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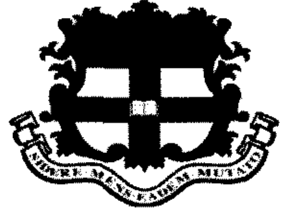

Faculty of Agriculture, Food and Natural Resources

The University of Sydney

Fusarium Species Associated with Grain Sorghum in Australia

by

Tijana Petrović

A thesis submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

May 2007

Certificate of Originality

The text of this thesis contains no material which has been accepted for the award of any other degree or diploma in any University and to the best of my knowledge, is original and contains no material previously published or written by another person, except where due reference is made.

Tijana Petrović

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"The important thing in science is not so much to obtain new facts as to discover new ways of thinking about them"

Sir William H. Bragg, Nobel Prize in Physics, 1915

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Dedicated to my beloved father Milko who passed away shortly after I had taken this path.

Abstract

The relative abundance and distribution of *Fusarium* species associated with grain sorghum were characterised and re-assessed in the sorghum-growing region of Goondiwindi-Moree-Quirindi, in the northern grain belt of eastern Australia. Nineteen *Fusarium* species, represented by 2152 isolates, were recovered from this region. Only five species were commonly recovered from the Goondiwindi, Moree and Quirindi areas, with *F. thapsinum* and *F. andiyazi* the dominant species associated with grain sorghum in these three areas. Both species were also prevalent in the various underground and aboveground plant parts of sorghum seedlings, physiologically mature sorghum and sorghum residues at Livingston Farm, Moree. Asymptomatic association of *F. thapsinum* with sorghum tissue was confirmed, whereas asymptomatic association of *F. andiyazi* was shown for the first time.

The dominance and relative abundance of *F. thapsinum* and *F. andiyazi* were correlated to agroclimatic areas. *Fusarium thapsinum* was more abundant in the hotter, drier Moree and Goondiwindi areas, whereas *F. andiyazi* was more abundant in the cooler, wetter Quirindi area.

Diagnostic morphological features of a subset of 64 *F. thapsinum* and 63 *F. andiyazi* isolates were evaluated using a polyphasic approach including sexual compatibility, AFLP fingerprinting and partial sequence analysis of the translation elongation factor 1-α gene. Both species are morphologically, biologically and genetically dissimilar to *F. verticillioides*, although all three species have been previously referred to as *F. moniliforme sensu lato* in Australia. *Fusarium andiyazi* was shown to be a distinct phylogenetic species, clearly separated from *F. thapsinum* and *F. verticillioides*, and phylogenetically most related to *F. brevicatenulatum*, *F. pseudoanthophilum* and *F.*

pseudonygamai (bootstrap value 94%). These species also share some morphological similarities.

Fusarium thapsinum was the most abundant species in the Goondiwindi and Moree areas and the second most abundant species in the Quirindi area. High levels of genetic diversity were shown for this species at various spatial levels of comparison (plant part, plot, geographic location, ecosystem/host and country/continent). Sexual reproduction possibly contributed to the high levels of genotypic diversity in the plant part, plot and geographic populations. The average number of asexual generations per sexual generation among the plant part, plot and geographic populations ranged from 125 to 249 (plant part and plot populations) and 145 to 290 (geographic populations). No genetic differentiation [$\theta(F_{ST}) = 0.006$] was observed among plant part populations due to extensive gene/genotype flow ($Nm = 32.4$–85.7). Genetic differentiation among plot and geographic populations may indicate that some populations experienced a lag in gene/genotype flow.

Fusarium thapsinum haplotypes were not differentiated on the basis of the ecosystem (agricultural/non-agricultural), host (grain sorghum, weed and native grasses) or global origin.

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List of Abbreviations

χ^2	Chi-square
ACE	Abundance-based coverage estimator
AFLP	Amplified fragment length polymorphism
ANOSIM	Analysis of similarities
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BLB	Black light blue
bp	Base pair
BSA	Bovine serum albumin
CA	Carrot agar
CI	Consistency index
CLA	Carnation leaf-piece agar
CO	Colorado
cv	Cultivar
D	Nei's unbiased genetic distance
df	Degree of freedom
diam	Diameter
DNA	Deoxyribonucleic acid
ELEM	Equine leukoencephalomalacia
FAM	6-carboxyfluorescine
FB1	Fumonisin B1
FB2	Fumonisin B2
FB3	Fumonisin B3
FGSC	Fungal Genetics Stock Centre
FHB	*Fusarium* head blight
Fpr.	F probability
f.sp.	Formae speciales
g, mg, μg, ng	Gram, milligram, microgram, nanogram
GCPSR	Genealogical concordance phylogenetic species
GMS	Grain mould severity
h	Nei's gene diversity
h, min, s	Hour, minute, second
H_2O	Water
ha	Hectare
HEX	Hexachloro-6-carboxyfluorescine
I	Genetic identity
ICE	Incidence-based coverage estimator
k	Speed
km, m, cm, mm	Kilometre, meter, centimetre, millimetre

KS	Kansas
KSU	Kansas State University
l, ml, μl	litre, millilitre, microliter
M	Mean number of female sterility mutations per strain
MAT	Mating type allele
$MgCl_2$	Magnesium chloride
MHC	Methuen handbook of colour
ML	Maximum likelihood
mM	Millimolar
MP	Maximum parsimony
MP X	Mating population X (X is from A to I)
MRC	Medical Research Council
NA	Data not available
NaOCl	Sodium hypochlorite
ND	Not detected
Ne(f)	Effective population number based on female fertility
Ne(mt)	Effective population number based on mating type
NJ	Neighbour joining
Nm	Estimate of gene flow
NMDS	Non-metric multidimensional scaling
NSW	New South Wales
NT	Northern Territory
NTSYSpc	Numerical taxonomy system for pc
n-UV	Near-ultraviolet
°C	Degree Celsius
P	Probability/significance level
PAUP	Phylogenetic analysis using parsimony
PCNB	Pentachloronitrobenzene
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
pmol	Picomole
PPA	PCNB peptone agar
PPE	Porcine pulmonary edema
PPS	Protein precipitate solution
QLD	Queensland
R	Coefficient of correlation
RBG	Botanic Gardens and Domain Trust
RI	Retention index
RNA	Ribonucleic acid
RNAse	Ribonuclease
SIMPER	Similarity percentage breakdowns

SNA	Synthetischer Nährstoffärmer agar
Sobs	Observed number of species
subsp.	Subspecies
SUPAMAC	Sydney University Prince Alfred Macromolecular
t	Ton
t	*t* Probability
Taq	*Thermus aquaticus*
TBE	Tris acetate-EDTA
TE	Tris-EDTA buffer
TEF	Translation elongation factor 1-α
TFPGA	Tools for population genetic analyses
tr	Trace
U	Unit
UPGMA	Unweighted pair group method with arithmetic mean
USA	United States of America
UV	Ultra violet
V	Volt
var.	Variety
$V_{(est)}$	Chao shared estimate
VCG	Vegetative compatibility group
w/v	Weight by volume
WA	Water agar
wk	Week
WP	Weighted parsimony
x *g*	Times gravity
$\theta(F_{ST})$	Coefficient of gene differentiation

List of Fungal Authorities

Alternaria alternata (Fries : Fries) von Keissler

Cercospora sorghi Ellis & Everh.

Claviceps africana Frederickson, Mantle & De Milliano

Curvularia lunata (Wakker) Boedijn

Fusarium acuminatum Ellis & Everhart

Fusarium andiyazi Marasas, Rheeder, Lamprecht, Zeller & Leslie

Fusarium anthophilum (A. Braun) Wollenweber

Fusarium arthrosporioides Sherbakoff

Fusarium avenaceum (Corda ex Fries) Saccardo

Fusarium beomiforme Nelson, Toussoun & Burgess

Fusarium brevicatenulatum Nirenberg & O'Donnell

Fusarium chlamydosporum Wollenweber & Reinking

Fusarium circinatum Nirenberg & O'Donnell emend. Britz, Coutinho, Wingfield & Marasas

(teleomorph: *Gibberella circinata* Nirenberg & O'Donnell emend. Britz, Coutinho, Wingfield & Marasas; MP H)

Fusarium commune Skovgaard, O'Donnell & Nirenberg

Fusarium compactum (Wollenweber) Gordon

Fusarium concentricum Nirenberg & O'Donnell

Fusarium culmorum (W. G. Smith) Saccardo

Fusarium denticulatum Nirenberg & O'Donnell

Fusarium dimerum Penzig

Fusarium equiseti (Corda) Saccardo

Fusarium fractiflexum Aoki, O'Donnell & Ichikawa

Fusarium fujikuroi Nirenberg

(teleomorph: *Gibberella fujikuroi* (Sawada) Ito in Ito & K. Kimura; MP C)

Fusarium gaditjirii Phan, Burgess & Summerell

(teleomorph: *Gibberella gaditjirii* Phan, Burgess & Summerell)

Fusarium globosum Rheeder, Marasas & Nelson

Fusarium graminearum Schwabe

(teleomorph: *Gibberella zeae* Schwein (Petch))

Fusarium hostae Geiser & Juba

Fusarium inflexum Schneider

Fusarium konzum Zeller, Summerell & Leslie

(teleomorph: *Gibberella konza* Zeller, Summerell & Leslie; MP I)

Fusarium kyushuense O'Donnell & Aoki

Fusarium langsethiae Torp & Nirenberg

Fusarium Link ex. Fr.

Fusarium longipes Wollenweber & Reinking

Fusarium miscanthi W. Gams, Klamer & O'Donnell

Fusarium moniliforme Sheldon *sensu lato*

Fusarium moniliforme Sheldon var. *subglutinans* Wollenweber & Reinking

Fusarium napiforme Marasas, Nelson & Rabie

Fusarium nygamai Burgess & Trimboli

(teleomorph: *Gibberella nygamai* Klaasen & Nelson; MP G)

Fusarium oxysporum f.sp. *vasinfectum* (Atk.) Snyder & Hansen

Fusarium oxysporum Schlechtendahl emend. Snyder & Hansen

Fusarium phyllophilum Nirenberg & O'Donnell

Fusarium poae (Peck) Wollenweber

Fusarium polyphialidicum Marasas, Nelson, Toussoun & van Wyk

Fusarium proliferatum (Matsushima) Nirenberg

(teleomorph: *Gibberella intermedia* (Kuhlman) Samuels, Nirenberg & Seifert; MP D)

Fusarium pseudoanthophilum Nirenberg, O'Donnell & Mubatanhema

Fusarium pseudograminearum Aoki &O'Donnell

Fusarium pseudonygamai Nirenberg & O'Donnell

Fusarium redolens Wollenweber

Fusarium sacchari (E. J. Butler) W. Gams

(teleomorph: *Gibberella sacchari* Summerell & Leslie; MP B)

Fusarium scirpi Lambotte & Fautrey

Fusarium semitectum Berkeley & Ravenel

Fusarium solani (Martius) Appel & Wollenweber emend. Snyder & Hansen

Fusarium sporotrichioides Sherbakoff

Fusarium subglutinans (Wollenweber & Reinking) Nelson, Toussoun & Marasas

(teleomorph: *Gibberella subglutinans* (Edwards) Nelson, Toussoun & Marasas; MP E)

Fusarium thapsinum Klittich, Leslie, Nelson & Marasas

(teleomorph: *Gibberella thapsina* Klittich, Leslie, Nelson & Marasas; MP F)

Fusarium tricinctum (Corda) Saccardo emend. Snyder and Hansen

Fusarium verticillioides (Saccardo) Nirenberg

(teleomorph: *Gibberella moniliformis* Wineland; MP A)

Macrophomina phaseolina (Tassi) Goidanich

Nigrospora sphaerica (Saccardo) Mason

Pratylenchus thornei Sher and Allen

Phytophthora infestans (Mont.) de Bary

Sclerotinia sclerotiorum (Lib.) de Bary

Sporisorium reilianum (Kuhn). Langdon and Fullerton

List of Botanical Species

Allium cepa L.

Andropogon gerardii Vitman

Arachis hypogaea L.

Asparagus officinalis L.

Austrostipa aristiglumis (F. Muell.) S. W. L. Jacobs & J. Everett

Avena sativa L.

Brassica napus L.

Cattleya Lindley

Coix gasteenii B. K. Simon

Cymbidium Swartz

Echinochloa crus-galli (L.) Beauv.

Gossypium hirsutum L.

Heteropogon triticeus (R. Br.) Stapf ex Craib

Hordeum vulgare L.

Livistona mariae F.Muell.

Musa paradisiaca L.

Oryza sativa L.

Panicum repens L.

Pennisetum typhoides (Burm. f.) Stapf & C. E. Hubb.

Phaseolus vulgaris L.

Pinus L.

Saccharum officinarum L.

Solanum tuberosum L.

Sorghum almum Parodi

Sorghum bicolor (L.) Moench. subsp. *bicolor*

Sorghum bicolor (L.) Moench. subsp. *drummondii* (Nees) de Wet

Sorghum bicolor (L.) Moench. subsp. *verticilliflorum* (Steud.) Piper

Sorghum brevicarinatum Snowden

Sorghum caffrorum (Thunb.) P. Beauv.

Sorghum halepense (L.) Pers.

Sorghum interjectum Lazarides

Sorghum miliaceum (Roxb.) Snowden

Sorghum sudanense (Piper) Stapf.

Sorghum verticilliflorum (Steud.) Stapf.

Sorghum vulgare Pers. cv. Saccaline

Sorghum vulgare Pers. var. *technicum* (Koern.) Jáv.

Striga hermonthica (Del.) Benth.

Triticum aestivum L.

Vicia faba L.

Zea mays L.

Chapter 1

Introduction

Grain sorghum (syn. cultivated sorghum) (*Sorghum bicolor* subsp. *bicolor*) is the fifth most important grain crop globally and the third most important grain crop in Australia. In many countries in Africa and Asia it is a staple food. However, in Australia, grain sorghum is used almost exclusively for livestock feed (1 million t) as well as in the pet food industry and a small quantity is being used in breakfast cereals and snack food. The remainder (750,000 t) is being exported. Grain sorghum is a main summer crop in the dryland cropping system of northeastern Australia. It has been commonly used as a break crop for crown rot fungus (*Fusarium pseudograminearum*) of wheat (*Triticum aestivum*) and has an added benefit in that it is resistant to root lesion nematode (*Pratylenchus thornei*).

A range of *Fusarium* species are associated with grain sorghum and some of the species are pathogenic, causing diseases such as seed dry rot or embryo death, seedling blight, root and stalk rot, peduncle and rachis blight, axillary-bud blight and leaf sheath blotch (Burgess *et al.* 1981; Ryley *et al.* 2002). *Fusarium* species are also associated with asymptomatic sorghum tissue and many are secondary colonizers of diseased or senescing tissues (Burgess and Trimboli 1986; Leslie *et al.* 1990; Trimboli and Burgess 1985). Some minor species probably have an important role in the infection process, competing with pathogenic isolates in plants (Leslie 2000). Therefore, the wide range of *Fusarium* species associated with grain sorghum need to be assessed prior to assist with mycotoxin risk management, breeding for resistance and development of integrated disease management strategies.

Fusarium thapsinum is considered the main pathogen of sorghum, and it produces a high level of moniliformin which can cause myocardial hypertrophy, haematological disorders, reduced performance and mortality of chickens, ducklings, pigs and rodents (Harvey *et al.* 1997; Ledoux *et al.* 1995). There are also several less pathogenic or opportunistic *Fusarium* species such as *F. nygamai*, that are also capable of producing considerable amounts of toxins (Leslie *et al.* 2005b).

The taxonomy of the *Fusarium* species associated with grain sorghum has been the subject of extensive research over the last 25 years. The discovery of new species and the division of old species are a feature of this period. The discovery of *F. nygamai* in Australia in the early 1980s (Burgess and Trimboli 1986) and *F. napiforme* in the Republic of South Africa in 1986 (Marasas *et al.* 1987) heralded major changes in the taxonomy of *Fusarium* species associated with grain sorghum and related crop and grass species. The taxonomy of the *Fusarium* species associated with sorghum has changed more than for the *Fusarium* species associated with other crops (Leslie and Marasas 2002). The use of molecular techniques, sexual compatibility studies and mycotoxin profiles have revealed that more *Fusarium* species are associated with grain sorghum than were differentiated using morphological markers alone (Klittich and Leslie 1992; Klittich *et al.* 1997; Mansuetus *et al.* 1997; Marasas *et al.* 2001). Consequently, *F. moniliforme sensu lato* was split into a number of new species including *F. thapsinum* (Klittich and Leslie 1992; Klittich *et al.* 1997), now considered the key stalk and root pathogen of grain sorghum; *F. verticillioides* (Seifert *et al.* 2003) regarded as the key stalk and ear rot pathogen of maize (*Zea mays*) and other *Fusarium* species. The newly recognized species *F. andiyazi* from grain sorghum also resembled *F. moniliforme sensu lato* but was differentiated as a new species using various markers (Marasas *et al.* 2001).

Molecular based taxonomic treatment of species in sections *Liseola* and *Dlaminia*, based on phylogenetic analyses of multiple genes, indicated possible biogeographic origins of these species (O'Donnell *et al.* 1998a). The species associated mainly with grain sorghum including *F. thapsinum*, *F. napiforme* and *F. nygamai* were postulated to belong to the 'African clade', with speculation that these species were dispersed with their host, grain sorghum, which is African in origin (Kimber 2000). This proposal, however, needs reappraisal. For example, *F. nygamai* is found in native grasslands, remote from grain sorghum cropping in Australia and there is no evidence that it was introduced with grain sorghum. It is most commonly associated with native millet *Panicum repens* (Amata 2005). There is no phylogenetic evidence for the origin of *F. andiyazi*, a species that together with *F. thapsinum* and *F. proliferatum* is commonly associated with grain sorghum (Summerell *et al.* 2003). *Fusarium proliferatum* an omnipresent species, associated with a wide range of hosts, as well as *F. sacchari,* which causes exotic disease Pokkah Boeng (twisted top) of grain sorghum were reported to belong to the 'Asian clade' (O'Donnell *et al.* 1998a).

The history of the Australian continent, the diverse agroclimatic conditions, and the use of various fodder sorghums from the early 1800's and grain sorghum from approximately 1908 (McDonald 1909) may have led to differing *Fusarium* communities being associated with grain sorghum throughout the sorghum producing regions. In Australia, grain sorghum is grown mainly as a dryland summer crop in the sub-tropical and tropical areas of northern New South Wales, and southern and central Queensland (Burgess *et al.* 1981; Wildermuth *et al.* 1997). Surveys conducted 25 years ago indicated that *F. moniliforme sensu lato* was the dominant *Fusarium* species associated with grain sorghum in the northeastern grain belt of Australia (Burgess and Trimboli 1986; Trimboli and Burgess 1985). *Fusarium nygamai* was also isolated

from grain sorghum but was less common than *F. moniliforme sensu lato* (Trimboli 1982).

Changes in the taxonomy of *Fusarium* species have led to a need to re-evaluate the spectrum and relative abundance of *Fusarium* species associated with sorghum in Australia using systematic surveys. Such information is important for the refinement of control measures for Fusarium stalk and root rot and for better understanding the risks of mycotoxin contamination. Furthermore, Marasas *et al.* (2001) recommended that "sorghum worldwide needs to be re-examined, not only to determine the frequency and distribution of *F. andiyazi*, but also to provide more information on *F. thapsinum* and to ensure that all of the *Fusarium* species common to sorghum have been properly identified and characterised".

The structure of *Fusarium* communities and the biological traits of the most dominant species associated with grain sorghum can be influenced by environmental factors. For example, the inbreeding potential of *F. thapsinum* populations has been shown to vary between climatically different countries such as South Korea and Tanzania, with higher inbreeding potential than the global *F. thapsinum* population (Leslie and Klein 1996; Lim *et al.* 2001; Mansuetus *et al.* 1997). The low inbreeding potential of the global population of *F. thapsinum* was attributed to the *F. thapsinum* population being clonal in nature and vegetatively propagated (Klittich and Leslie 1988; Leslie and Marasas 2002). Furthermore, molecular analyses of populations from different climatic regions, hosts and substrates, can reveal the nature of diversity and adaptation in *Fusarium* species (Burgess *et al.* 1996).

