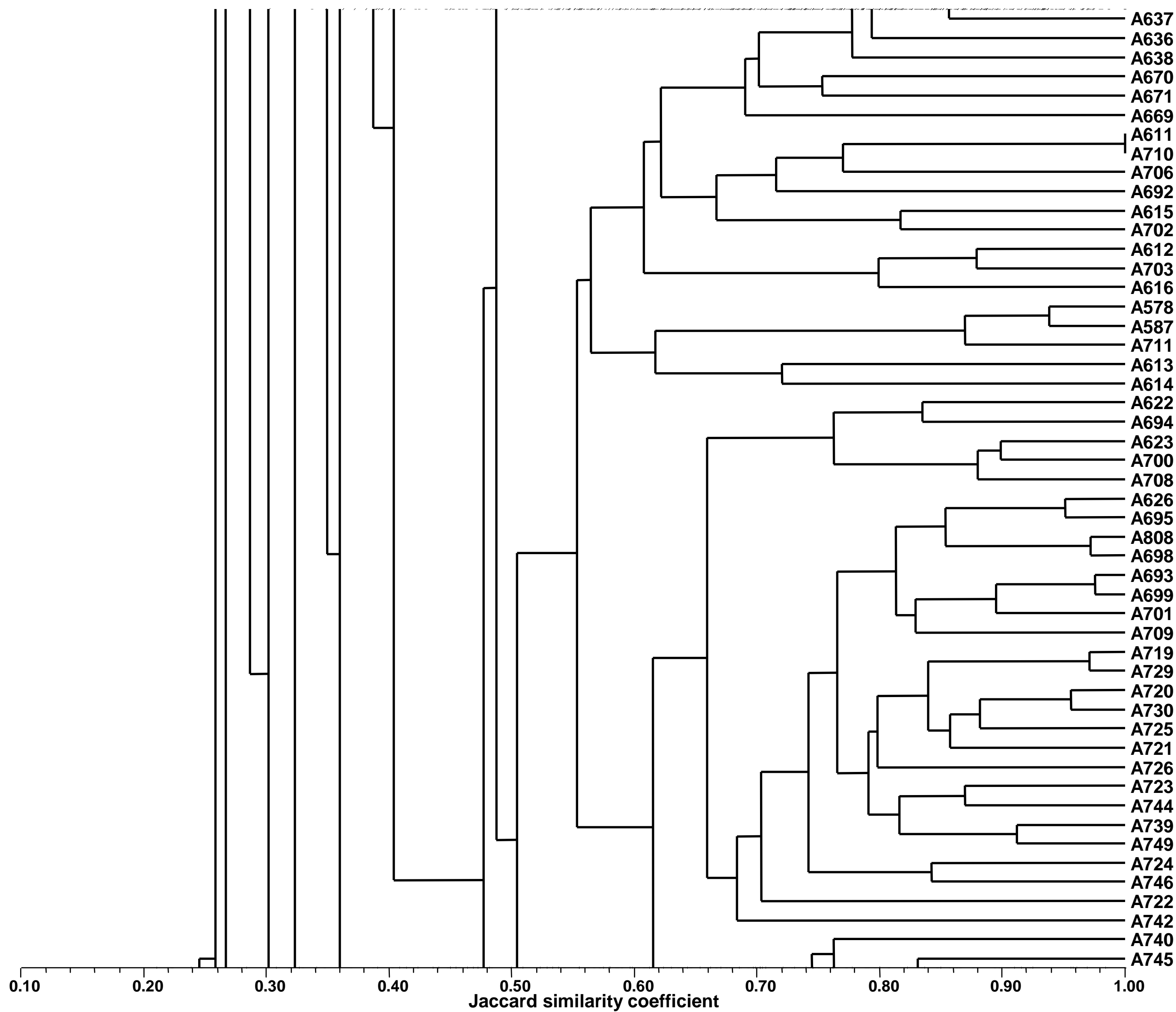
Jaccard similarity coefficient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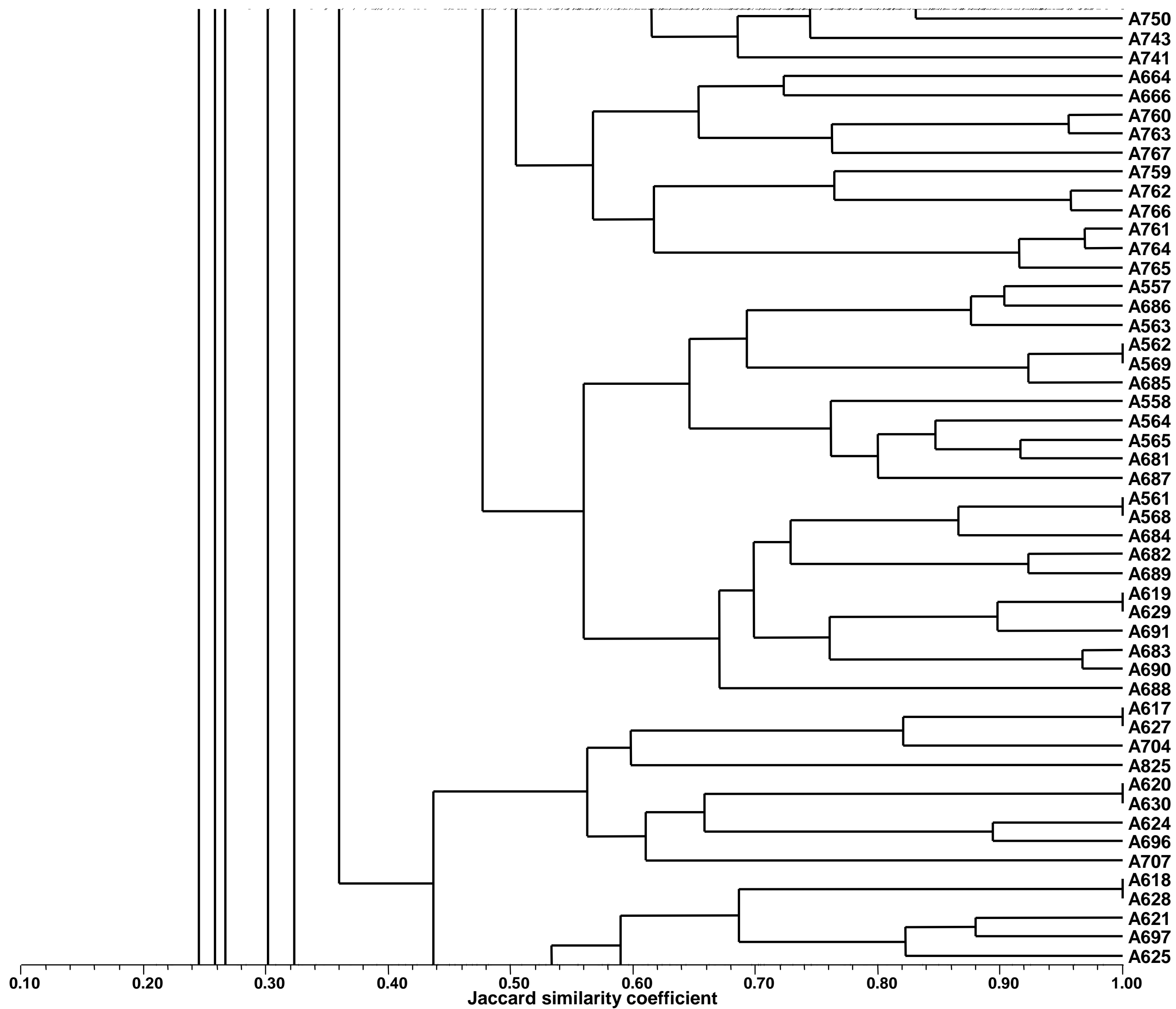