The main goals of the studies reported in this thesis were to characterise and re-assess the relative abundance and distribution of *Fusarium* species in the grain sorghum-growing region of Gooondiwindi–Moree–Quirindi in the northern grain belt of eastern

Australia. Four hypotheses were proposed as the basis for these studies. Firstly, that *F. thapsinum* is the dominant species associated with Fusarium stalk rot of grain sorghum in the Goondiwindi-Moree-Quirindi areas of the northern grain belt of eastern Australia. Secondly, that *F. andiyazi* is not associated with grain sorghum in Australia. Thirdly, that the populations of *F. thapsinum* in the Goondiwindi-Moree-Quirindi areas are genetically similar. Fourthly, that the Australian population of *F. thapsinum* is an integral part of an international panmictic population of this species.

Chapter 2

Literature Review

2.1 Importance of *Fusarium* species

The genus *Fusarium* comprises many economically important species, having direct effects on human welfare and agriculture. Direct effects include health hazards to humans and animals caused by mycotoxins, yield loss and reduced product quality in a wide range of crops and pasture plants (Marasas and Nelson 1987; Nelson *et al.* 1994).

Direct effects on agriculture are of great concern as it has been estimated that 25% of the world food crop is affected by mycotoxins (Charmley *et al.* 1995). For example, the *Fusarium* toxin fumonisin B_1 is commonly present in maize grain and can cause equine leukoencephalomalacia (ELEM), porcine pulmonary edema (PPE) and hepatic syndrome in swine, poor performance in poultry and alteration in hepatic and immune function in cattle (Logrieco *et al.* 2002).

Fusarium species are widely distributed and representatives occur in climatic regions worldwide. Indeed, the importance of Fusarium diseases was recognized even before the genus *Fusarium* was described. For example, one of the first descriptions of ear rot of maize caused by *F. moniliforme sensu lato* was written by a Franciscan friar in Mexico in the sixteen century (Booth 1984). The economic importance of *Fusarium* is illustrated by the following examples. Fusarium stalk rot of maize and sorghum has often been reported as the main problem in some growing seasons in many regions. Pammel *et al.* (1916) reported losses in maize in excess of US $15 million in Iowa in the 1914 growing season. The same authors, also made the first report of Fusarium stalk rot of sorghum occurring in Kansas and Iowa. In 1996, Fusarium stalk rot of

sorghum caused more than US $80 million in grain yield losses in Kansas alone (Waniska *et al.* 2001). Grain mould caused by one of the most common *Fusarium* species on sorghum, *F. thapsinum*, and other fungi causes losses of US $130 million per annum worldwide (Bandyopadhyay *et al.* 2002).

2.2 Recognition of *Fusarium* Species

2.2.1 Morphological and Physiological Species Recognition

Morphological identification is the first step in species recognition. A morphological species is defined as a group of isolates that share a set of morphological characters and are distinguished from other groups because of this set of characters.

Since the genus *Fusarium* was proposed in 1809 by Link, the main task for taxonomists has been to establish morphological and cultural criteria for species identification. In the first 100 years, species were delineated primarily according to the host plant on which they were collected, leading to the establishment of almost 1000 named *Fusarium* species. At the beginning of the 1900s Appel and Wollenweber (1910) established morphological and cultural criteria for species identification, re-defined the genus and reduced the number of *Fusarium* species through a process of rejection and consolidation.

Further systematic work by Wollenweber was focused on determining consistent and reliable morphological characters for species identification and relating a *Fusarium* anamorph to its teleomorph (Wollenweber 1913; Wollenweber 1914). The result of this work was the consolidation of the number of taxa to 65 species, 55 varieties and 22 forms arranged in 16 sections (Wollenweber and Reinking 1935).

All subsequent taxonomic systems have been based on Wollenweber and Reinking's monograph *"Die Fusarien"*. Some of these taxonomic systems relied on classical

morphological and physiological characterisation of species, leading to a re-examination of *Fusarium* species and establishing 73 species and 26 varieties (Gerlach and Nirenberg 1982). In contrast, other taxonomic systems based more on the physiological characterisation of species led to the drastic reduction in the number of species. Snyder and Hansen (1940, 1941, 1945) reduced the number of species in the genus *Fusarium* to nine. Snyder and Hansen's taxonomic work gave no relevant information about the ecology and biogeography of *Fusarium* species (Leslie *et al.* 2001).

Booth combined the best aspects of Wollenweber and Reinking's, and Snyder and Hansen's systems in his taxonomic treatment, which was widely used (Booth 1971). Booth (1971, 1984) listed most of the known *Gibberella* species, recognising the value of perithecia in routine identification of some *Fusarium* species. Booth also recognized the mode of production of microconidia as a useful character in the taxonomy of some *Fusarium* species and emphasised the taxonomic value of the morphology of the conidiogenous cell. He recognized 44 *Fusarium* species and 7 varieties in his taxonomy treatment.

In the past 25 years, two additional taxonomic systems derived from Snyder and Hansen's work have been proposed. The systems of Nelson *et al.* (1983) and Burgess *et al.* (1994) incorporate key aspects of the taxonomies developed by Wollenweber and Reinking, Snyder and Hansen, and Booth. Their species concepts were based on morphological criteria and the examination of populations of cultures from diverse geographic regions and substrates, and pre-dated more recent findings using molecular markers.

Morphological identification of species is now used for the routine grouping of similar isolates, with their final identification based on molecular methods or sexual

compatibility tests if appropriate and standard tester strains are available. Furthermore, it remains important to carefully delineate the morphological characters of both newly recognized or insufficiently described species as morphology remains the primary basis for putative identification. This is especially true when sorting cultures derived from large scale surveys.

2.2.2 Importance of the Teleomorph and Biological Species Recognition

Both teleomorph and anamorph, or the pleomorphic holomorph (the whole organism), provide the basis for a natural classification of *Fusarium* species and can be correlated to the phylogenetically based classification.

Studies on the sexual stage can lead to a better understanding of the biology and population processes of the species. Biological species are defined as interbreeding populations (Mayr 1992) and often correspond to morphologically and ecologically distinct groups where gene flow, or lack of it, may cause such correspondence (Mayr 1982).

Sexual compatibility studies contributed significantly to understanding the species in section *Liseola* (Hsieh *et al.* 1977; Kuhlman 1982; Leslie 1991; Leslie 1995), for resolving mechanisms of inheritance of mating types and sex (Gordon 1961) and estimating population size parameters (Leslie and Klein 1996). Delineation of biological species [syn. mating populations (MPs)] has been correlated with the major host, in some species. Hsieh *et al.* (1977) demonstrated that segregation of isolates into MP A, MP B and MP C was based on host affinities. Further research confirmed the link between host and mating population with the segregation of isolates of *F. subglutinans* from maize into MP E (Leslie 1991; Leslie 1995). Similarly, many isolates of *F. moniliforme sensu lato* from maize and sorghum segregated into MP A

and MP F (Klittich *et al.* 1997). However, MP D was not delineated based on host preference (Kuhlman 1982).

One of the common species on grain sorghum, *F. nygamai*, has been established as MP G. An indication that the geographic distribution of mating types affects the results of mating studies (Gordon 1954) can be related to MP G. Klaasen and Nelson (1996) produced fertile perithecia of MP G by crossing isolates of *F. nygamai* from several geographically diverse locations, noting that only isolates from geographically distant locations (*i.e.* Australia and Sub-Saharan Africa) were sexually compatible. However, recent re-examination of *F. nygamai* isolates has shown that the results of previous sexual compatibility studies of MP G, even between tester strains, are not reproducible (Leslie *et al.* 2005b).

Recently, two additional mating populations have been established. Mating population H (*F. circinatum*) is an economically important species, which is apparently restricted to pines (*Pinus* spp.). Mating population I (*F. konzum*) is a species from prairie grasses in the USA (Britz *et al.* 1999; Nirenberg and O'Donnell 1998; Zeller *et al.* 2003). More recently sexual compatibility studies contributed to the differentiation and description of *Gibberella gaditjirii* from tropical tall grass (*Heteropogon triticeus*) in Australia (Phan *et al.* 2004).

2.2.3 Recognition Based on Metabolites

Biological or physiological traits such as the production of mycotoxins and secondary metabolites can be used as markers for *Fusarium* species identification. Differences in secondary metabolite production have been suggested as taxonomic characters for distinguishing *Fusarium* species (Thrane 2001). Type and level of secondary metabolite, and toxicity to experimental animals have been shown, for example, to be a good criterion for distinguishing two mating populations considered as sibling

species, MP A (*F. verticillioides*) and MP F (*F. thapsinum*) (Leslie *et al.* 1996). More recently, the production of fumonisins and/or moniliformin and duckling toxicity have been shown to be a good criterion for identification of morphologically similar species, *F. nygamai* and *F. pseudonygamai* (Leslie *et al.* 2005b).

The production of secondary metabolites such as moniliformin, fumonisins, beauvericin, fusaproliferin and gibberellic acid have provided a better understanding of relationships between mating populations (Desjardins *et al.* 2000; Moretti *et al.* 1996). The development of standard metabolite profiles and/or chemotypes for each *Fusarium* species can enable the use of chemotaxonomy as an integral part of defining a species (Thrane 2001).

2.2.4 Molecular and Phylogenetic Species Recognition

The application of PCR-based techniques has had a dramatic impact on systematics and applied studies in *Fusarium* research. Molecular identification based on genotyping and sequencing of one or more conserved genes has increased the accuracy of species identification.

Genotyping methods provide unique band patterns useful for identifying and comparing genetic individuals within a species. For example, species such as *F. moniliforme sensu lato* and *F. subglutinans* associated with maize ear rot have been diagnosed based on species-specific PCR assays (Möller *et al.* 1999). Furthermore, species from section *Liseola* have been identified based on genotyping techniques in concert with other methods, *eg*. sexual compatibility tests (Amoah *et al.* 1995; Voigt *et al.* 1995). Comparing large numbers of isolates against representative strains of all mating populations is considered critical for validating the diagnoses of species in section *Liseola* (Schlacht *et al.* 1997). Summerell *et al.* (2003) suggested that genotyping diagnoses should be based on a large number of isolates of the target

species, or that the target species should be tested against representative strains of a large number of species. An example of the value of genotyping methods is the delineation of *F. andiyazi* based on AFLP fingerprinting patterns compared to fingerprints of all species in sections *Liseola* and *Dlaminia*. It is likely that the development and deployment of molecular diagnostics for some *Fusarium* species, and relevant genotypic databases, will provide challenges and advantages for mycologists in the coming years (Geiser 2003; Summerell *et al.* 2003).

Molecular phylogenetic analysis is the most valuable approach for species identification as it leads to the establishment of an objective classification of the genus *Fusarium* and its teleomorphs. This approach has allowed for resolution of the phylogenetic relationships between *Fusarium* and its teleomorphs in the Hypocrealean genera, among sections and between intra-specific taxa. It has also facilitated better understanding of species concept and mechanisms of evolutionary change in *Fusarium* populations (O'Donnell 1996; O'Donnell and Cigelnik 1997; O'Donnell *et al.* 1998b; O'Donnell *et al.* 2000). However, the validity of such studies is still dependent on the study of populations of individuals from diverse geographic origins and substrates.

Phylogenetic species are defined as the smallest aggregation of populations (sexual) or lineages (asexual) diagnosable by a unique combination of character states in comparable individuals (semaphoronts) (Nixon and Wheeler 1990). Phylogenetic species recognition in *Fusarium* has relied mainly on the genealogical concordance phylogenetic species recognition (GCPSR) (Geiser *et al.* 2004). This method is based on comparison of DNA sequence differences between two or more gene genealogies and identifies shared partitions among them as markers for species boundaries.

The presence of "cryptic species" within economically important pathogens of maize (*F. subglutinans*) and wheat (*F. graminearum*) has been revealed by application of GCPSR. Steenkamp *et al.* (2002) divided *F. subglutinans sensu stricto* (MP E) into several phylogenetic lineages that were further subdivided into a number of reproductively isolated groups. This finding suggested that existing *F. subglutinans* populations were in the process of divergence and that each of the resulting lineages were undergoing separation into distinct taxa. O'Donnell *et al.* (2004) defined nine phylogenetically distinct species within *F. graminearum*, but two of these species are interfertile (Cumagun *et al.* 2004), indicating that delineation of *Fusarium* species should not be based solely on a molecular phylogenetic approach.

Molecular phylogenetic analysis based on the comparison of more than one gene genealogy has delineated many new species and resolved the taxonomic status of some morphologically misidentified species. A gene genealogy is a tree-like representation of the history of descent from the ancestral sequence of one or more loci (genomic regions) (Carbone and Kohn 2004) and can reflect the relationships of a part of the species genome. The application of this approach has led to the differentiation of 26 new species in sections *Liseola* and *Dlaminia* ("*Gibberella fujikuroi* complex") and delineated taxa such as *F. miscanthi*, *F. hostae* and *F. commune* (Gams *et al.* 1999; Geiser *et al.* 2001; Nirenberg and O'Donnell 1998; O'Donnell *et al.* 1998a; Skovgaard *et al.* 2003). Re-identification of morphologically misidentified *Fusarium* species and the establishment of the new phylogenetic species, *F. kyushuense* and *F. fractiflexum*, revealed the complex nature of trichothecene-producing species and species associated with pitch cancer disease of pine (Aoki and O'Donnell 1998; Aoki *et al.* 2001)

Apart from comparison of two or more gene genealogies for species recognition, sometimes species can be identified accurately using a single DNA sequence marker as long as background phylogenetic analyses have been performed using the marker along with others, thereby validating its diagnostic utility (Geiser 2003). Geiser *et al.* (2004) demonstrated high phylogenetic utility of the translation elongation factor 1-α (TEF) gene for identification of species from sections *Liseola* and *Dlaminia* due to the high level of sequence polymorphism among closely related species. This implies that correct identification of a known species in these sections can be performed using this gene alone, or as a support for additional genotyping data.

2.2.5 Current Approaches to **Fusarium** *Identification*

"... Multidisciplinary or polyphasic taxonomic studies are the way of the future"

Editors of *Studies in Mycology*, 2000

The correct identification of *Fusarium* species, especially those in the section *Liseola*, cannot rely on the set of characters obtained by application of a single method. Combinations of morphological, sexual compatibility, molecular (fingerprinting and/or sequencing data) and mycotoxin production data can help to resolve the taxonomical status of species. For example, the taxonomy of *F. avenaceum*, *F. arthrosporioides* and *F. tricinctum* was re-examined by applying a polyphasic approach to identification (Yli-Mattila *et al.* 2002). Similarly, Summerell *et al.* (2003) proposed this approach to the identification of species in section *Liseola*, and related species. The authors considered the polyphasic approach as a stepwise process, beginning with morphological identification, followed by sexual compatibility tests, DNA fingerprinting, DNA sequencing and finally with morphological re-examination. By utilising this strategy, *Gibberella konza* (Zeller *et al.* 2003) and *G. gaditjirii* (Phan *et al.* 2004) were described.

The outcome of using combined diagnostic markers is a detailed characterisation of the biology of species and clarification of the relationships between different species. It seems that an integrated or multidisciplinary approach to species identification, as was recently demonstrated in the European *Sporotrichiella* project (Schmidt *et al.* 2004; Torp and Nirenberg 2004), will be the future of *Fusarium* taxonomy.

2.3 Biogeography and Ecology of *Fusarium* Species

Distribution patterns of many *Fusarium* species can be attributed to ecological factors on local scales, although other events, such as continental drift or human-mediated movement of plants may have preceded current species distributions. The geographic distribution of *Fusarium* species can indicate their evolutionary history, adding an additional component to phylogenetic systematics (O'Donnell *et al.* 1998a; O'Donnell *et al.* 2000).

O'Donnell *et al.* (1998a) speculated that Africa may have been the ancestral continent of *Fusarium* species from the "*Gibberella fujikoroi* complex" and that all species, except *F. verticillioides* and *F. denticulatum*, and their hosts, had been moved to other continents. It was postulated that the African, American and Asian phylogeographic clades of these species had evolved as a result of evolutionary processes. It is likely that the geographic distribution of *Fusarium* species that are exclusively associated with non-agricultural plants will provide more information about the evolutionary history of *Fusarium* species. This is in comparison to species associated with agricultural plants, as the latter have been moved between continents by human activity. Thus, Leslie *et al.* (2004b) assumed that *G. konza*, recovered only from native grasslands in Kansas, would belong to the "American" clade, since the species is phylogenetically most closely related to the "American" species *G. subglutinans*.

Studies on the *Fusarium* species associated with crops have largely been focussed on the geographical and ecological distribution of species (Backhouse *et al.* 2004; Pettitt *et al.* 2003; van Leur and Bailey 2000; Waalwijk *et al.* 2003). However, there have been extensive studies on *Fusarium* species from grassland and other non-agricultural soils, especially in Australia and the Republic of South Africa, over a long period of time (Burgess *et al.* 1988; Burgess and Summerell 1992; Marasas *et al.* 1988; Sangalang *et al.* 1995). More recently, attention has shifted to the *Fusarium* species associated with native plants (Leslie *et al.* 2004b; Phan *et al.* 2004; Zeller *et al.* 2003).

The role of some *Fusarium* species in the epidemiology of diseases is determined by their ecological and geographical distribution. Abundance and distribution are influenced by environmental conditions, primarily temperature and moisture. For example, in Europe studies have shown that species of the section *Liseola* cause pink fusariosis of maize ears, and the disease is dominant in the drier and warmer areas of southern Europe (Logrieco *et al.* 2002). However, a red fusariosis of maize ears caused by species in section *Discolor* is dominant in the northern/central areas of Europe that are characterised by frequent rainfall and low temperatures in the summer and early autumn (Logrieco *et al.* 2002). A similar distribution pattern of red *Fusarium* species associated with asparagus (*Asparagus officinalis*) was also found in Québec province in Canada (Vujanovic *et al.* 2006). Authors reported that red *Fusarium* species were more abundant in the cooler northern regions than in the warmer southern-central regions of Québec province.

The role of some *Fusarium* species in the production of mycotoxins in infected plants is also determined by their ecological and geographical distribution. As reviewed by Logrieco *et al.* (2002), various mycotoxins are associated with pink and red fusarioses of maize ears in different climates in Europe. Danielsen and Jensen (1998) found that

climatic differences in Costa Rica caused significant differences in average fumonisin content.

Edaphic factors and agro-technical practices may also account for the geographical distribution and abundance of *Fusarium* species and therefore the ecology of disease expression. Vujanovic *et al.* (2006) found that the complex of species associated with *Fusarium* crown and root rot of asparagus, *F. proliferatum*, *F. oxysporum* and *F. redolens*, were more abundant in less acidic soils of southern regions compared to the more acidic soils of the northern regions of Québec, Canada. The planting date of the crop can be positively correlated with *Fusarium* disease expression and abundance of *Fusarium* species. Positive correlations between the first planting date of grain sorghum and severe expression of grain mould caused by *F. thapsinum* were demonstrated during the rainy seasons in Morelos, Mexico (Montes-Belmont *et al.* 2003).

Optimal conditions for growth and reproduction vary widely in *Fusarium* species, influencing disease expression, species abundance and distribution. For example, the recently described species *F. langsethiae*, associated with wheat (*Triticum aestivum*), oat (*Avena* sativa) and barley (*Hordeum vulgare*) ears in the northern/central areas of Europe, has slower growth than the morphologically similar *F. poae*, and the metabolically similar *F. sporotrichioides* (Torp and Nirenberg 2004). Therefore, its potential to cause disease in different ecological and geographic areas of Europe might be underestimated as the other species are more competitive.

2.4 Cultivated Sorghum

2.4.1 Importance of Sorghum Worldwide and in Australia

Cultivated sorghum is the fifth most important cereal worldwide, and is mostly grown in regions with low rainfall and drought, from 40°S to 45°N latitude. Australia is the

fifth most important producer of grain sorghum, with grain sorghum the third most grown cereal in Australia (Maunder 2002).

Cultivated sorghum is a crucial human food and animal feedstuff in many African and Asian countries, and the main source of alcoholic beverages in some countries (Maunder 2002). However, Australian grown sorghum is used almost exclusively as feed for livestock, with a small quantity used for breakfast cereals and snack food (Ryley *et al.* 2002).

2.4.2 Classification and Domestication of Cultivated Sorghum

Grain sorghum (*Sorghum bicolor* subsp. *bicolor*) is a cultivated sorghum and belongs to the sub-genus Eu-Sorghum, which is one of the five sub-genera of the genus *Sorghum*. All species of the sub-genus Eu-Sorghum are African in origin. "Wild" relatives of cultivated sorghum include the annual weed grasses *S. bicolor* subsp. *drummondii* and *S. bicolor* subsp. *verticilliflorum* (Dahlberg 2000). Four races of "wild" weed grass *S. bicolor* subsp. *verticilliflorum*, considered to be essentially well-defined ecotypes (de Wet 1978), are the parental races of four of the five cultivated races of *S. bicolor* subsp. *bicolor* (Doggett 1988).

Species in the subgenus Eu-Sorghum have been domesticated more than once in Africa, as well as in South Arabia and India (Kimber 2000). However, the most accepted "Late hypothesis" suggests that domestication happened in the core area of diversity of "wild" weed grass relatives of *S. bicolor* in the eastern Sahara-Nile Valley-Lake Chad-inland Niger delta, in roughly 1000 B.C. (Kimber 2000).

Species in the subgenus Eu-Sorghum were introduced to Australia following the first European settlement in 1788 (Doggett 1988). The available data indicate that nine *Sorghum* species were cultivated or/and naturalized in Australia (Table 2-1). These

Sorghum species have been used as fodder sorghums, except for broom millet (*S. vulgare* var. *technicum*), which has been used in the broom industry.

Table 2-1 *Sorghum* species introduced to Australia and utilized as fodder sorghum[a]

Sorghum species	Common name	Geographic origin
S. almum† (*S. bicolor* x *S. halepense*)	Columbus grass	Argentina
S. bicolor‡	Grain sorghum, Cultivated sorghum, Sorghum	Ethiopia
S. brevicarinatum	Lunsamba	Central Africa, Indian Ocean Islands
S. halepense†	Johnson grass	Southern Euroasia – east to India
°*S. miliaceum*†	Unknown	India – northern & eastern
S. sudanense‡	Sudan grass	Sudan
S. verticilliforum▼	Wild sorghum	Tropical & subtropical southern Africa
S. vulgare cv. Saccaline	Sweet sorghum, Sorgho	Eastern African coast
**S. vulgare* var. *technicum*	Broom millet, Broomcorn	Mediterranean area (Italy)

[a]After Jackson and Jacobs (1985)

†perennial sorghum; ‡annual sorghum; ▼annual or weak biannual; °species morphologically similar to *S. halepense*, occupying north-west Pakistan to India, whereas *S. halepense* occupies Mediterranean to northern India; *not fodder sorghum

The first sorghum introduced to Australia was most likely broom millet (*S. vulgare* var. *technicum*) in the early 1800's (Figure 2-1). However, grain sorghum seed was probably introduced around 1908 from Texas, USA (McDonald 1909). Grain sorghum production in Australia commenced in 1938 (Jackson and Jacobs 1985).

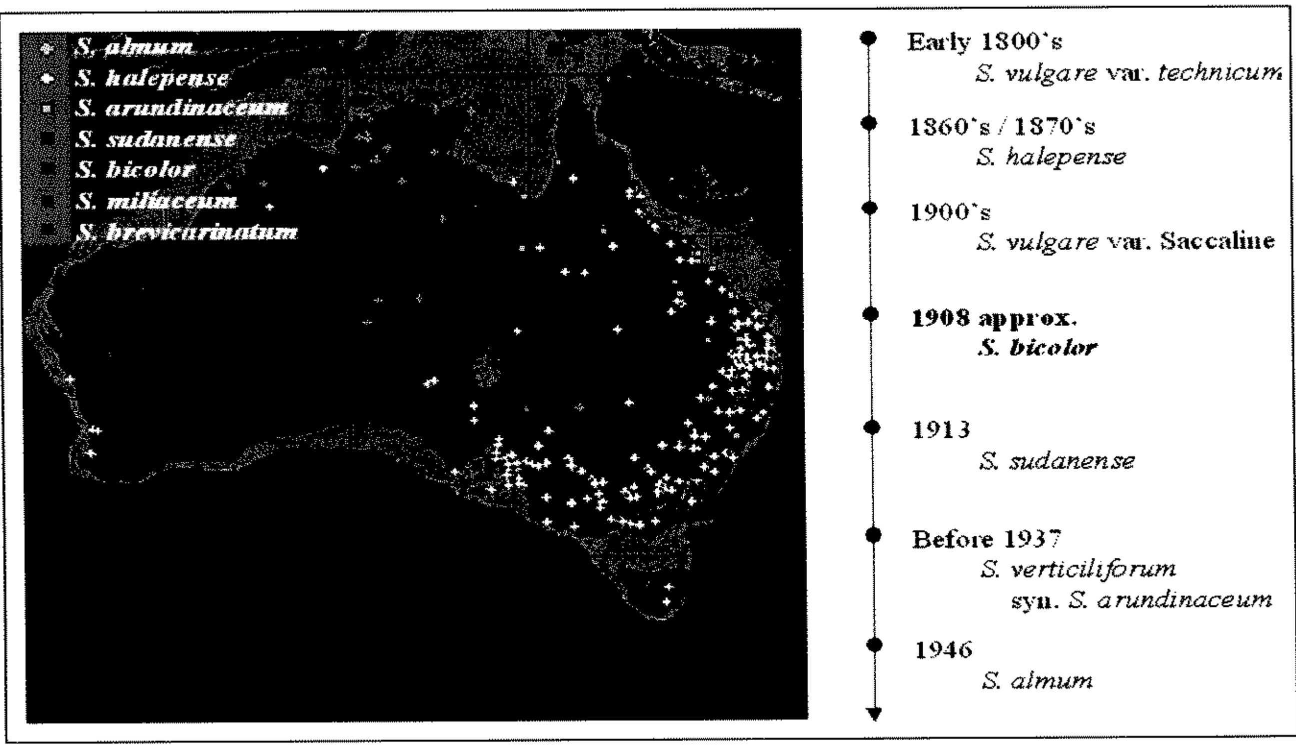


Figure 2-1 Distribution of introduced *Sorghum* species and time scale of their introduction to Australia. Distribution of introduced *Sorghum* species was generated from the *Australia's Virtual Herbarium* (www.anbg.gov.au/cgi-bin/avh.cgi)

Currently, grain sorghum hybrids are grown on approximately 600,000 ha in northeastern Australia (Figure 2-1), with the major production in central and southern Queensland (approx. 60%) and northern New South Wales (approx. 38%). Some of the short-day-length cultivars are grown in irrigated areas on the Ord River in Western Australia (approx. 2000 ha) (*Australian Bureau of Statistics*).

As a summer crop, grain sorghum is grown under dryland conditions with unreliable rainfall of 500 to 700 mm per annum. These conditions can lead to drought stress, increasing susceptibility to stress related diseases such as stalk and root rot (Burgess *et al.* 1981; Ryley *et al.* 2002). The fourth most important disease of grain sorghum in Australia is Fusarium stalk rot, which causes significant yield loss in some regions depending on climatic conditions (Trimboli and Burgess 1985).

2.5 *Fusarium* Species Associated with Grain Sorghum

2.5.1 *Taxonomy of the Most Important* Fusarium *Species Associated with Grain Sorghum*

The taxonomy of the *Fusarium* species associated with grain sorghum has changed remarkably over the past 25 years, more so than the *Fusarium* species associated with any other crop. Until 1980, the most frequently isolated *Fusarium* species from diseased sorghum were identified as *F. moniliforme sensu lato* (Pammel *et al.* 1916) and *F. moniliforme* var. *subglutinans* (Tarr 1962). Both species have been the subject of much taxonomic research (Table 2-2) (Burgess and Trimboli 1986; Klittich and Leslie 1992; Klittich *et al.* 1997; Leslie *et al.* 2005a; Marasas *et al.* 1987; Marasas *et al.* 2001).

Two simultaneous, independent surveys conducted in Australia and Africa in the 1980s, resulted in the discovery of *F. nygamai* (Burgess and Trimboli 1986) and *F. napiforme* (Marasas *et al.* 1987). Both species morphologically resemble *F. moniliforme sensu lato* but they also produce chlamydospores. Sufficient morphological variation existed to delineate the species, but their placement in the existing *Fusarium* classification system was uncertain. Both species shared morphological characters from sections *Liseola* and *Elegans*, indicating that they were good candidates for future molecular and phylogenetic analyses. *Fusarium nygamai* and *F. napiforme* were placed into the newly established section *Dlaminia* (Kwasna *et al.* 1991).

Similar systematic surveys of *Fusarium* species in the USA were carried out in 1986, resulting in a re-evaluation of *F. moniliforme sensu lato* (MP A) from sorghum and maize (Leslie *et al.* 1990; Leslie and Plattner 1991). Physiological markers (sexual compatibility, vegetative compatibility, culture pigmentation) indicated that *F.*

moniliforme encompassed two sibling species and in 1992 this species was split into two species with the same name, but different mating ability, *i.e.* *F. moniliforme* MP A and *F. moniliforme* MP F (Klittich and Leslie 1992). Subsequently, in 1997 *F. moniliforme* MP F was defined as *F. thapsinum* (teleomorph *Gibberella thapsina*) based on morphological, physiological, cytological and molecular markers (Klittich *et al.* 1997). Species *F. moniliforme* MP A, known predominantly as a maize pathogen with fumonisin-producing ability, was designated as *F. verticillioides* in 2003 (Seifert *et al.* 2003).

In 2001, another species morphologically resembling *F. verticillioides* was described (Marasas *et al.* 2001). The identification of *F. andiyazi* using molecular markers was followed by the formal description of the species. Morphological examination of *F. andiyazi* indicated that this species shared some characters with *F. nygamai* and *F. napiforme*, and also with *F. verticillioides*. These characters placed *F. andiyazi* into the section *Dlaminia*, together with *F. nygamai* and *F. napiforme* from sorghum (Rheeder *et al.* 2002). The teleomorph of *F. andiyazi* is unknown.

Fusarium moniliforme var. *subglutinans* was the second species considered as a major cause of disease in sorghum, with the taxonomic status of this species being resolved only recently (Leslie *et al.* 2005a; Samuels *et al.* 2001). In the past, *F. moniliforme* var. *subglutinans* encompassed two species with one teleomorph (*G. fujikuroi* var. *subglutinans*) (Nirenberg 1976; Kuhlman 1982). Sexual compatibility studies split this species into two, as *F. sacchari* (MP B; *G. sacchari*) (Kuhlman 1982; Leslie *et al.* 2005a) and *F. subglutinans* (MP E; *G. subglutinans*) (Leslie 1995; Samuels *et al.* 2001).

Table 2-2 Taxonomic changes of *Fusarium* species commonly associated with grain sorghum

Species	[a]Year	[b]Host	Method	Country	Author
F. moniliforme	1914	Sorghum	Morphology	USA (KS)	Pammel *et al.* 1916
F. nygamai	1980	Sorghum, soil	Morphology	Australia (NSW, QLD)	Burgess & Trimboli 1986
		French beans		Australia (QLD)	
		Root debris, Soil		Republic of South Africa	
		Soil		Thailand	
		Soil		Puerto Rico	
F. napiforme	1985	Pearl millet – grains	Morphology	Namibia, Republic of South Africa	Marasas *et al.* 1987
		Sorghum - grains		Namibia	
		Soil debris		Australia (QLD)	
F. thapsinum	1990 -1993	Sorghum – stalk	Sexual compatibility tests	Egypt	Klittich & Leslie 1992; Klittich *et al.* 1997
	1991	Maize – stalk		Egypt	
	1987	Sorghum – seed	VCGs	Philippines	
	1990	Sorghum – root, crown, seed, soil debris	Hygromycin resistance	Republic of South Africa	
	1988	Banana – stalk	Mycotoxins	Thailand	
	1985	Sorghum – seed	Electrophoretic karyotype	USA	
	1985 - 1988	Sorghum – stalk	Isozymes	USA	
	1985	Maize – seed	ITS restriction fragments	USA	
	1987	Maize – stalk		USA	
	1986	Peanut soil debris		USA	
	1989	Sorghum – stalk		USA	
	1997	Big bluestem		USA	Leslie *et al.* 2004b
F. andiyazi	1990	Sorghum plants	AFLP	Republic of South Africa	Marasas *et al.* 2001
	1991	Sorghum – grain	Sexual compatibility	USA (CO)	
	1990	Sorghum – grain	tests	Ethiopia	
	1988	Sorghum – grain	Morphology	Nigeria	

Table 2-2 (*Continued*)

Species	[a]Year	[b]Host	Method	Country	Author
F. moniliforme var. *subglutinans*	[c]NA	Sorghum	NA	NA	NA
F. sacchari	1984	Sorghum	Sexual compatibility tests	Brasil	Leslie *et al.* 2005a
	1981	Sugarcane		China	
	2002	Sorghum – stalk		El Salvador	
	NA	Orchids		Germany	
	NA	Sugarcane		India	
	2002	Maize		Mexico	
	1988	Sorghum – leaf		Philippines	
	1988	Sorghum – stalk		Philippines	
	1988	Sorghum – seed		Philippines	

[a]Date of survey and recovery of the species
[b]Host and substrate from which the species was recovered
[c]NA - data not available

2.5.2 **Fusarium** ***Species Associated with Sorghum in Australia***

A number of *Fusarium* species have been reported as colonizers of grain sorghum, broom millet (*S. vulgare* var. *technicum*) and forage sorghum in the sorghum growing areas of New South Wales (NSW), Queensland (QLD), Western Australia and Victoria (Burgess and Trimboli 1986; Chambers 1961; McKnight 1963; Norris 1949; Shivas 1989; Trimboli and Burgess 1985; Warcup and Talbot 1981; Woodcock 1983). Apart from *F. nygamai*, which was initially from grain sorghum in Australia (Burgess and Trimboli 1986), and then worldwide, all other species have been described worldwide and then in Australia. The most common *Fusarium* species on sorghum in Australia has been identified as *F. moniliforme sensu lato*. Since 1931, when this species was identified as a cause of root and stalk rot of grain sorghum in costal areas of NSW (Noble 1932), and later in Kununurra (Western Australia) (Norris 1949), the species has been regularly reported as a major pathogen of grain sorghum in NSW and QLD (Burgess *et al.* 1981; Henzell *et al.* 1984; Ryley *et al.* 2002; Trimboli and Burgess 1982; Trimboli and Burgess 1985)

The second most frequently isolated species, *F. subglutinans* (syn. *F. moniliforme* var. *subglutinans*), whose taxonomy has also changed was identified as a cause of stalk rot of grain sorghum in Western Australia (Shivas 1989) and Victoria (Woodcock 1983). Recently, the teleomorph of this species has been observed on old sorghum stalks in Spring Ridge, NSW (Burgess *et al.* 2002).

Fusarium graminearum (*Gibberella zeae*) was recorded as a cause of stalk rot of broom millet in Western Australia (Chambers 1961), and as a colonizer of the basal stalk of grain sorghum in NSW (Trimboli and Burgess 1985). Abundant perithecia of this species have been observed on old grain sorghum stalks in Spring Ridge, NSW (Burgess *et al.* 2002).

Fungi acting as causal agents of root rot of forage sorghum in Victoria (Clarke 1983), pre-emergence and seedling root rot of grain sorghum, and mouldy head of grain sorghum in QLD (McKnight 1963) were identified as *Fusarium* spp. Other *Fusarium* species, such as *F. culmorum*, *F. dimerum* and *F. oxysporum* were associated with root rot of grain sorghum in Western Australia (Shivas 1989), whereas *F. equiseti* and *F. semitectum* were associated with basal stalk rot of grain sorghum in NSW (Trimboli and Burgess 1985). *Gibberella fujikuroi* was recorded on *Sorghum* spp. in South Australia (Warcup and Talbot 1981).

2.5.3 Host Range and Implications of **Fusarium** *Species Common to Grain Sorghum*

Some *Fusarium* species are found more commonly on sorghum than any other crop. Strains of *F. thapsinum* are usually associated with grain sorghum, whereas strains of *F. verticillioides* are most commonly associated with maize (Leslie and Marasas 2002). It is likely that *F. thapsinum* is mainly associated with monocotyledonous plants such as grain sorghum, maize, banana (*Musa paradisiaca*) and, more recently a

prairie grass, *Andropogon gerardii* (Klittich *et al.* 1997; Leslie *et al.* 2004b). Also, *F. thapsinum* has been recovered from sorghum and peanut (*Arachis hypogaea)* soil debris (Table 2-2).

Fusarium species with limited host ranges and substrates include the recently described *F. andiyazi* recovered only from sorghum; *F. napiforme* recovered from pearl millet (*Pennisetum typhoides*) and sorghum (*Sorghum caffrorum*) grains, and soil debris from one grassland site; *F. sacchari* recovered from sugarcane (*Saccharum officinarum*), sorghum, maize and orchids (*Cattleya* spp.) (Leslie *et al.* 2005a; Marasas *et al.* 1987; Marasas *et al.* 2001; Sangalang *et al.* 1995).

On the other hand, two *Fusarium* species associated with sorghum, *F. proliferatum* and *F. nygamai*, have broad host ranges and substrates, including monocotyledonous and dicotyledonous plants, and diverse types of soils (Brayford 1997b; Desjardins *et al.* 2000; Laday *et al.* 2004; Leslie *et al.* 2004b). The initial host range and substrates of *F. nygamai* included grain sorghum, French beans (*Phaseolus vulgaris*), and soils from grasslands and different crops (Burgess and Trimboli 1986). Subsequent studies indicated that millet, rice (*Oryza sativa*), broad beans (*Vicia faba*) and the parasitic weed *Striga hermonthica* are also hosts of *F. nygamai* (Balmas *et al.* 2000; Brayford 1997b; Kurmut *et al.* 2002; Marasas *et al.* 1988).

An understanding of the host range and substrate of *Fusarium* species is an essential prerequisite for the development of disease management strategies, especially in rotational cropping systems.

2.5.4 *Importance of* **Fusarium** *Species on Grain Sorghum as Plant Pathogens and Mycotoxin Producers*

Several *Fusarium* species can be involved with Fusarium diseases of sorghum, including root and stalk rot, grain mould, grain weathering and exotic disease Pokkah Boeng (twisted top), making identification of the primary pathogen difficult (Claflin and Giorda 2002; Prom *et al.* 2003). *Fusarium* species affect sorghum severely under stressful environmental conditions. Thus, manifestation of Fusarium stalk rot is enhanced by hot dry weather followed by wet, cool conditions in the later growth stages of sorghum (Trimboli and Burgess 1985; Zummo 1983), whereas grain mould is severe when the grain matures under wet conditions (Garud *et al.* 2000). Yield reductions are attributed indirectly to lodging, and directly, to poor filling of seeds. Reduction in grain yield varies from 5 to 10%, but in some localities it can reach 100% (Claflin 2000; Claflin and Giorda 2002). In addition, certain *Fusarium* strains are capable of producing mycotoxins, which can be formed in pre-harvest infected plants, or in stored grains (Leslie 2000). However, the dominant *Fusarium* species on sorghum, *F. thapsinum* does not produce significant amounts of mycotoxins (Leslie *et al.* 2005b).

Fusarium species can live asymptomatically in sorghum plants as endophytes (Leslie *et al.* 1990; Leslie 2000) but their role and the nature of their association with sorghum have not been studied. The pathogenicity of the most common *Fusarium* species associated with sorghum, *i.e. F. thapsinum* (Jardine and Leslie 1992; Leslie *et al.* 2005b; Little and Magill 2003), *F. andiyazi* and *F. nygamai* (Leslie *et al.* 2005b) has been confirmed either in seedlings or mature plant tests under controlled conditions (Table 2-3). The pathogenic nature of *F. moniliforme sensu lato* on sorghum was also confirmed at different developmental stages (Trimboli and Burgess

1983). Today these isolates would probably be considered as either *F. thapsinum* or *F. andiyazi*.