Jaccard similarity coefficient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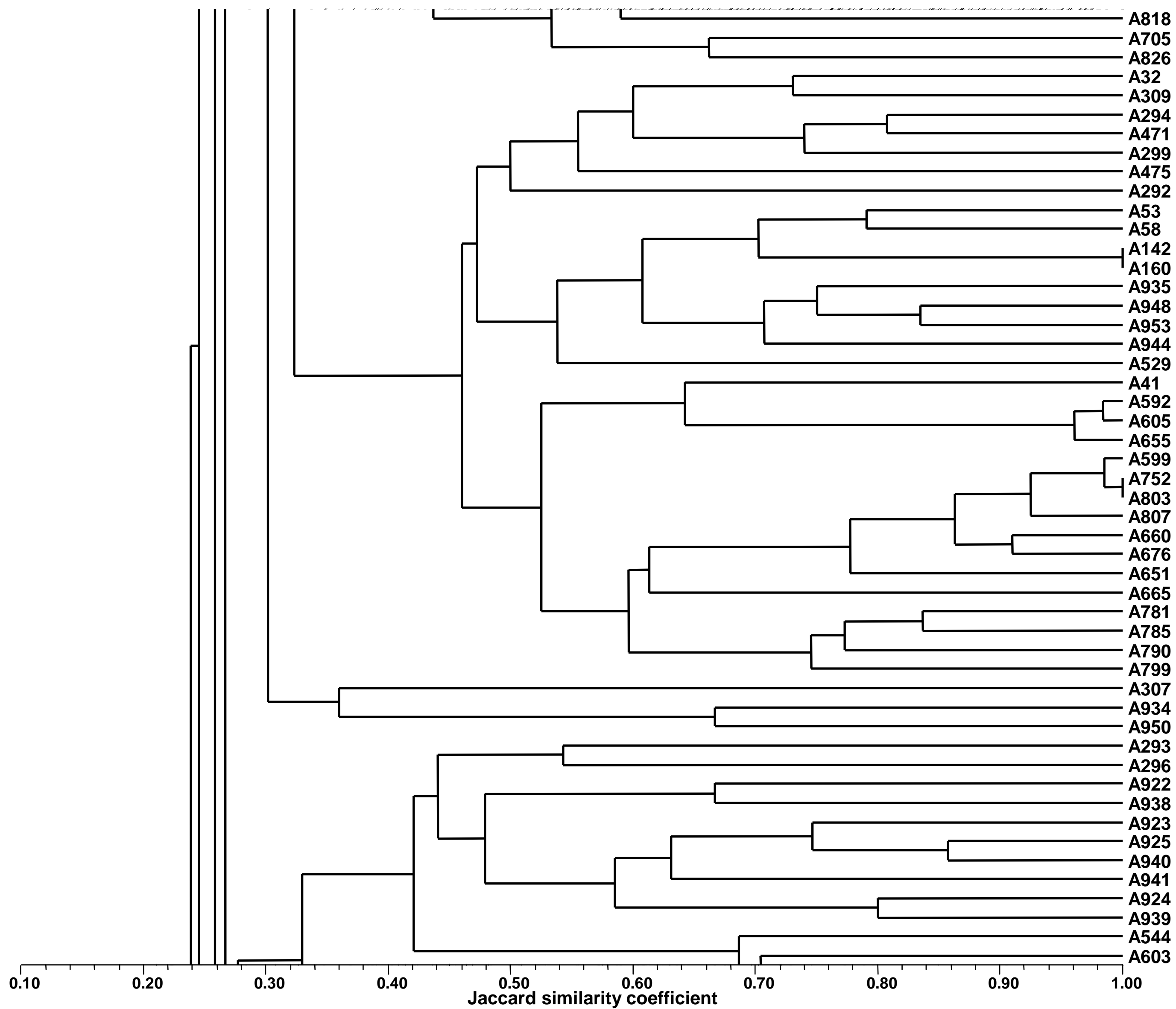
Jaccard similarity coefficient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