Table 2-3 Toxigenic *Fusarium* species associated with grain sorghum

Species	*Fumonisins			Moniliformin	Beauvericin	Fusaproliferin	†Pathogenicity
	FB_1	FB_2	FB_3				
F. andiyazi	[a]Tr	[b]ND	ND	ND	[c]NA	NA	+++
F. napiforme	+	NA	NA	[d]+	NA	NA	NA
F. nygamai	+	+	+	+	+	ND	+
F. proliferatum	+	+	+	+	+	+	NA
F. thapsinum	Tr	Tr	Tr	+	ND	ND	++++
F. sacchari	+	NA	NA	+	+	[e]-	NA
F. subglutinans	+	NA	NA	+	+	+	NA
‡*F. verticillioides*	+	+	+	ND	ND	ND	++

*The most common forms of fumonisin (*ie.* FB_1 is the most prevalent form)
†Rated on a scale of + to ++++ with + = least pathogenic and ++++ = most pathogenic
‡Not frequently associated species with sorghum
[a]Tr - trace; [b]ND - not detected; [c]NA - data not available
[d]+ = produce toxin; [e]- = no toxin

The relative importance of the recently described species *F. andiyazi* in sorghum diseases is not clear, however sorghum seedling pathogenicity tests demonstrated that *F. andiyazi* is significantly less virulent than *F. thapsinum*, but more virulent than *F. nygamai* and *F. verticillioides* (Leslie *et al.* 2005b). Unfortunately, evaluating the aggressiveness of each *Fusarium* species is problematic, as it is difficult to ensure that the disease observed is due to the strain used for inoculation and not due to the strains asymptomatically present in the plant (Leslie *et al.* 2005b). The pathogenic nature of *F. proliferatum* and *F. sacchari*, from sorghum has been confirmed under either field conditions as a part of resistance breeding programs (Tesso *et al.* 2004; Tesso *et al.* 2005) or under greenhouse conditions in order to determine host range (Nirenberg 1976). The pathogenic nature of *F. napiforme* has not been studied (Brayford 1997a).

The production of toxins by *Fusarium* species and their accumulation in sorghum grain is an important sorghum–*Fusarium* interaction, since sorghum grain is used for

human consumption and animal feed. The most frequently associated species with sorghum, *F. thapsinum*, produces either trace or low levels of fumonisins but high levels of moniliformin (Leslie *et al.* 2005b; Rheeder *et al.* 2002). Moniliformin has no known potential to cause human health problems compared to fumonisins, but diets that contain this compound are toxic to ducklings, rodents, chickens and pigs (Harvey *et al.* 1997; Ledoux *et al.* 1995). *Fusarium andiyazi* does not produce either fumonisins or moniliformin, and as such this species is unlikely to be a threat to human and animal health. Unique to the *Fusarium* species associated with sorghum (Table 2-3), *F. proliferatum* produces all four toxins and has the potential to be toxic to animals if contaminated sorghum is present in their diet.

Knowledge on the pathogenicity and toxicity of these common *Fusarium* species on sorghum can be applied to delineating these species, especially if a wide range of strains are considered from different environmental conditions.

A new study of *Fusarium* species associated with grain sorghum in Australia is necessary following the recent major changes in the taxonomy of *Fusarium* species. The presence of relevant new species, reported on sorghum overseas, have not been assessed in Australia. An understanding of the occurrence and distribution of the species considered to cause stalk and root rot will contribute to improved disease management practices and assist sorghum breeders in the development of stalk rot resistant hybrids. Consequently, an intensive study was undertaken of *Fusarium* species associated with grain sorghum at different growth stages in the 2001/2002 growing season at Livingston Farm, Moree. This study provided detailed information on the nature and occurrence of *Fusarium* species. Subsequently a survey was undertaken of *Fusarium* species associated with senescent sorghum stalks in the 2002/2003 growing season in two agroclimatic regions, namely the Goondiwindi and

Quirindi areas of the northern grain belt of eastern Australia. This mycogeographic study was designed to provide a better understanding of the distribution and abundance of the dominant species (Chapter 6), their ecological role (Chapters 4 and 5) and the population structure of *F. thapsinum* (Chapter 7).

Chapter 3

General Materials and Methods

3.1 Growth and Processing of Isolates for DNA Extraction

Selected isolates were grown either in liquid SNA (Schilling 1996) (Appendix 1) or on solid PDA. Plates were incubated for 5 - 6 days at 25°C in complete darkness until abundant mycelia were produced. The mycelia from liquid SNA or solid PDA were transferred into a sterile 1.5 ml Eppendorf tube or a 1.5 ml Simport Micrewtube®, respectively.

3.2 Molecular Methods

3.2.1 Extraction and Estimation of Genomic DNA

Genomic deoxyribonucleic acid (DNA) was extracted using a modified FastDNA® Kit (Qbiogene, Inc., U.S.A.) protocol. All solutions except stock of Binding Matrix were prepared and sterilized following Sambrook *et al.* (1989) and are listed in Appendix 2.

Eppendorf tubes with harvested mycelia from liquid SNA were first centrifuged for 2 min at 12,000 x *g* and excess liquid was decanted. Approximately 0.2 g of the mycelia was then transferred from the Eppendorf tube into a sterile Simport Micrewtube® that contained approximately 0.1 g of white quartz sand (Sigma) (-50 + 70 mesh) and a ceramic bead. A 1 ml aliquot of cell lysis solution (extraction buffer) was added to each tube. The Simport tubes were placed in a homogeniser and shaken twice at 3.0 k for 30 s. The tubes were then cooled on ice for 2 min.

The mixture was then centrifuged at 12,000 x *g* for 5 min to separate the aqueous phase (supernatant) from the organic phase (pellet). A 750 µl aliquot of the

supernatant was transferred to a new sterile 1.5 ml Eppendorf tube. Proteins in the supernatant were precipitated by adding 125 µl of protein precipitate solution (PPS) and inverting tubes for approximately 4 min on a rotator. The tubes with mixture were then centrifuged at 14,000 x *g* for 5 min.

A 750 µl aliquot of protein-free supernatant that contained the DNA was transferred to a new 1.5 ml Eppendorf tube, and 700 µl of Binding Matrix was added to the protein-free supernatant and the tube contents were rotated for at least 20 min. This process allowed the negatively charged DNA to bind to small positively charged silicon particles in the Binding Matrix.

The matrix was then centrifuged at 10,000 x *g* for 15 s. The supernatant was decanted and the pellet resuspended in 800 µl of salt/ethanol wash solution (SEWS). The DNA was then repelleted at 10,000 x *g* for 30 s. The supernatant was decanted and the pellet was dried at room temperature for 30 min.

Finally, the DNA pellet was resuspended in 120 µl of sterile tris-EDTA (TE) buffer with ribonuclease (RNAse) (10 µg/ml). This process allowed the TE buffer to neutralize the silicon particles of the binding matrix and DNA molecules, releasing the bonds between them. To active the RNAse in order to degrade any ribonucleic acid (RNA) molecules present, tubes were incubated in a water bath at 37°C for 60 min. The tubes were centrifuged at 14,000 x *g* for 2 min to allow precipitation of silicon particles and elution of the DNA in TE buffer with RNAse.

A 90 µl aliquot of supernatant with DNA was transferred to a new 0.6 ml Eppendorf tube and stored at -20°C. The concentration and integrity of extracted genomic DNA was assessed using agarose gel electrophoresis. A quality control assay was performed using 100 bp DNA ladder (Promega) or HYPERLADDER I™ (Bioline).

Each of 5 µl of the 100 bp DNA ladder and 5 µl of the genomic DNA were mixed with 1 µl of blue/orange 6x loading dye and subjected to electrophoresis in single lanes on a 2% agarose gel (w/v). Electrophoresis was done at 80 V, in 1 × tris acetate-EDTA (TBE) running buffer, for 75 min. Double-stranded DNA was detected by staining the agarose gel with ethidium bromide in 1 x TBE buffer for 20 min. The gels were visualized and photographed on a ultra violet (UV) transilluminator.

Intensity of the DNA band was compared with the intensity of the 500 bp DNA fragment of the 100 bp DNA Ladder and DNA concentration was thus estimated.

3.2.2 PCR-Based Determination of Mating Type Alleles

Prior to sexual compatibility studies, mating type alleles (*MAT-1* and *MAT-2*) of selected isolates were determined using the PCR technique described by Steenkamp *et al.* (2000) and Kerényi *et al.* (1999). PCR-based identification of mating type alleles served to reduce the number of crosses of putative isolates with available tester strains.

The Gfmat1a and Gfmat1b (Steenkamp *et al.* 2000) and, GfHMG1 and GfHMG2 (Kerényi *et al.* 1999) primers were used in a co-amplification reaction to amplify an approximately 200 bp fragment from *MAT-1* mating type strains and a 262 bp fragment from *MAT-2* mating type strains. The primer sequences are listed in Table 3-1.

PCR amplifications of the approximately 200 bp fragment from *MAT-1* mating type strains were performed in 25 µl reaction mixtures containing 1 × PCR buffer (Promega), 2.5 mM $MgCl_2$ (Sigma), 0.2 mM of each deoxynucleoside triphosphate (Promega), 0.1 µM of each primer (Gfmat1a and Gfmat1b) (Sigma), 1 U of *Taq* DNA polymerase (Promega) and 1 µl of approximately 50 ng of diluted template DNA.

Amplification conditions for PCR were 94°C for 5 min (initial denaturation) followed by 35 cycles of 92°C for 30 s, 65°C for 30 s, and 72°C for 30 s, and a final extension at 70°C for 5 min. All amplifications were performed either in a Perkin Elmer 2400 Gene Amp PCR System or an Apollo™ ATC 401 thermal cycler.

PCR amplifications of the 262 bp fragment from *MAT-2* mating type strains were performed in 25 µl reaction mixtures containing the same concentrations of reagents as for the *MAT-1* reactions, except that 0.25 mM of each deoxynucleoside triphosphate (Promega) and primers GfHMG1 and GfHMG2 (Table 3-1) were used. Amplification conditions for PCR of the *MAT-2* allele were 95°C for 3 min (initial denaturation), followed by 30 cycles of 94°C for 1 min, 65°C for 30 s and 72°C for 1 min, and a final extension at 72°C for 9 min.

An 8 µl aliquot of each amplification product was mixed with 2 µl of dye and electrophoresed at 80 V in 1.5% agarose gel with TBE buffer and ethidium bromide (10mg/ml).

Table 3-1 Primer and adapter oligonucleotide sequences

Primer or adapter oligonucleotide	Sequence (5' to 3')
Mating type primers	
GFHMG1	ACCGTAAGGAGCGTCACCATT
GFHMG2	GGGGTACTGTCGGCGATGTT
Gfmat1a	GTTCATCAAAGGGCAAGCG
Gfmat1b	TAAGCGCCCTCTTAACGCCTTC
Adapters	
*Eco*RI adapters:	
EA1	CTCGTAGACTGCGTACC
EA2	AATTGGTACGCAGTCTAC
*Mse*I adapters:	
MA1	GACGATGAGTCCTGAG
MA2	TACTCAGGACTCAT
Preamplification primers	
*Eco*RI+0	GACTGCGTACCAATTC
*Mse*I+0	GATGAGTCCTGAGTAA
Amplification (Selective) primers	
*Eco*RI+GG	GACTGCGTACCAATTCGG
*Mse*I+CT	GATGAGTCCTGAGTAACT
Translation elongation factor-1α	
ef1	ATGGGTAAGGA(A/G)*GACAAGAC
ef2	GGA(G/A)GTACCAGT(G/C)*ATCATGTT

*( ) indicates degenerate bases

3.2.3 AFLP Procedures

The AFLP fingerprinting protocol was based on the technique modified by Zeller *et al.* (2000).

A 5 μl aliquot (approximately 100 ng) of genomic DNA was digested with 1.2 U of the *Eco*RI (Promega) and 0.4 U of the *Mse*I (New England Bioscience) restriction enzymes for 7 h at 37°C in a 20 μl reaction mixture that contained 1 x one-phor-all buffer (Pharmacia Biotechnologies) and 1 x bovine serum albumin (BSA) (Promega).

After digestion, *EcoR*I and *Mse*I double stranded-adapters (Table 3-1) were ligated to the sticky ends of the restriction fragments at 22°C overnight. A 5 µl aliquot of the reaction mixture containing approximately 4 pmol *Eco*RI adapter (Sigma), 20 pmol *Mse*I adapter (Sigma), 1mM adenosine triphosphate (ATP) (Amersham Biosciences), 0.5 U T4 DNA ligase (USB) and 1 x T4 ligase buffer (USB) or 1x one-phor-all buffer (Pharmacia Biotechnologies) were added to the mixture of the digested DNA fragments. To check that complete digestion and ligation were obtained, 6 µl of digestion and ligation reaction mixtures for all isolates were run on a 1.5% agarose gel at 65 V for 75 min. A genomic DNA sample (2 µl) was loaded in a single lane as a positive control. After ligation, the reaction mixture was diluted 1:10 in sterile distilled H_2O and stored at –20°C for use in AFLP PCRs.

The AFLP pre-selective amplification was done using the *Eco*RI/*Mse*I primer combination with no additional selective nucleotide (Table 3-1). The pre-selective amplification reaction was performed in a final volume of 15 µl. Each reaction mixture contained 1 x PCR buffer (Promega), 2.0 mM $MgCl_2$ (Sigma), 0.2 mM of each deoxynucleoside triphosphate (Promega), 10 pmol of the *Eco*RI and *Mse*I primers, 0.5 U of *Taq* polymerase (Promega), and 5 µl of diluted ligation product. The pre-selective amplification reaction conditions were 20 cycles consisting of 94°C for 30 s (denaturation), 56°C for 1 min (annealing) and 72°C for 1 min (extension). To check the pre-selective amplification of DNA fragments were performed and the quality of the PCR product, 5 µl of pre-selective amplification PCR products of all isolates were run on a 2% agarose gel at 80 V for 60 min. The pre-selective amplification PCR product was diluted 1:20 or 1:50 in TE buffer and stored at –20°C for use in the selective amplification.

Selective amplifications were carried out with *Eco*RI and *Mse*I primers containing two selective nucleotides at the 3' ends (Table 3-1), with *Eco*RI primer labelled at the 5' end with fluorescent dye either hexachloro-6-carboxyfluorescine (HEX) or 6-carboxyfluorescine (6-FAM). Each selective amplification was performed in a final volume of 15 μl, containing 1 x PCR buffer (Promega), 2.0 mM $MgCl_2$ (Sigma), 0.17 mM of each deoxynucleoside triphosphate (Promega), 6 pmol of *Eco*RI+GG and *Mse*I+CT primers, 0.75 U of *Taq* polymerase (Promega) and 5 μl of diluted pre-selective amplification product. The selective amplification conditions were 13 cycles of 94°C for 60 sec, 65°C for 60 s, decreasing 0.7°C every cycle, and 72°C for 90 s, followed by 23 cycles consisting of 94°C for 30 s, 56°C for 30 s and 72°C for 60 s.

The selective amplification products and a molecular weight marker were electrophoresed at 80 V for 90 min in 2% agarose gel. Based on the intensity of fragments, selective amplification products were diluted two to tenfold in sterile distilled H_2O. Diluted products were separated using vertical gel electrophoresis with an ABI PRISM® 3700 DNA Analyser (Applied Biosystems Inc., U.S.A) at SUPAMAC (Sydney University Prince Alfred Macromolecular Analysis Centre).

3.2.4 DNA Sequencing

A portion of the translation elongation factor 1-α (TEF) gene was sequenced and analyzed to identify a group of isolates to species level. This gene was selected because of the high level of sequence polymorphisms in this gene among closely related *Fusarium* species, and is the marker of choice as a single-locus identification tool for *Fusarium* (Geiser *et al.* 2004). DNA sequences were generated according to the procedures described by O'Donnell *et al.* (2000) and Geiser *et al.* (2004).

Amplification of the approximately 700 bp fragment of the TEF gene in selected isolates was performed in 50 μl reaction mixtures containing 1 x PCR buffer (Promega), 2.5mM $MgCl_2$ (Sigma), 1 mM of each of deoxynucleoside triphosphate (Promega), 0.19 μM of each primer (efl and ef2), 1.25 U of *Taq* polymerase and approximately 50 ng of genomic DNA. Amplification conditions for PCR were 94°C for 60 sec (initial denaturation) followed by 35 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 50 s, and a final extension at 72°C for 3 min.

Amplification products were purified with the Wizard® SV Gel and PCR Clean-Up System (Promega, Australia) according to the manufacturer's instructions. Primers efl and ef2 were used to sequence both strands of the TEF gene region, which provides an internal check for sequence quality. DNA sequencing was conducted and analyzed using an ABI PRISM® 3700 automated DNA Sequencer (Applied Biosystems Inc., U.S.A) at SUPAMAC.

3.3 Sexual Compatibility Tests

Selected putative isolates from which DNA was extracted and mating type alleles were determined were included in sexual compatibility tests with morphologically similar standard mating population tester strains for species identification. Putative isolates identified as *F. thapsinum* were also assessed for female fertility as a component of the population studies on this species.

Tester strains used in this study were obtained from the Fungal Genetics Stock Center (FGSC) (Department of Microbiology, University of Kansas Medical Center, Kansas City): MP A (*G. moniliformis*) [FGSC 7600 (*MATA-1*) and FGSC 7603 (*MATA-2*)], MP B (*G. sacchari*) [FGSC 7611 (*MATB-1*) and FGSC 7610 (*MATB-2*)], MP D (*G. intermedia*) [FGSC 7615 (*MATD-1*) and FGSC 7614 (*MATD-2*)], MP E (*G.*

subglutinans) [FGSC 7616 (*MATE-1*) and FGSC 7617 (*MATE-2*)] and MP F (*G. thapsina*) [FGSC 7057; KSU4094 (*MATF-1*) and FGSC 7056; KSU4093 (*MATF-2*)].

Some of the crosses of putative *F. thapsinum MAT-1* isolates were performed with the highly female fertile *MAT-2* isolate (1050) obtained during this project due to the apparent female loss of fertility in the tester strain FGSC 7056 (*MATF-2*). Frequent lyophilisation and subculturing of tester strain FGSC 7056 (*MATF-2*) may have led to this loss of fertility.

Sexual compatibility tests were conducted according to the procedure described by Klittich and Leslie (1988). Firstly, selected putative isolates served as males and the standard tester strains served as the female parents. Putative isolates (males) were used for fertilization of tester strains (females) with opposite mating type alleles.

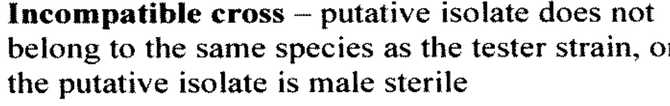
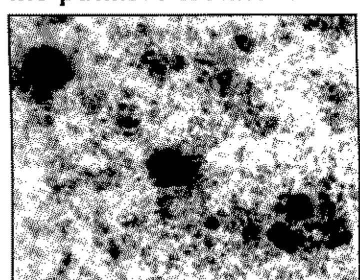
All isolates were grown, and crossings were performed, in 6 cm diameter Petri plates. Standard tester strains and selected putative isolates were grown on CLA at 25°C light / 20°C dark with a 12 h photoperiod for six and 13 days, respectively. Six-day-old standard tester strains were subcultured from CLA to carrot agar (CA) and incubated at 25°C in the dark for six to seven days. Thirteen-day-old putative isolates grown on CLA were used for preparation of spore and mycelial suspensions. Three to four carnation fragments with sporodochia and mycelia were transferred into 10 ml of sterile distilled H_2O and vortexed. An aliquot of the suspension (0.8 ml or 1 ml) was poured over the tester strain mycelia and spread with a glass rod until all of the mycelium was wet. Positive and negative controls (two replicates of each) were also included. Tester strains with opposite mating type alleles were crossed with each other as a positive control. Each of the tester strains was also fertilized with sterile distilled

H_2O as a negative control. Also, each tester strain was fertilized to itself to exclude the possibility of homothallic crosses or contamination of tester strains.

Fertilized cultures were incubated at approximately 21°C with a 12 h photoperiod for six weeks. Incubation temperature fluctuated during the day but never exceeded 25°C.

Male fertility crosses of putative isolates were performed at least twice and were examined weekly to observe if the isolates produced perithecia with exuding ascospores. The crosses were examined under a dissecting microscope four and six weeks after fertilization. A cross was considered compatible and fertile if a moderate to abundant number of perithecia with oozing cirri developed in the culture (Table 3-2).

Table 3-2 Scoring of sexual compatibility of putative isolates and tester strains

Compatible cross - putative isolate belongs to the same species as the tester strain	 **Incompatible cross** – putative isolate does not belong to the same species as the tester strain, or the putative isolate is male sterile 
Male fertility	Male fertility
Tester strain ♀ x Putative isolate ♂	Tester strain ♀ x Putative isolate ♂
MAT-1 x *MAT-2*	*MAT-1* x *MAT-2*
MAT-2 x *MAT-1*	*MAT-2* x *MAT-1*
Female fertility	Female fertility
Putative isolate ♀ x Tester strain ♂	
MAT-1 x *MAT-2*	Not required
MAT-2 x *MAT-1*	

Putative isolates that resulted in fertile perithecia when crossed with tester strains were considered to be male fertile. Their female fertility was checked in the reciprocal

crosses, reversing the male and female role of putative isolates and tester strains. This time, selected putative isolates served as females whereas standard tester strains served as the male parents. Tester strains (males) were used for fertilization of putative isolates (females) with the opposite mating type allele. The same procedure of fertilization and incubation of crosses described above were applied to these crosses.

Female fertility crosses of putative isolates with tester strains of *G. thapsina* were performed three times because of the low number of female fertile isolates and sparse perithecia with exuding ascospores in some crosses. The crosses were examined under a dissecting microscope three, four and six weeks after fertilization.

Chapter 4

Comparison of *Fusarium* Communities Associated with Different Plant Parts and Crop Stages in Grain Sorghum

4.1 Introduction

Fusarium species are associated with grain sorghum through all growth stages either as primary or secondary colonizers. Primary colonizers are the pathogenic species that cause seedling diseases, poor stand establishment, root and stalk rot, exotic disease Pokkah Boeng (twisted top), and panicle and seed diseases (grain mould, grain weathering, head blight) (Burgess *et al.* 1981; Forbes *et al.* 1992; Giorda *et al.* 1995; Giorda 2002; Leslie and Mansuetus 1995; Pande and Karunakar 1992; Tarr 1962). Apart from Pokkah Boeng, which is caused mainly by *F. sacchari* (Leslie *et al.* 2005a; Priode 1933; Zummo 1983), the other sorghum diseases are considered complex in nature, as several *Fusarium* species can be involved in pathogenesis (Giorda 2002; Karunakar *et al.* 1993; Mansuetus *et al.* 1997; Marley *et al.* 2002). *Fusarium* species also can be associated with asymptomatic sorghum tissue (Leslie 2000; Reed *et al.* 1983; Trimboli and Burgess 1982), but the nature of this association is poorly understood.

Environmental conditions, such as rainfall distribution during the growing season and cultivar have a major influence on both colonisation by *Fusarium* species and disease severity (Giorda 2002; Pande and Karunakar 1992). For example, in some humid areas, such as those in Venezuela, *F. moniliforme sensu lato* invades stems and the axis of the panicles, reducing grain formation and causing plants to collapse (Malaguti 2002). In other humid areas worldwide, a complex of species causes grain mould of sorghum (Bandyopadhyay *et al.* 1991; Garud *et al.* 1998; Garud *et al.* 2000). In dry

areas, such as those in northeastern Australia, *F. moniliforme sensu lato* is associated with sorghum roots and stalks, and occasionally causes blight of sorghum heads (Trimboli and Burgess 1985).

Infection at different root and stalk loci of the sorghum plant may occur early in the growing season, but expression of symptoms, such as stalk rot, may be delayed until the plant is exposed to stress factors, such as drought (Dodd 1978; Partridge *et al.* 1983). Infection of sorghum roots and stalks may be initiated by inoculum in residues or from the planting of contaminated seed (Khune *et al.* 1984).

Other fungal species such as *Macrophomina phaseolina* and *Nigrospora sphaerica* are also common colonizers of sorghum roots and stalks (Pande and Karunakar 1992; Trimboli and Burgess 1982). Studies conducted in NSW revealed that *F. moniliforme sensu lato* was an early colonizer whereas *M. phaseolina* and *N. sphaerica* were later, secondary colonizers of sorghum stalks (Trimboli and Burgess 1982).

In the past, the nature of the association between *Fusarium* species and sorghum plants has been studied throughout the growing seasons based on the morphological identification of *Fusarium* species (Leslie *et al.* 1990; Partridge *et al.* 1983; Reed *et al.* 1983; Trimboli and Burgess 1982; Trimboli and Burgess 1985). The temporal and spatial dynamics of *Fusarium* colonization of sorghum tissue was studied either under conventional cultivation (Trimboli and Burgess 1985), or in no-tillage cropping systems (Reed *et al.* 1983). More recently, the identification of *Fusarium* species from the section *Liseola* associated with sorghum seeds have been studied using the biological identification of species through sexual compatibility tests (Mansuetus *et al.* 1997).

The taxonomic status of many *Fusarium* species associated with grain sorghum has changed significantly over the past decade (Klittich and Leslie 1992; Klittich *et al.* 1997; Marasas *et al.* 2001). These changes led Marasas *et al.* (2001) to recommend that "sorghum worldwide needs to be re-examined ... to determine the frequency and distribution of *F. andiyazi*, ... to provide more information on *F. thapsinum* and to ensure that all of the *Fusarium* species common to sorghum have been properly identified and characterised".

The study reported in this chapter was designed to test the hypotheses that *F. thapsinum* is the dominant species associated with sorghum irrespective of the plant part; that *F. thapsinum* is the dominant species associated with sorghum irrespective of the crop stage; that the diversity and abundance of *Fusarium* species are similar in various plant parts of sorghum; and that the diversity and abundance of *Fusarium* species are similar in the different crop stages of sorghum.

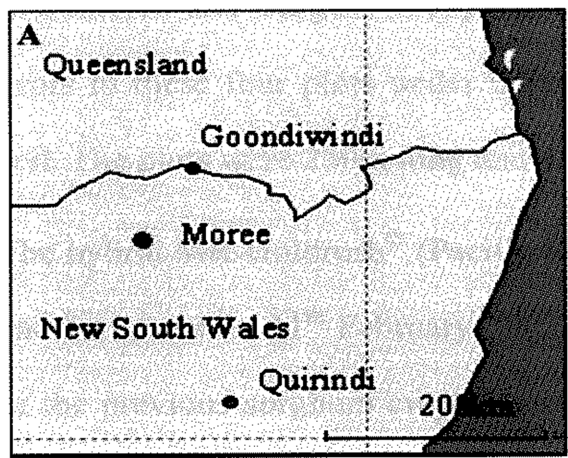
A further outcome of this study was the collection of isolates for studying on the population structure of *F. thapsinum*, as reported in Chapter 7.

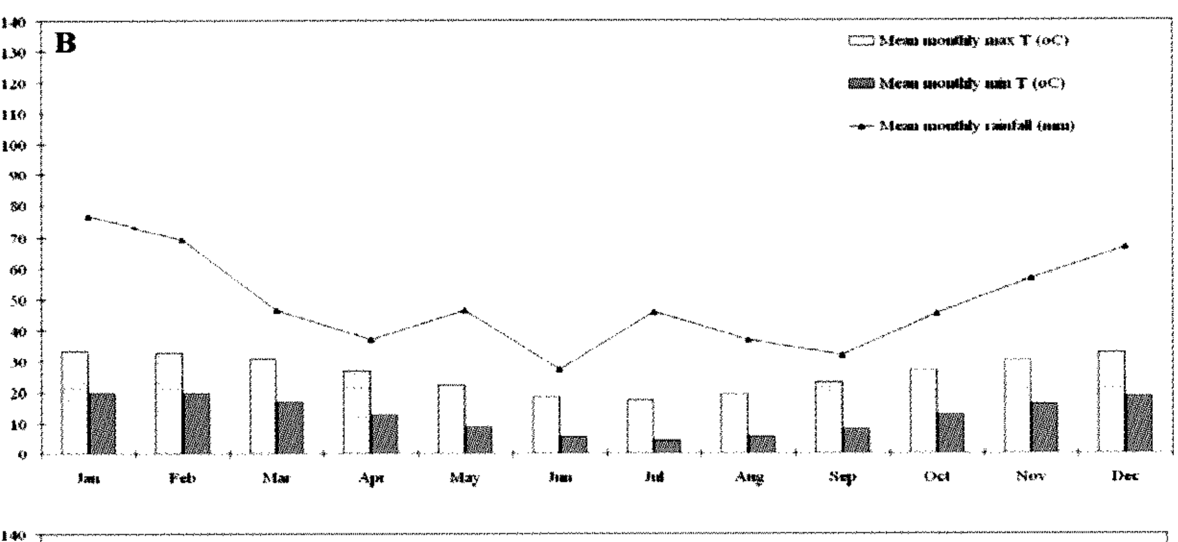
4.2 Materials and Methods

4.2.1 Climatic data and field sampling

Samples of grain sorghum were collected at the University of Sydney's Livingston Farm, Moree, in northwestern NSW, Australia in the 2001/2002 growing season. Moree is in the northern summer dominant rainfall region of the eastern grain belt of Australia (Figure 4-1A), with an annual average rainfall of 585 mm. Moree has a long warm to hot summer, with a mean minimum temperature of 18-20°C and a mean maximum temperature of 33°C. Winter is characterised by cool clear days and cold, sometimes frosty nights, with an average minimum temperature of 4-5°C and an average maximum temperature of 17-19°C (Figure 4-1B). From September 2001 till

March 2002 (the 2001/2002 crop season), the total rainfall at Livingston Farm was 402.1mm (Figure 4-1C).





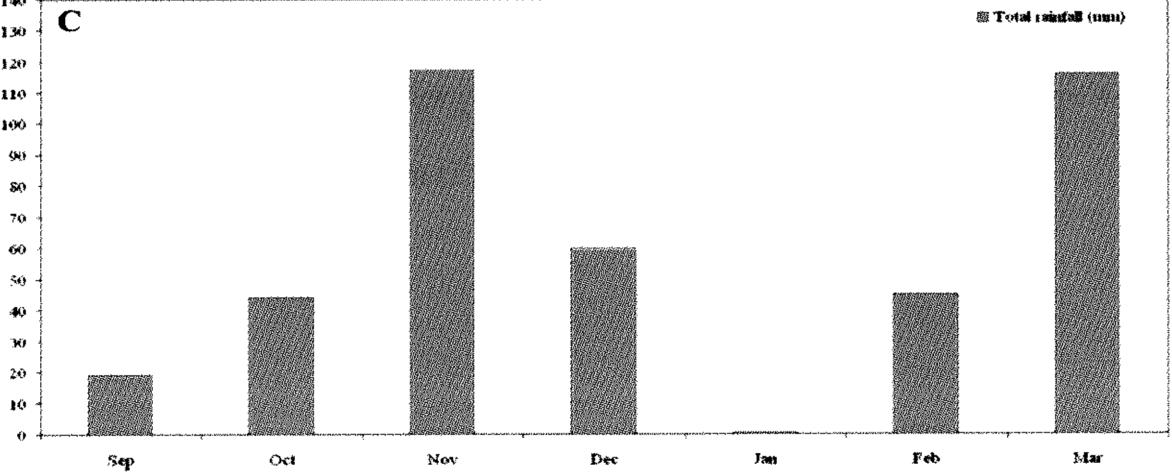


Figure 4-1 Map of part of eastern Australia showing the location of Livingston Farm, Moree (A) and climatic data for Moree (B). Total monthly rainfall at Livingston Farm in the 2001/2002 growing season (C) (*Australian Government Bureau of Meteorology*)

Samples of grain sorghum were collected from four plots, designated D, F, M and S, which are part of a long-term trial on stubble management and disease (Figure 4-2A) (Summerell and Burgess 1988). Grain sorghum had been grown continuously for 20 years in these four plots under reduced tillage on a neutral self-mulching grey clay soil. The plots were 250m long and 45m wide.

The hybrid MR-Goldrush© (Pacific Seeds Pty Ltd) was sown on 20th September 2001 and harvested on 11th February 2002. Sorghum was planted directly into the residues of the previous sorghum crop (Figure 4-3A). Fifty sorghum plants were collected at random from each plot at three crop stages: seedling (developmental stage 5.1 - main shoot and one tiller), physiological maturity (developmental stage 11.4 - grains at physiological maturity) and residue (twelve weeks after harvest) (Dahlberg and Frederiksen 2000) (Table 4-1). Sampling began at least 25 m from the end of each plot and 13 m from the side of the plot (Figure 4-2B) to avoid edge effects. One plant was collected from each third row crossed along a zig-zag sampling transect (Figure 4-2C).

Whole seedlings were sampled at five weeks after planting (Figure 4-3A, B), followed by sampling at 18 weeks after planting (20 days before harvest) (Figure 4-3C, D) and at twelve weeks after harvest (Figure 4-3E, F).

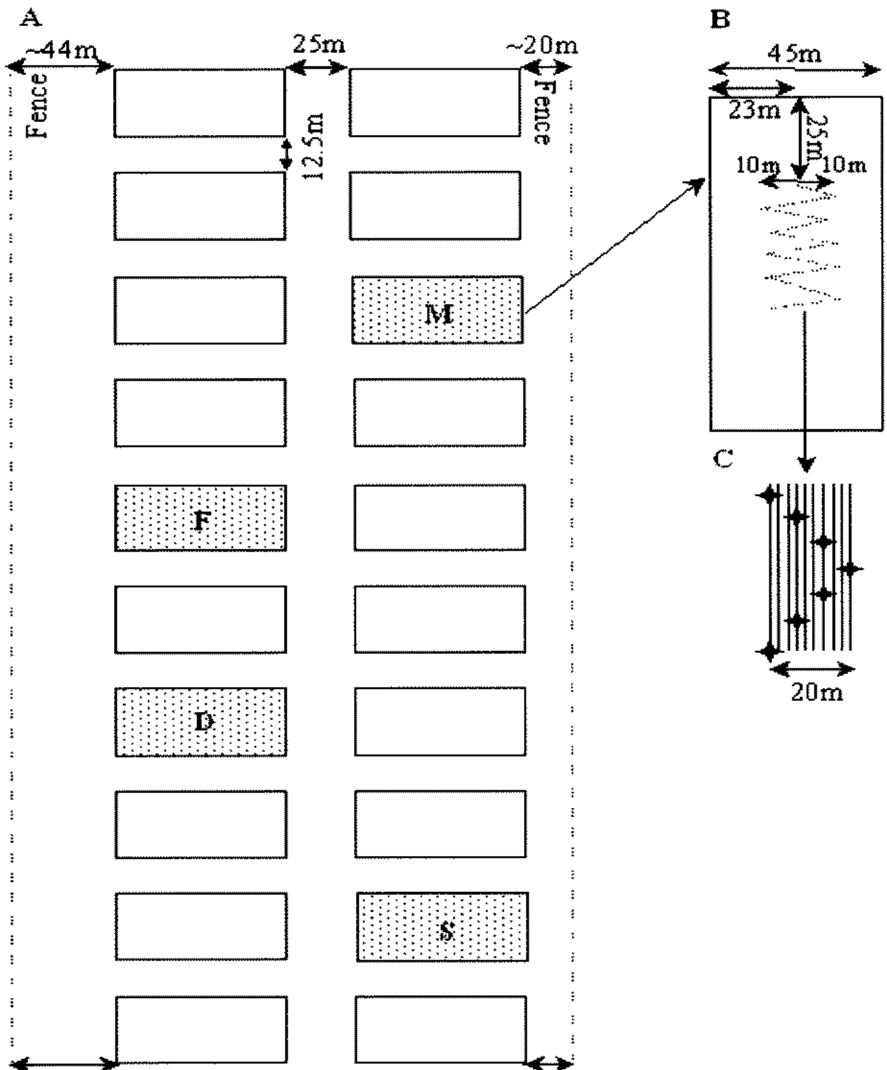


Figure 4-2 Position of four sorghum plots within a long-term trial at Livingston Farm, Moree (A), sampling strategy within a plot (B) and the zig-zag sampling transect (C)

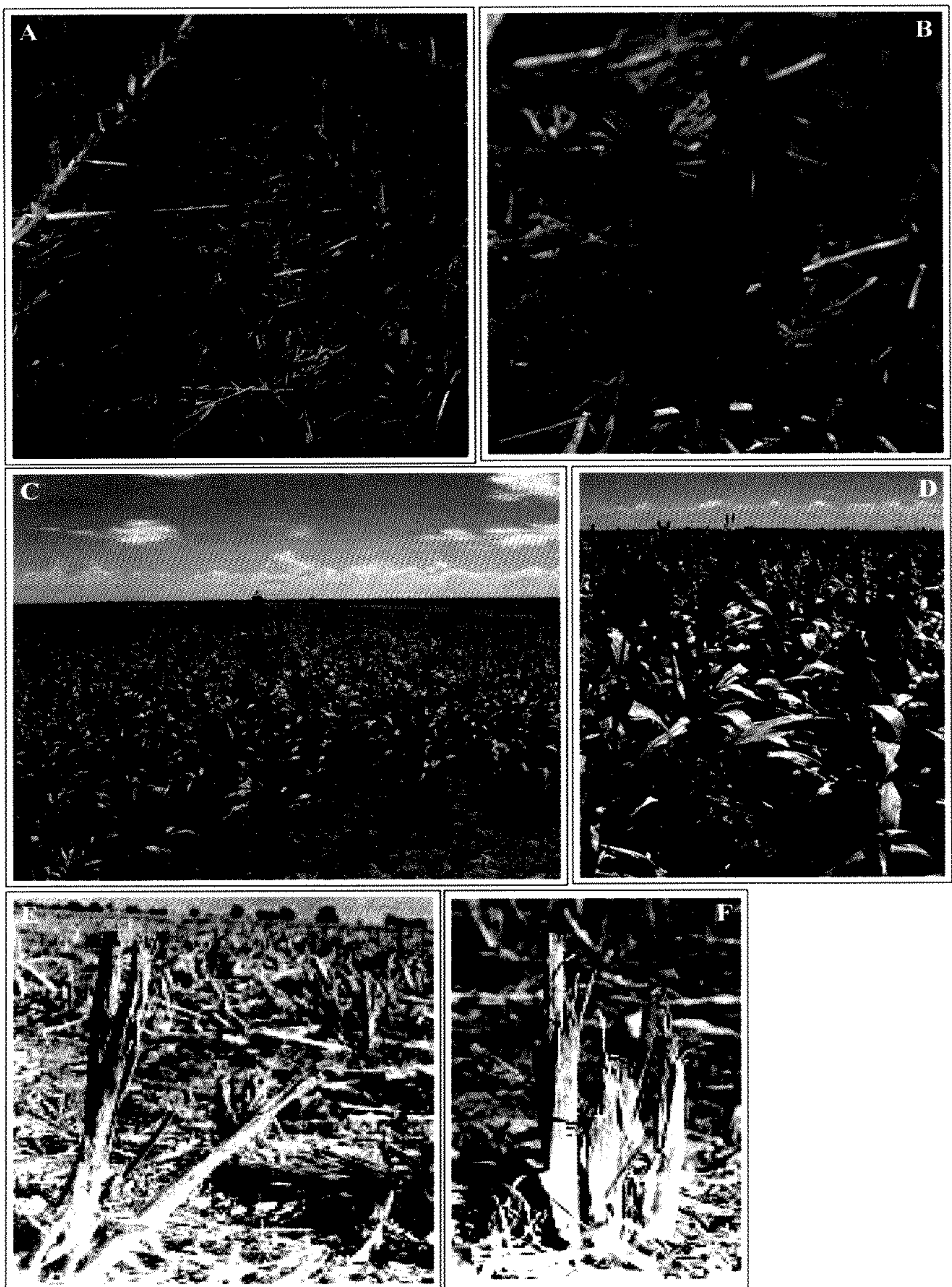


Figure 4-3 Crop stages of grain sorghum sampled at Livingston Farm, Moree, in the 2001/2002 growing season. Seedlings (A, B), physiologically mature plants (C, D) and residues (E, F). Sorghum stalks and residues from the 2000/2001 growing season were also present on the soil surface (A)

Table 4-1 Sampling dates, crop stages and sample number of grain sorghum at Livingston Farm, Moree, in the 2001/2002 growing season

Sampling				Plant organs						
					Root			Stalk		
Time	Date	Crop stage[†]	Trial plots	Mesocotyl[‡]	Asymptomatic	Symptomatic	Crown	4th node	6th node	Total number of fragments
1st	25/10/2001	Seedling	D	50	50	50	50			800
			F	50	50	50	50			
			M	50	50	50	50			
			S	50	50	50	50			
2nd	21/01/2002	Physiological maturity	D	19	50	50	50	50	50	1052
			F	5	50	50	50	50	50	
			M	15	50	50	50	50	50	
			S	13	50	50	50	50	50	
3rd	6/05/2002	Residue	D			50	50	50		600
			F			50	50	50		
			M			50	50	50		
			S			50	50	50		
			Total	252	400	600	600	400	200	2452

[†]Crop stages of sorghum after Dahlberg and Frederiksen (2000): Seedling (5.1 – main shoot and one tiller); Physiological maturity (11.4 – grains at physiological maturity); Residue (residues of harvested plants)
[‡]Mesocotyl – this part of sorghum sampled where attached to the plant stalk

4.2.2 *Isolation and purification of isolates*

Sorghum roots were washed under tap water to remove soil, soaked in diluted detergent LOC® (1 ml / 5 l) for 15 min and then washed with tap water for 3 min. Plants were placed on a paper towel to air dry. The stalks of physiological mature plants and residues were washed and leaves were removed before surface sterilising with 70 % ethanol.