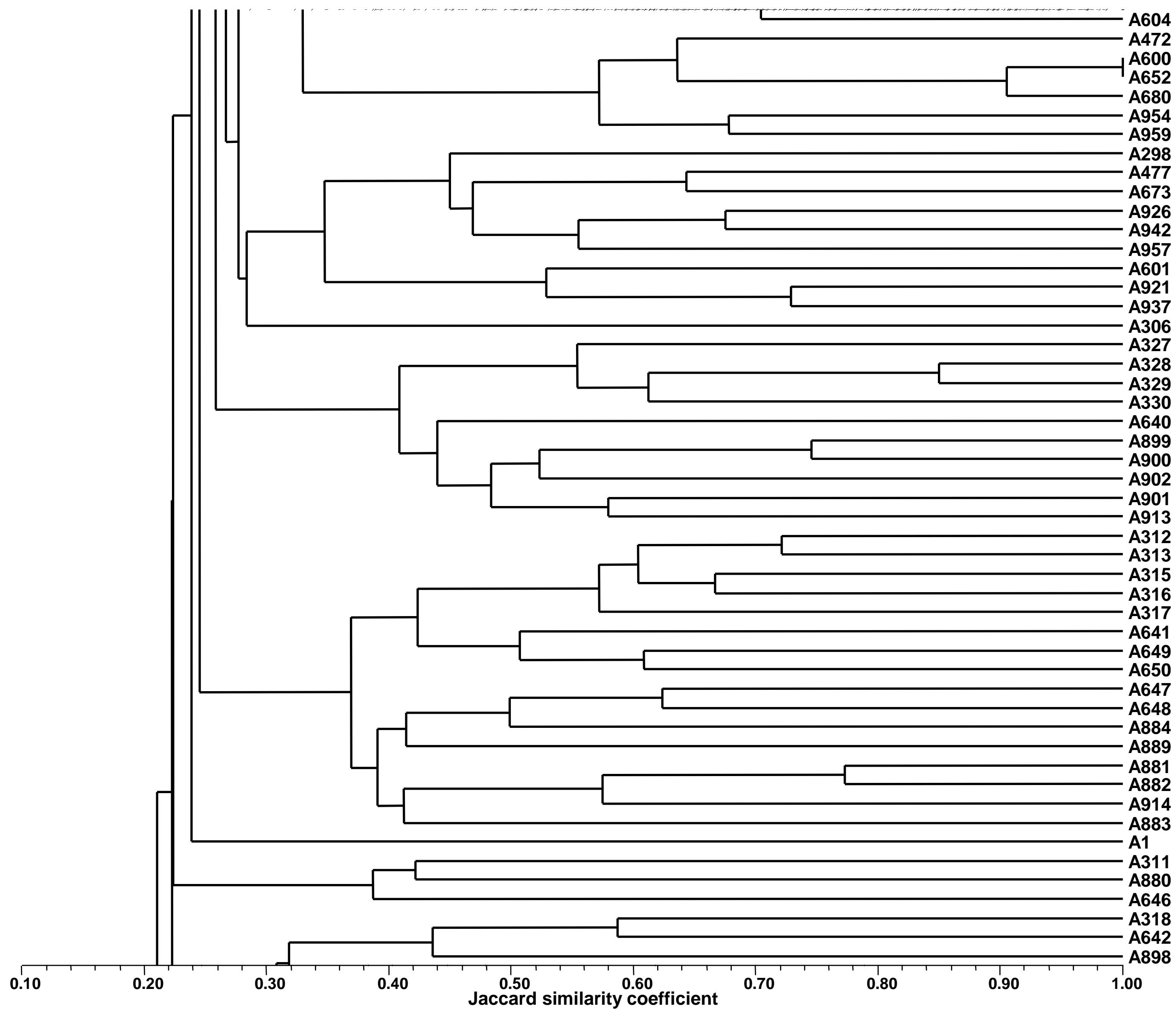
1.00
Jaccard similarity coefficient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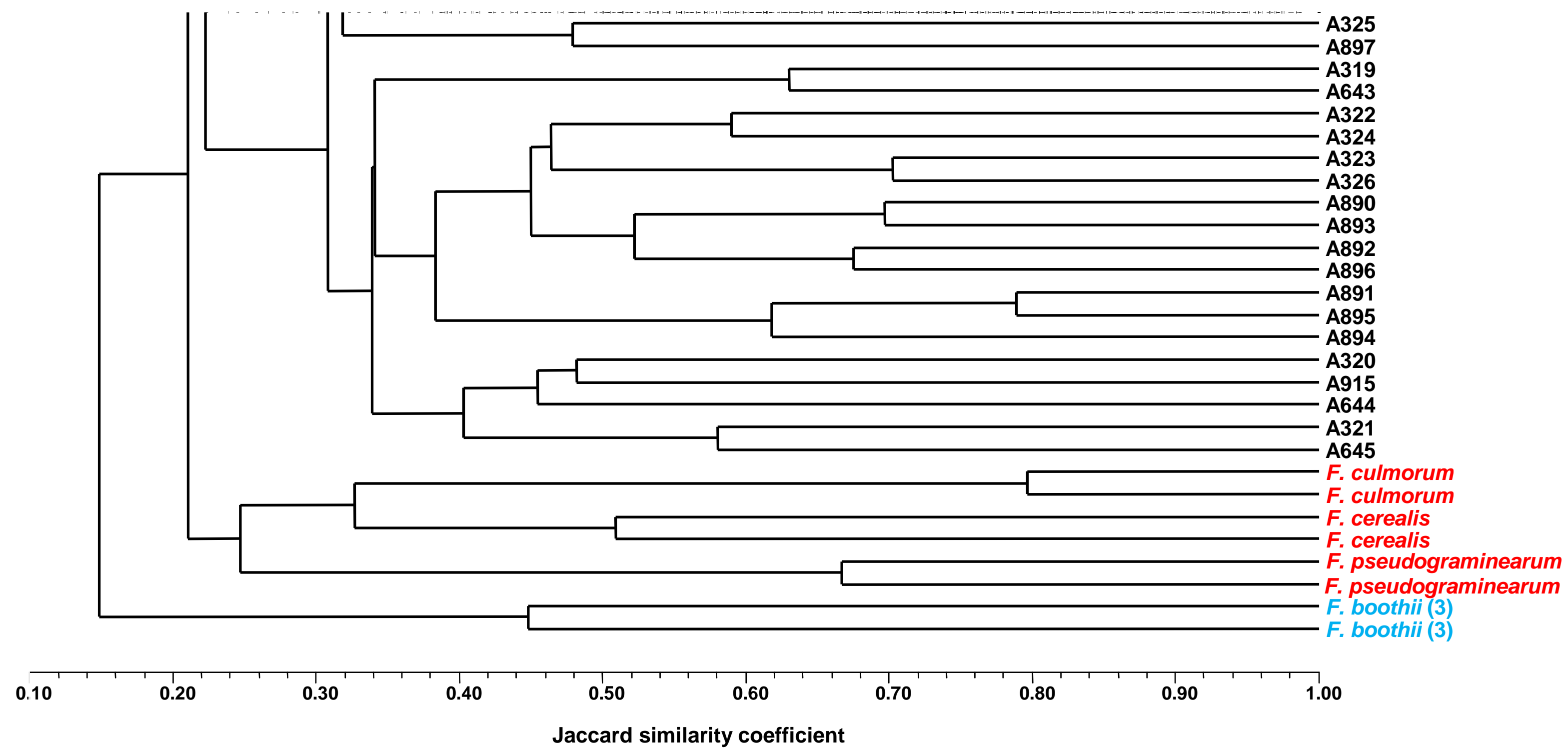
Jaccard similarity coefficient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A645
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F. culmorum
F. cerealis
F. cerealis
F. pseudograminearum
F. pseudograminearum
F. boothii (3)
F. boothii (3)
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0.20
0.30
0.40
0.50
0.60
0.70
0.80
0.90
1.00
Jaccard similarity coefficient