Different plant parts were selected for isolation depending on the crop stage (Table 4-1; Figure 4-4). The mesocotyl, asymptomatic and symptomatic crown root tissue and crown were selected from sorghum seedlings for *Fusarium* isolation. Asymptomatic and symptomatic crown root tissue, the crown, the fourth and sixth nodes of the stalk

and the mesocotyl if present, were selected from physiologically mature plants for *Fusarium* isolation. Symptomatic crown root tissue, the crown and the fourth node of stalk were selected from sorghum residues, for *Fusarium* isolation.

The selected plant parts from seedlings and physiological mature plants were removed from each plant and surface sterilized in a 1.2 % solution of NaOCl for 30 s, rinsed twice in sterile distilled water and air-dried. Symptomatic crown roots, the crown and the fourth node of stalk from sorghum residues were soaked longer (2 min) in a 1.2 % NaOCl solution, rinsed twice in sterile distilled water and air-dried.

A segment of each plant part was plated on *Fusarium*-selective medium, PCNB-peptone agar (PPA) (Burgess *et al.* 1994) (Figure 4-4; Appendix 1). Plates were incubated for 5 days under an alternating 25°C light / 20°C dark temperature regime with a 12 h photoperiod (Burgess *et al.* 1994). Colonies of *Fusarium* species were subcultured onto carnation leaf agar (CLA) (Fisher *et al.* 1982) (Appendix 1) and incubated for 7 - 10 days under the above conditions. Isolates were purified using the single-spore technique on water agar (WA) (Burgess *et al.* 1994) and colonies were established on each of three media, CLA, potato dextrose agar (PDA) and low-nutrient synthetischer nährstoffärmer agar (SNA) with a piece of ca. 0.5 x 1 cm sterile filter paper (Nirenberg 1976) (Appendix 1).

Cultures on CLA and PDA were incubated under the same conditions as isolation plates and subcultures, while the cultures on SNA were incubated at 25°C in complete darkness (Aoki and Nirenberg 1999). After 10 to 14 days of incubation, morphological characters were examined on CLA and SNA, and cultural characters were assessed on PDA.

Isolates were stored temporarily as mycelium with spores on a plug of WA in sterile 1.5 ml Eppendorf tubes at 4°C. Selected isolates were preserved for further work either by freezing in 15% glycerol at -70°C (Summerell *et al.* 2003) or by lyophilisation (Burgess *et al.* 1994).

4.2.3 Identification of isolates

Morphological identification - Isolates that resembled species from sections *Liseola* and *Dlaminia* were morphologically identified based on the characters and key devised by Nirenberg and O'Donnell (1998). All other isolates were identified based on the species descriptions of Burgess *et al.* (1994) and Gerlach and Nirenberg (1982). Different keys were applied to accommodate latest changes in taxonomy of *Fusarium* species in sections *Liseola* and *Dlaminia*, and to incorporate morphological criteria for other *Fusarium* sections established by applied mycologists (Burgess *et al.* 1994) and classical taxonomist (Gerlach and Nirenberg 1983), providing more accurate morphological identification of species. *Fusarium* species in sections *Liseola* and *Dlaminia*, characterized by production of microconidia in chains ("chain-producing" species) or false heads on monophialides and polyphialides, require rigorous examination of morphological characters due to species similarity.

Sexual compatibility - A subset of 510 isolates (390 isolates from physiologically mature plants and 120 isolates from sorghum residue) of morphologically identified, *F. thapsinum* (362 isolates) and *F. andiyazi* (148 isolates), were used in sexual compatibility tests with tester strains of *G. thapsina* (MP F) and *G. moniliformis* (MP A). An additional subset of 30 isolates morphologically identified as *F. proliferatum* isolated from physiologically mature sorghum were used in sexual compatibility tests with tester strains of *G. intermedia* (MP D). PCR-based determination of mating type alleles was performed prior to the sexual compatibility tests (*see* Chapter 3). Sexual

compatibility tests were conducted based on the protocol described by Klittich and Leslie (1988) (*see* Chapter 3).

Molecular identification - The identity of 127 putative isolates of *F. thapsinum* (64 isolates) and *F. andiyazi* (63 isolates) from the original subset (510 isolates) was further confirmed using AFLPs. The identity of three isolates of *F. proliferatum* out of the original subset (30 isolates) was further confirmed using phylogenetic analysis of a portion of the TEF gene (*see* Chapter 6).

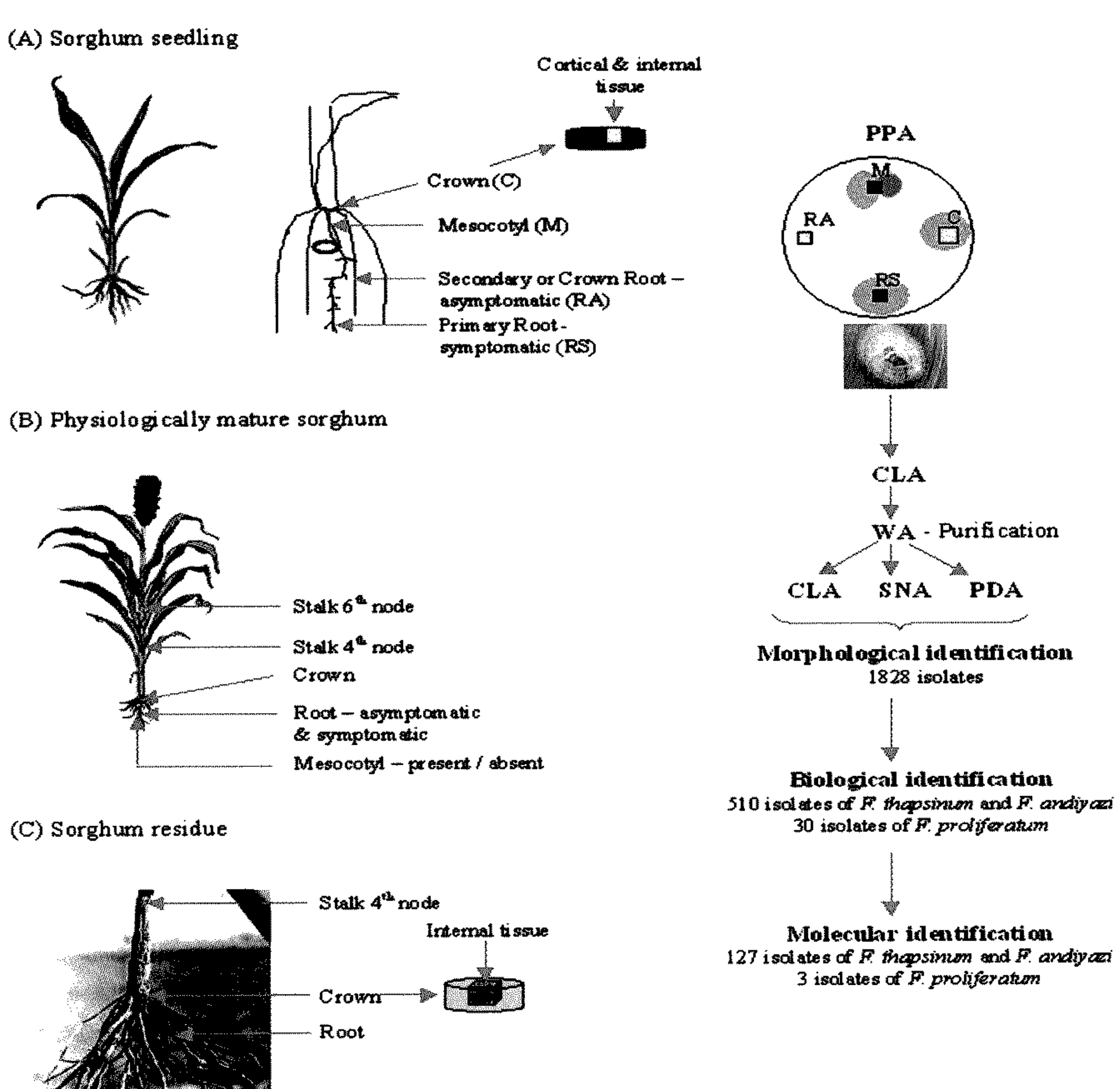


Figure 4-4 Procedure for isolation, purification and identification of *Fusarium* isolates from grain sorghum sampled at three crop stages at Livingston Farm, Moree

4.2.4 Data analysis

The community of *Fusarium* species associated with any of the sorghum plant parts was named after that plant part (*i.e. Fusarium* community from the crown). *Fusarium* communities were analysed at two levels: (1) *Fusarium* communities associated with a plant part within each crop stage and (2) *Fusarium* communities across crop stages (irrespective of plant part). The four plots were used as replicates for both levels of analyses.

Fusarium species recovered from mesocotyls of physiologically mature plants were considered only in the analysis of communities across crop stages. As many plants had decomposed mesocotyls, the sample size of this plant part (52) was unequal in comparison to the samples from the other plant parts from physiologically mature plants (*i.e.* 200 of each plant part). This discrepancy in sample size interfered with comparisons among plant parts from physiological mature plants and therefore, the mesocotyl isolates were excluded from analysis at this level.

Abundance of Fusarium species - Percentage abundance (Ho *et al.* 2001) was used to determine the dominance of *Fusarium* species among plant part or crop stage and is defined as:

$$P_i = \frac{n_i}{\sum_{i=1}^{S} n_i} x100$$

P_i - the percentage of abundance of the ith species
n_i - the number of samples with i^{th} species
S - the number of species found in all plant parts at each crop stage (or in all crop stages)

Abundance data, as expressed by counts of isolates for each species were analysed using an analysis of variance (one-way and two-way ANOVA), χ^2 goodness of fit and a contingency table based on the maximum likelihood (ML) method. Analysis of variance was used to test mean abundance and the interaction between two variables

(*eg.* number of *Fusarium* species and crop stage). The χ^2 goodness of fit and contingency table method based on ML were performed to test the significance of the abundance of *Fusarium* species in relation to plant part and crop stage in GenStat version 8.1 (VSN International Ltd.).

Diversity measurements - The diversity of the *Fusarium* communities associated with either plant parts or crop stages were assessed using non-parametric measures of diversity, namely species richness, Simpson's index and Simpson's measure of evenness. Binary data matrices based on presence or absence of a species from each site were used to compute these indices and species richness estimators (ACE and ICE) in EstimateS version 7 (Statistical estimation of species richness and shared species from samples) (Colwell 2005). Diversity indices and standard deviations of species richness estimators (ACE and ICE) were computed based on 1000 randomizations of samples without replacement. Abundance-based coverage estimator (ACE) and incidence-based coverage estimator (ICE) were chosen because they place weight on rarely isolated species, which can provide useful information on undescribed species (Chazdon *et al.* 1988). Species richness estimators were based either on species with ≤ ten individuals per sample (ACE) or on species found in ≤ 10 sampling units (ICE).

Estimates of species richness were analysed graphically using a species richness accumulation curve, which involved plotting the estimated and observed number of species (Sobs) as a function of the cumulative number of sorghum plants.

Species richness emphasises the number of species as a component of diversity, whereas the Simpson's index and evenness measures tend to emphasize the level of abundance of the most common species (Magurran 2003). Species richness is the number of species in the community, whereas evenness is the distribution of the

individuals in the community among the species. Simpson's index indicates the probability that any two individuals drawn at random from an infinitely large community belong to a different species.

Similarity of the communities – The diversity of *Fusarium* communities associated with plant parts at each crop stage and between crop stages was measured by comparing diversity indices and estimating similarity coefficients.

The similarity of *Fusarium* communities associated with plant parts at each crop stage and between crop stages was measured by the number of shared species [Chao shared estimate ($V_{(est)}$)], the Bray-Curtis similarity coefficient and multivariate analysis [non-metric multidimensional scaling (nMDS) and analysis of similarities (ANOSIM for the 1-way layout)]. Apart from the number of shared species, all similarity analyses of communities were performed using Primer-E version 5 (Clarke and Warwick 2001).

Chao shared estimate ($V_{(est)}$) estimates the number of species shared between two sampling areas. The Bray-Curtis similarity coefficient was used as it is one of the few measures that satisfy most important criteria for biological data on community structure (Clarke and Warwick 2001). The abundance similarity matrix based on the Bray-Curtis coefficient was calculated between every pair of samples (or sites) without transformation of data. Non-metric multidimensional scaling (nMDS) was performed based on an abundance similarity matrix. Graphical representation gives the rank of similarities between samples and relative values of similarity among samples. Analysis of similarity for a 1-way layout was used to estimate the level of similarity between samples. This test takes into account the observed differences between samples and differences among replicates within samples and calculates the significance level.

4.3 Results

Sorghum seedlings did not develop obvious symptoms of disease. However, some red to brown lesions were present on the mesocotyl and primary roots. Physiologically mature plants either had no visible expression of symptoms, having hard dark to pale green stalks with no change in the structure of the internal tissue, or had obvious symptoms of stalk rot, with soft pale straw coloured stalks (Figure 4-5A, B) with reddening of the internal, disintegrated tissue (Figure 4-5C). Residue of harvested plants were either firm, or soft due to disintegrated stalk tissue.

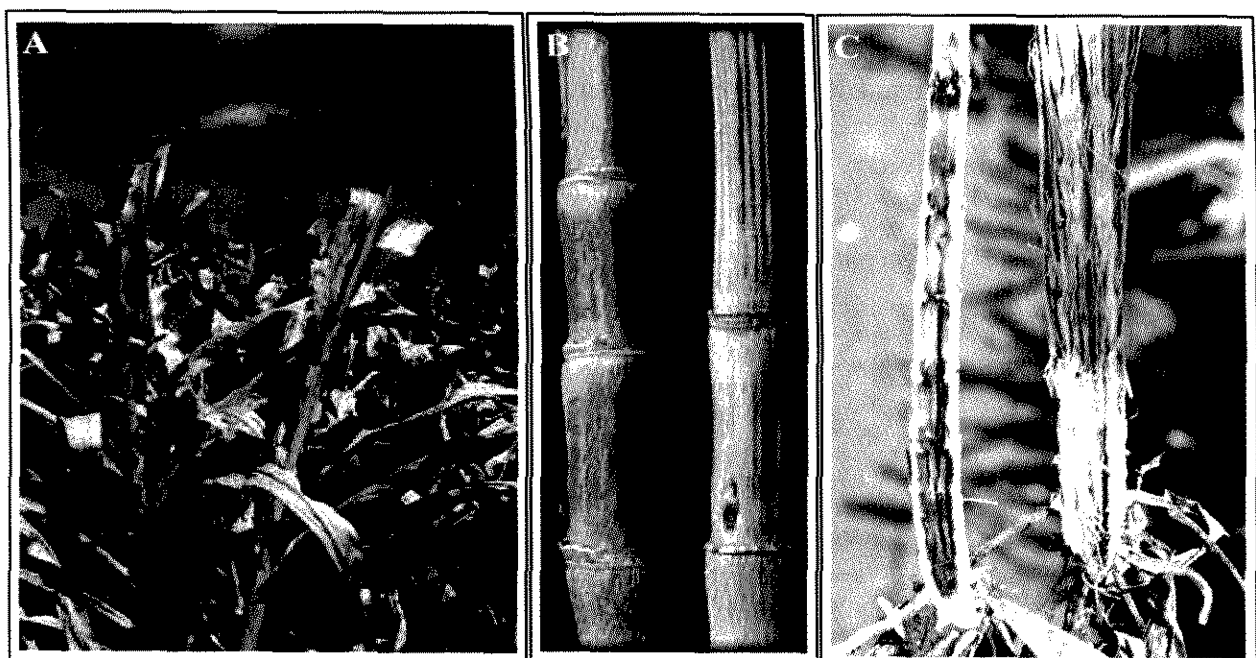


Figure 4-5 Symptoms of Fusarium stalk rot in physiologically mature grain sorghum plants at Livingston Farm, Moree in the 2001/2002 growing season (A, B). Disintegration of the internal stalk tissue (C) (*Courtesy* Prof. L.W. Burgess)

4.3.1 **Fusarium** ***species associated with different plant parts of sorghum***

4.3.1.1 *Fusarium* species associated with different parts of sorghum seedlings

Abundance of Fusarium species - A total of 361 isolates were recovered from the mesocotyls, asymptomatic and symptomatic root tissue, and crowns of seedlings. The abundance of *Fusarium* species was significantly influenced by the plant part ($F_{pr.} < 0.001$, df = 3). In total, 144 isolates (39.9%) were recovered from mesocotyls, 143 isolates (39.6%) were recovered from the symptomatic root tissue, 53 isolates (14.7%) were recovered from crowns, and 21 isolates (5.8%) were recovered from asymptomatic root tissue (Figure 4-6). Significant differences in the mean abundance of *Fusarium* species using plots as the replicates were not found between the most abundant *Fusarium* communities from mesocotyls and symptomatic root tissue as well as between the least abundant communities from crowns and asymptomatic root tissue.

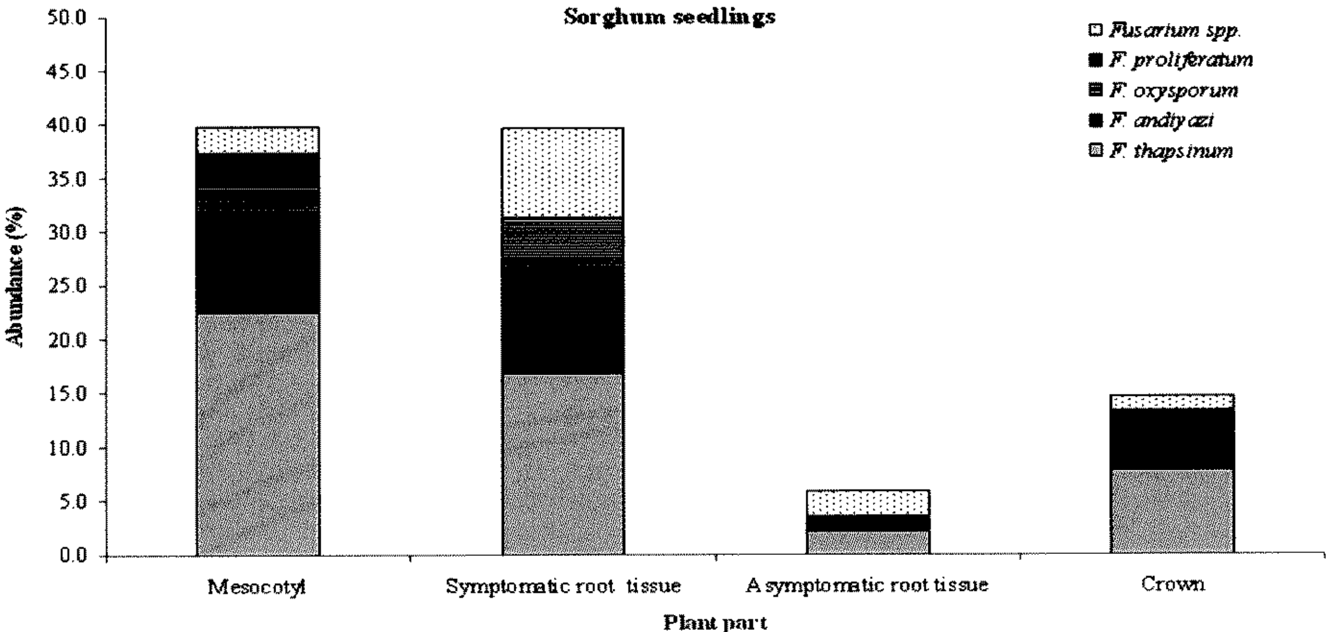


Figure 4-6 Abundance of *Fusarium* species expressed as a percentage of the total abundance of the species recovered from four parts of sorghum seedlings. *Fusarium* species with abundance less then 2% were consolidated into *Fusarium* spp.

Eleven *Fusarium* species were recovered from sorghum seedlings, and the number of species recovered from different plant parts was similar ($F_{pr.}$ = 0.071, df = 3). *Fusarium thapsinum* and *F. andiyazi* formed an important part of the *Fusarium* communities associated with mesocotyls (31.9%) and symptomatic root tissue (26.6%). These species were significantly less abundant in asymptomatic root tissue and crowns, but still dominant. *Fusarium proliferatum* and *F. oxysporum* were the next most abundant species with uneven distribution across seedling plant parts (Figure 4-6).

Diversity measurements - Ten species were recovered from the 200 mesocotyl and symptomatic root tissue samples. Six species were obtained from the 200 asymptomatic root and crown tissue samples. The species accumulation curves for *Fusarium* communities from asymptomatic and symptomatic root tissue and crowns reached an asymptote, but curve for the *Fusarium* community from the mesocotyls did not (Figure 4-7).

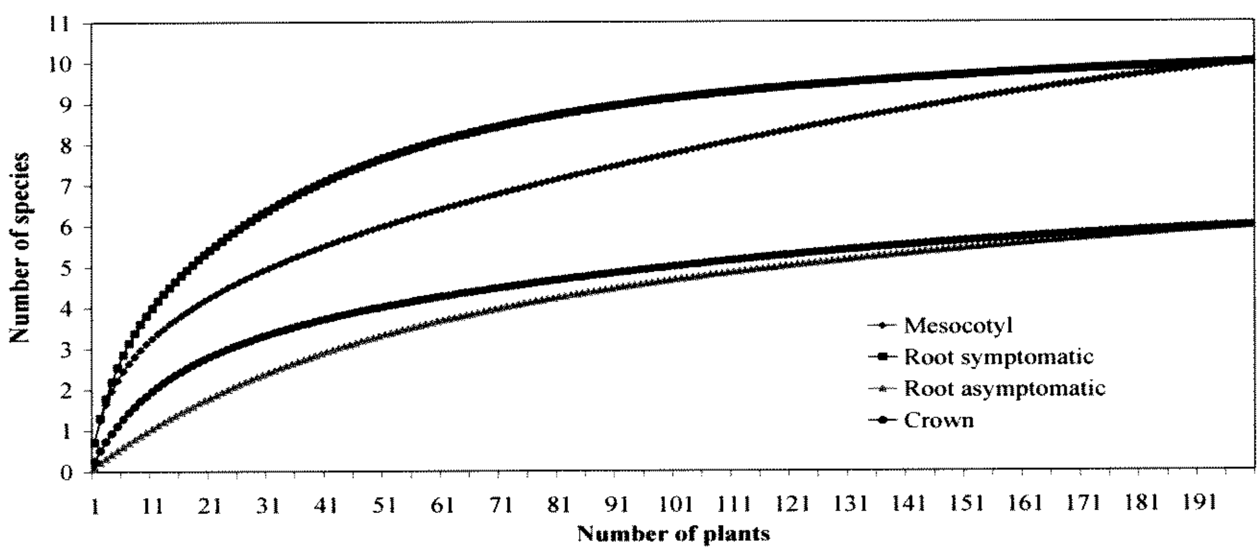


Figure 4-7 Species accumulation curves for *Fusarium* species isolated from mesocotyls, asymptomatic and symptomatic root tissue, and crowns of sorghum seedlings in the 2001/2002 growing season at Livingston Farm, Moree

The abundance-based coverage estimators (ACE and ICE) predicted species richness for the *Fusarium* community from mesocotyls at approximately 15 species, which is five species more than the actual number of species isolated. The same estimators predicted species richness for the *Fusarium* community from symptomatic root tissue as 10.27, which is similar to the actual number of species (10) recovered. Both estimators predicted approximately 7 and 7.5 species for the communities from asymptomatic root tissue and crowns (Table 4-2).

Values for Simpson's index of diversity ranged from a minimum of 2.64, for the mesocotyl *Fusarium* community, to a maximum of 4.67 for the asymptomatic root tissue community (Table 4-2). The evenness index indicates that isolates are the least evenly distributed between species in the *Fusarium* community from the mesocotyl (0.26) and most evenly distributed among species in the *Fusarium* community from the asymptomatic root tissue (0.78) (Table 4-2).

Table 4-2 Diversity and richness of the *Fusarium* communities associated with different sorghum seedling plant parts at Livingston Farm, Moree

Plant part	Fragments	Species richness	Estimators of species richness		Simpson's index	Evenness index
			ACE	ICE		
Mesocotyl	200	10	15.48 ± 0.97	15.23 ± 0.92	2.64 ± 0.02	0.26
Asymptomatic root tissue	200	6	7.56 ± 0.29	7.56 ± 0.29	4.67 ± 0.09	0.78
Symptomatic root tissue	200	10	10.27 ± 0.1	10.26 ± 0.1	3.87 ± 0.03	0.39
Crown	200	6	7.13 ± 0.75	7.04 ± 0.69	2.91 ± 0.04	0.48

Similarity of Fusarium *communities from seedlings* - Five species were common amongst the four *Fusarium* plant part communities, namely *F. thapsinum*, *F. andiyazi*, *F. proliferatum*, *F. oxysporum* and *F. equiseti*. The maximum number of shared species (nine species) was recorded between the mesocotyl and symptomatic root tissue *Fusarium* communities (Table 4-3).

The Chao shared species estimation $V_{(est)}$ suggested an approximately similar number of shared species to the number of observed species (Table 4-3). However, the estimation of the shared species between the most abundant *Fusarium* communities (from the mesocotyl and symptomatic root tissue) was 15.3, with six species more than the observed number. This discrepancy is attributed to the presence of 'hidden' species not recovered from the mesocotyls during this survey (Chao *et al.* 2000).

Table 4-3 Similarity of the *Fusarium* communities based on the number of shared species recovered from mesocotyls, asymptomatic and symptomatic root tissue, and crowns of sorghum seedlings

Plant part	Fragments	Species richness	Shared observed species (†Chao shared estimate $V_{(est)}$)		
			Mesocotyl	Asymptomatic root tissue	Symptomatic root tissue
Mesocotyl	200	10			
Asymptomatic root tissue	200	6	6 (9)		
Symptomatic root tissue	200	10	9 (15.3)	6 (6.5)	
Crown	200	6	5 (5.9)	6 (6.8)	5 (5.3)

†Chao shared estimate $V_{(est)}$ is given in the brackets

Multivariate analyses, nMDS ordination and ANOSIM, revealed differences among *Fusarium* plant part communities from sorghum seedlings. The most distinct difference was between the *Fusarium* community from asymptomatic root tissue and the remainder of the *Fusarium* communities. The nMDS ordination showed separation of the *Fusarium* asymptomatic root tissue community from the other *Fusarium* plant part communities (Figure 4-8). The grouping of the communities is supported by a stress value of 0.05, indicating a good ordination of communities with no prospect of a misleading interpretation (Clarke and Warwick 2001).

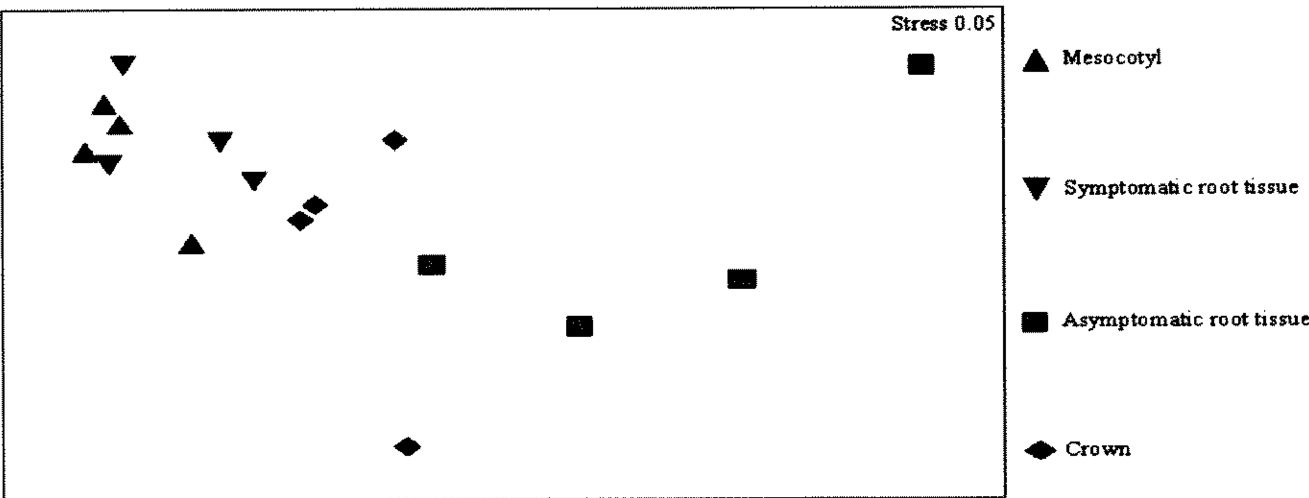


Figure 4-8 Two-dimensional nMDS configuration of the *Fusarium* communities from mesocotyls, asymptomatic and symptomatic root tissue, and crowns of sorghum seedlings

ANOSIM analysis confirmed the pattern of nMDS ordination for *Fusarium* plant part communities showing differences among them (global R = 0.474) at a significance level of P = 0.001. Pairwise comparisons between *Fusarium* plant part communities indicated that the most different, and therefore clearly separated communities, were the mesocotyl and asymptomatic root tissue communities (R = 0.844), followed by the asymptomatic and symptomatic root tissue communities (R = 0.802) (Appendix 3.1). However, mesocotyl and symptomatic root tissue communities were the most similar (R = -0.031).

4.3.1.2 *Fusarium* species associated with different plant parts from physiologically mature sorghum

Abundance of Fusarium species - A total of 768 isolates were recovered from asymptomatic and symptomatic root tissue, crowns, and the fourth and sixth nodes of sorghum stalks. The abundance of *Fusarium* species recovered was significantly influenced by the plant part from which it was isolated ($F_{pr.} < 0.001$, df = 4). The greatest number of isolates was recovered from crowns [238 isolates (31.0%)], followed by the sixth node [173 isolates (22.5%)], and the fourth node [167 isolates

(21.7%)]. The least number of isolates was recovered from asymptomatic root tissue [46 isolates (6%)] and symptomatic root tissue [144 isolates (18.8%)] (Figure 4-9). There were significant differences in the mean abundance of *Fusarium* species between the communities from the crowns and symptomatic root tissue, as well as between the communities from the asymptomatic root tissue and all other plant part communities.

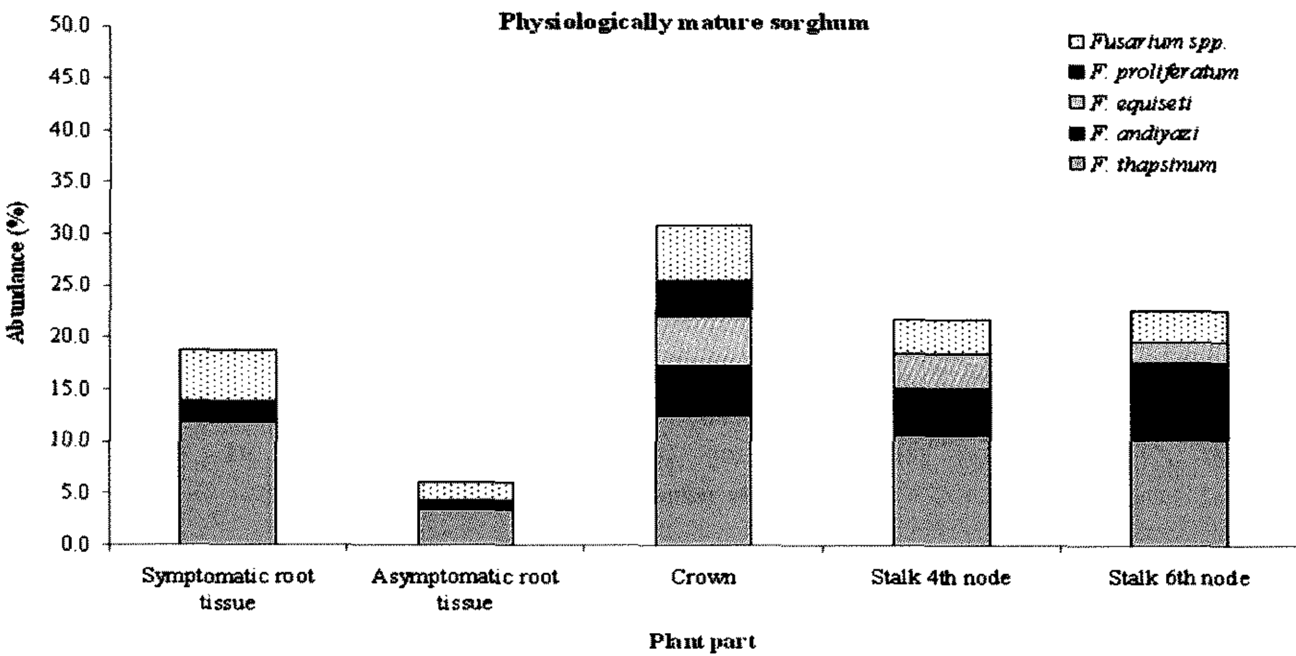


Figure 4-9 Abundance of *Fusarium* species expressed as a percentage of the total abundance of all species recovered from all plant parts in physiologically mature sorghum plants. *Fusarium* species with abundance less then 2% are consolidated into *Fusarium* spp.

Seventeen species were recovered from physiologically mature sorghum, but the number of species isolated from various plant parts differed significantly ($F_{pr.} = 0.02$, df = 4). The largest number of species was recovered from the crown (15 species) with the least number of species recovered from asymptomatic root tissue (7 species).

Fusarium thapsinum and *F. andiyazi* formed an important part of the *Fusarium* communities associated with symptomatic root tissue (13.9%), crowns (17.3%), the

fourth node (15.1%) and the sixth node (17.6%). These species were significantly less abundant in asymptomatic root tissue (4.3%).

Fusarium equiseti and *F. proliferatum* were the next most abundant species, with the highest abundance in the crowns (8.3%). Unlike *F. proliferatum*, *F. equiseti* was also abundant in the stalk nodes (Figure 4-9).

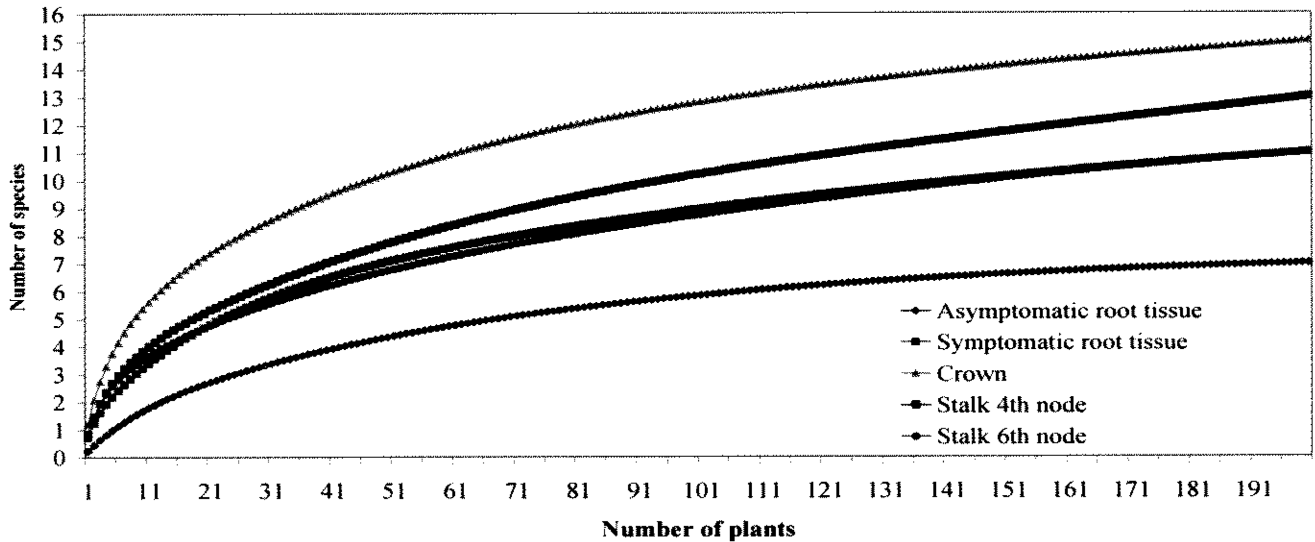


Figure 4-10 Species accumulation curves for *Fusarium* species isolated from symptomatic and asymptomatic roots, crowns, fourth and sixth node of stalks from physiologically mature sorghum plants in the 2001/2002 growing season at Livingston Farm, Moree

Diversity measurements - The total number of species obtained from the 200 asymptomatic root tissue, symptomatic root tissue, crowns, fourth and sixth nodes was seven, 11, 15, 13 and 11, respectively. The species accumulation curve for the *Fusarium* community from asymptomatic root tissue reached an asymptote, but the accumulation curves for the other communities did not (Figure 4-10).

The abundance-based coverage estimators (ACE and ICE) of species richness predicted species richness for all plant part communities at a higher level than the observed number of species, except that from the asymptomatic root tissue (Table 4-4). The largest discrepancy between the observed and estimated number of species

was in the *Fusarium* community from the fourth node, with 13 observed versus approximately 17 estimated species.

Values for Simpson's index of diversity ranged from a minimum 2.38, recorded for the symptomatic root tissue *Fusarium* community, compared to a maximum 4.41 for the crowns community (Table 4-4). The evenness index indicates that isolates are the least evenly distributed between species in the *Fusarium* community from symptomatic root tissue (0.22) and most evenly distributed among species in the community from asymptomatic root tissue (0.41) (Table 4-4).

Table 4-4 Diversity and richness of the *Fusarium* communities associated with different physiologically mature sorghum plant parts at Livingston Farm, Moree

Plant part	Fragments	Species richness	Estimators of species richness		Simpson's index	Evenness index
			ACE	ICE		
Asymptomatic root tissue	200	7	7.54 ± 0.18	7.52 ± 0.18	2.87 ± 0.04	0.41
Symptomatic root tissue	200	11	13.72 ± 0.37	13.69 ± 0.37	2.38 ± 0.02	0.22
Crown	200	15	16.64 ± 0.31	16.57 ± 0.31	4.41 ± 0.03	0.29
Stalk 4th node	200	13	17.25 ± 0.45	17.08 ± 0.44	3.27 ± 0.02	0.25
Stalk 6th node	200	11	14.41 ± 0.57	14.27 ± 0.55	3.13 ± 0.01	0.28

Similarity of Fusarium *communities in physiologically mature plants* - Six species were common amongst the *Fusarium* communities from asymptomatic and symptomatic root tissue, crowns and the fourth and sixth nodes. These species were *F. thapsinum, F. andiyazi, F. proliferatum, F. equiseti, F. oxysporum* and *F. semitectum*. The maximum number of shared species (11 species) was recorded between the crown and fourth node, and crown and sixth node *Fusarium* communities.

The Chao shared species estimation $V_{(est)}$ indicated a larger number of species shared among the most abundant communities than was observed (Table 4-5). This

discrepancy is again attributed to the presence of 'hidden' species not recovered from the crowns and the fourth and sixth nodes during this survey (Chao *et al.* 2000).

Table 4-5 Similarity of the *Fusarium* communities based on the number of shared species recovered from asymptomatic and symptomatic roots, crowns, fourth and sixth node of physiologically mature sorghum

Plant part	Fragments	Species richness	Shared observed species (†Chao shared estimate $V_{(est)}$)			
			Asymptomatic root	Symptomatic root	Crown	Stalk 4th node
Root asymptomatic	200	7				
Root symptomatic	200	11	6 (6)			
Crown	200	15	7 (7.57)	11 (15.34)		
Stalk 4th node	200	13	7 (8.13)	8 (9.67)	11 (16.02)	
Stalk 6th node	200	11	6 (6.69)	8 (9.65)	11 (15.68)	10 (12.97)

†Chao shared estimate $V_{(est)}$ is given in the brackets

Multivariate analysis, nMDS ordination and ANOSIM, detected some differences between *Fusarium* plant part communities. The nMDS ordination showed clear differentiation of the *Fusarium* community from asymptomatic root tissue compared to the other *Fusarium* plant part communities (Figure 4-11).

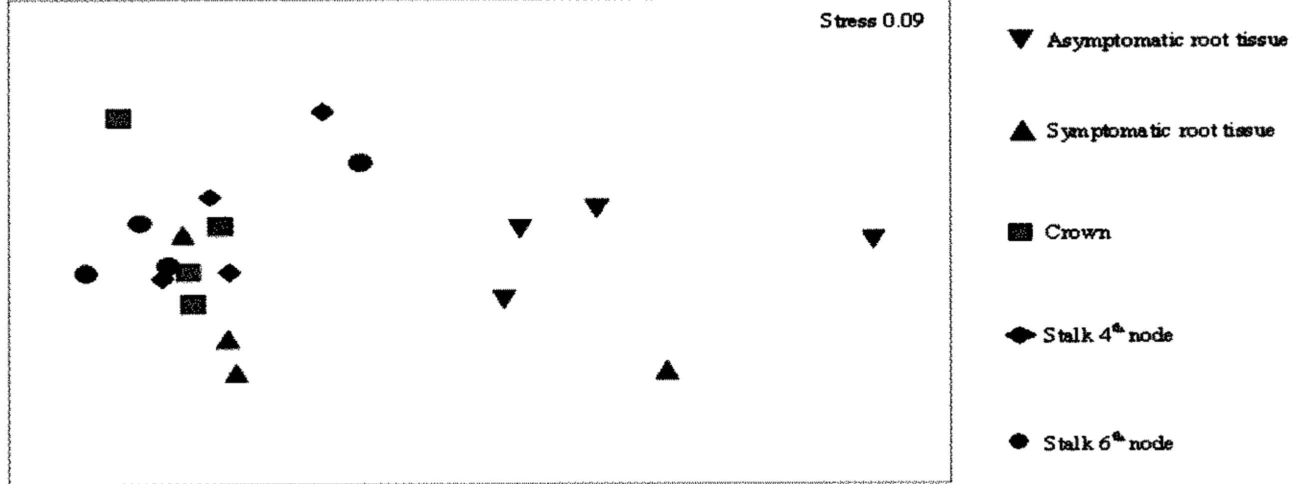


Figure 4-11 Two-dimensional nMDS configuration of *Fusarium* communities from asymptomatic and symptomatic roots, crowns, fourth and sixth nodes of stalks of physiologically mature sorghum

A stress value of 0.09 supported this grouping of communities from different plant parts.

ANOSIM analysis identified a lower degree of separation for the five *Fusarium* plant part communities (global R = 0.327) at a significance level of P = 0.003 (Figure 4-11; Appendix 3.2). Pairwise comparison between *Fusarium* plant part communities indicated that the symptomatic root tissue and crown communities were the most similar, least separated communities (0.052), followed by symptomatic root tissue and the fourth node (0.083) communities and the fourth and sixth node (-0.094) communities.

The most dissimilar communities, clearly separated communities were from asymptomatic roots and crowns (0.896), asymptomatic root tissue and the fourth node (0.781) and asymptomatic root tissue and the sixth node (0.781) (Appendix 3.2).

4.3.1.3 *Fusarium* species associated with different parts of sorghum residues

Abundance of Fusarium *species* - A total of 647 *Fusarium* isolates were recovered from the root tissue, crown and the fourth node of sorghum residues. The abundance of the *Fusarium* communities associated with different parts of sorghum residues were significantly different ($F_{pr.}$ = 0.006; df = 2). In total, 172 isolates (26.6%) from root tissue, 198 isolates (30.6%) from crowns and 277 isolates (42.8%) from the fourth node were recovered from residues (Figure 4-12). Significant differences in the mean abundance of *Fusarium* species were found between the most abundant *Fusarium* community from the fourth node, and the remaining communities.

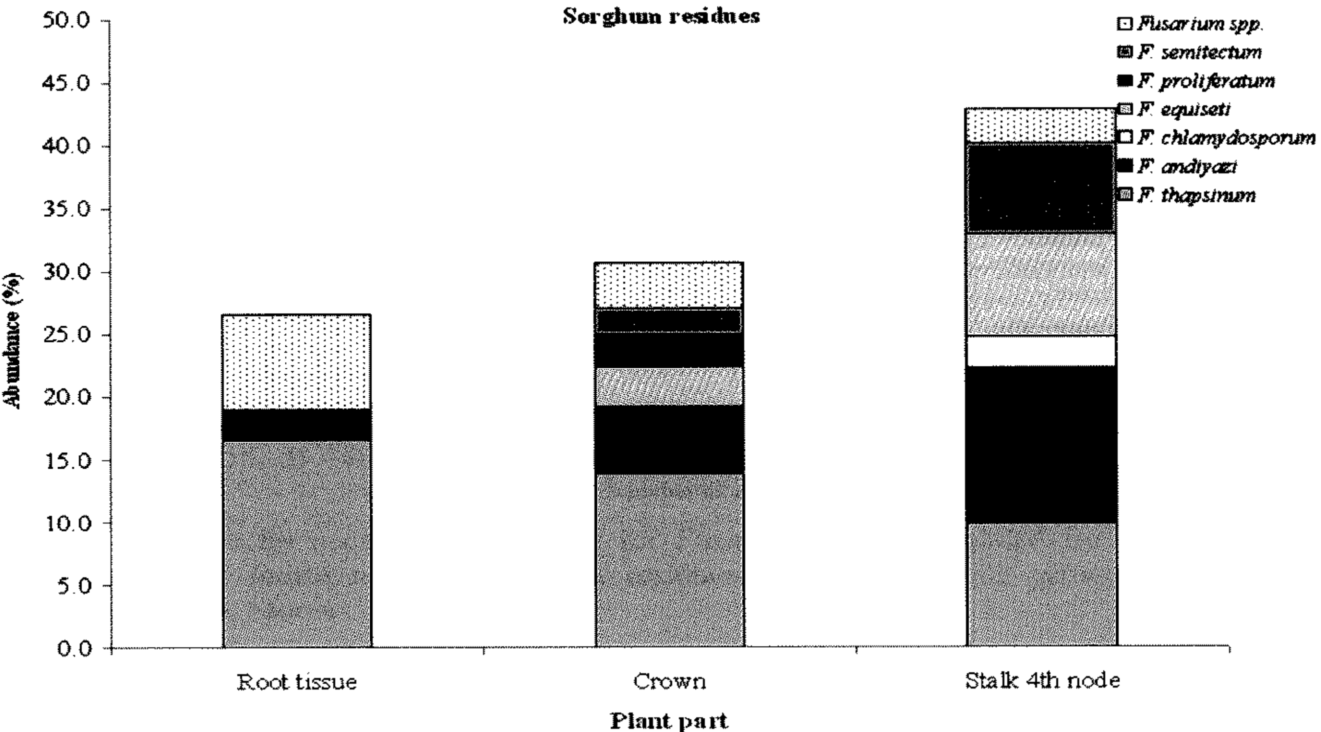


Figure 4-12 The abundance of *Fusarium* species expressed as a percentage of the total abundance of the species recovered from root tissue, crown and the fourth node of the sorghum residues. *Fusarium* species with abundance less then 2% are consolidated into *Fusarium* spp.

Thirteen species were recovered from sorghum residues, with no difference in the number of species from root tissue, crowns and stalks ($F_{pr.}$ = 0.532, df = 2). *Fusarium thapsinum* and *F. andiyazi* formed an important part of the *Fusarium* communities associated with root tissue (19.0%), crowns (19.2%) and fourth node (22.3%). *Fusarium equiseti*, *F. semitectum* and *F. proliferatum* were also abundant species, primarily colonizing the crowns and stalks of sorghum residues (Figure 4-12).

Diversity measurements – The total number of species recovered from the 200 roots, crowns and fourth nodes was 12, nine and nine, respectively. The species accumulation curves for the *Fusarium* communities from crowns and the fourth node reached an asymptote, whereas the accumulation curve for the root tissue community did not (Figure 4-13).

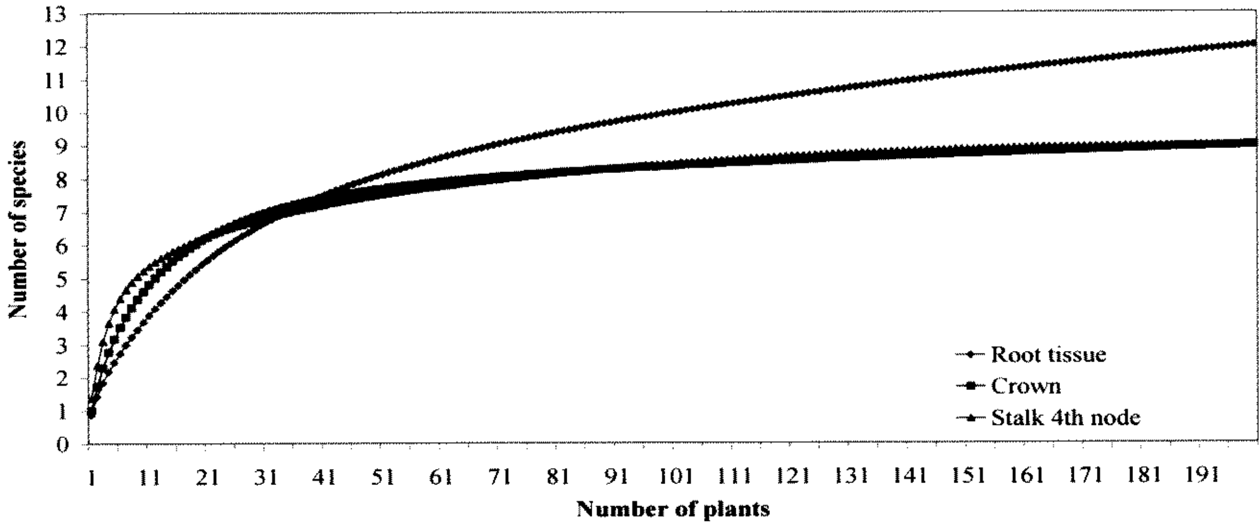


Figure 4-13 Species accumulation curves for *Fusarium* species isolated from root tissue, crowns and the fourth node of sorghum residues in the 2001/2002 growing season at Livingston Farm, Moree

The abundance-based coverage estimators (ACE and ICE) of species richness predicted species richness for the *Fusarium* communities from crown and stalk residues similar to the actual number of nine species recovered (Table 4-6). For the community from root tissue, both estimators predicted approximately 14.5 species in comparison to the 12 observed species.

Values for Simpson's index of diversity ranged from a minimum of 2.47 recorded for the root tissue community, compared to a maximum of 4.92 for the fourth node community (Table 4-6). The evenness index indicates that isolates are the least evenly distributed between species in the *Fusarium* community from root tissue (0.21) and most evenly distributed among species in the community from the fourth node (0.55) (Table 4-6).

Table 4-6 Diversity and richness of the *Fusarium* communities associated with different sorghum residue plant parts at Livingston Farm, Moree

Plant part	Fragments	Species richness	Estimators of species richness		Simpson's index	Evenness index
			ACE	ICE		
Root tissue	200	12	14.66 ± 0.43	14.55 ± 0.42	2.47 ± 0.02	0.21
Crown	200	9	9.83 ± 0.15	9.72 ± 0.13	3.84 ± 0.03	0.43
Stalk 4th node	200	9	9.02 ± 0.13	9.02 ± 0.13	4.92 ± 0.00	0.55

Similarity of the communities - Six species were common amongst the *Fusarium* communities from roots, crowns and the fourth node. These species were *F. thapsinum*, *F. andiyazi*, *F. proliferatum*, *F. equiseti*, *F. semitectum* and *F. chlamydosporum*. The maximum number of shared species (nine species) was recorded between the root tissue and crown communities. Unexpectedly, the least number of shared species (six species) was recorded between the most abundant *Fusarium* communities from the fourth node and crown communities (Table 4-6).

The Chao shared species estimation $V_{(est)}$ suggested a similar number of species shared between the root tissue and the other two communities from residues, compared to the observed number of species (Table 4-7). However, the estimated number of shared species among *Fusarium* communities from crowns and the fourth node was nil, indicating that the *Fusarium* community from stalks is quite different from the community from crowns.

Table 4-7 Similarity of the *Fusarium* communities based on the number of shared species recovered from root tissue, crowns and fourth node of sorghum residues

Plant part	Fragments	Species richness	Shared observed species (†Chao shared estimate $V_{(est)}$)	
			Root	Crown
Root	200	12		
Crown	200	9	9 (9.78)	
Stalk 4th node	200	9	8 (8.70)	6 (0)

†Chao shared estimate $V_{(est)}$ is given in the brackets

Multivariate analysis, nMDS ordination and ANOSIM, detected differences between the *Fusarium* communities from root tissue, crowns and the fourth node. The nMDS ordination showed clear differentiation of the *Fusarium* community from the fourth node compared to both the root tissue and crown communities (Figure 4-14). Grouping of the communities from the three parts of sorghum residues is supported by a stress value of 0.06.

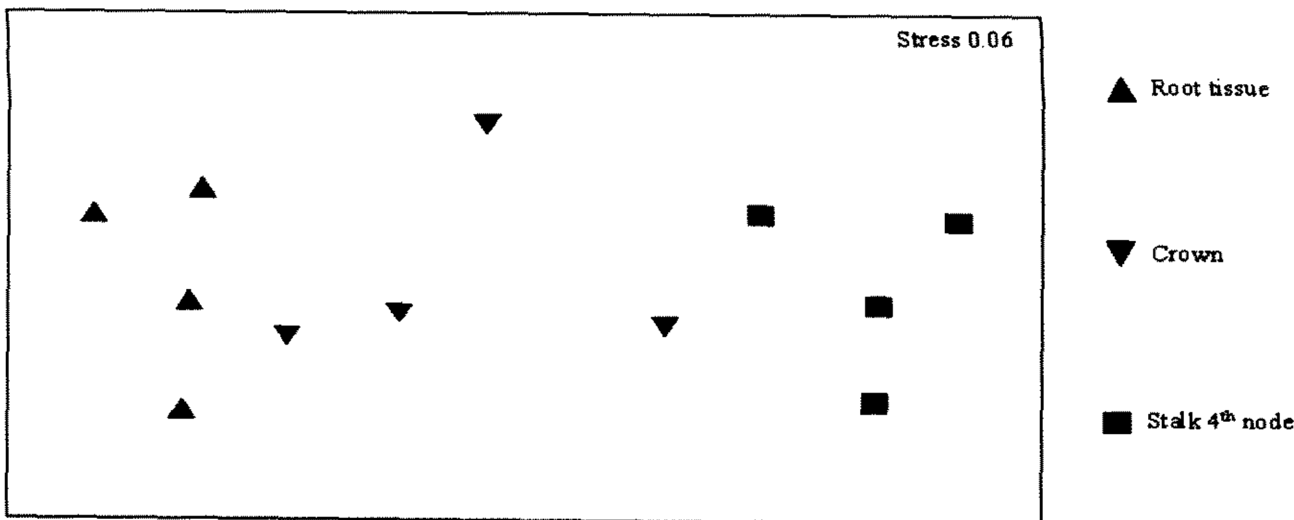


Figure 4-14 Two-dimensional nMDS configuration of the *Fusarium* communities from root, crown and fourth node of stalk of sorghum residues

ANOSIM analysis confirmed the pattern of nMDS ordination for *Fusarium* communities from root tissue, crowns and the fourth node, indicating significant differences between them (global R = 0.67) at a level of P = 0.001. Pairwise tests showed that the most different, and therefore clearly separated *Fusarium* communities were the root and fourth node communities (R = 1), followed by the crown and 4th node (0.656) (Appendix 3.3). The most similar, and therefore least separated *Fusarium* communities were the root and crown communities (R = 0.281) (Figure 4-14).

4.3.2 **Fusarium** ***species associated with sorghum in different crop stages***

Abundance of Fusarium species - A total of 1828 isolates representing 17 species were recovered from 2452 segments from 600 sorghum samples collected at the three crop stages (Table 4-8). The abundance of *Fusarium* species, the number of species and segments from which they were obtained was significantly associated with the sorghum crop stage (χ^2 = 133.85, df = 6, P < 0.001). In total, 361 isolates comprising 11 species were recovered from sorghum seedlings, 820 isolates comprising 17

species were recovered from physiologically mature plants and 647 isolates comprising 13 species were recovered from sorghum residue (Table 4-8).

Table 4-8 Number of *Fusarium* isolates, species, segments and samples recovered from three sorghum crop stages at Livingston Farm, Moree

	Crop stage		
	Seedling	Physiological maturity	Residue
Isolates	361	820	647
Species	11	17	13
Segments	800	1052	600
Samples	200	200	200
χ^2	133.85		
df	6		
P	< 0.001		

The number of species recovered was significantly influenced by the sorghum crop stage ($F_{pr.} = 0.032$, df = 2). The number of species recovered from seedlings differed significantly to recovery from physiologically mature plants. The spectrum of species was similar over all crop stages, although the distribution of isolates among the species was significantly uneven (Table 4-9).

Table 4-9 Percentage abundance of *Fusarium* species† from sorghum seedlings, physiologically mature sorghum and sorghum residues from Livingston Farm, Moree

	Crop stage		
Species	Seedling	Physiological maturity	Residue
F. acuminatum	0.3	0.3	0.0
F. andiyazi	4.7	8.8	7.1
F. avenaceum	0.0	0.1	0.1
F. chlamydosporum	0.1	1.0	1.5
F. compactum	0.4	0.2	0.2
F. culmorum	0.0	0.2	0.0
F. equiseti	0.7	5.0	4.6
F. graminearum	0.0	0.1	0.0
F. nygamai	0.2	0.4	0.5
F. oxysporum	1.6	1.3	0.4
F. semitectum	0.1	1.5	3.3
F. scirpi	0.0	0.2	0.2
F. solani	0.4	0.4	1.1
F. polyphialidicum	0.0	0.2	0.2
F. proliferatum	1.5	2.7	1.8
F. pseudograminearum	0.0	0.5	0.0
F. thapsinum	9.7	22.1	14.3
Total	19.7	44.9	35.4
†χ^2	453.80	1342.78	631.41
df	7	10	8
P	< 0.001	< 0.001	< 0.001

†Performed chi-square goodness of fit (the maximum likelihood method)

Fusarium thapsinum was the most abundant species isolated from all three sorghum crop stages and almost twice as abundant as *F. andiyazi* (Figure 4-15). Two species, *F. thapsinum* and *F. andiyazi* comprised 14.4%, 30.9% and 21.4% of the *Fusarium* communities from seedlings, physiologically mature plants and sorghum residues, respectively (Figure 4-15). The abundance of the third most common species, *F. equiseti*, increased towards sorghum maturity, whereas the abundance of the fourth

most common species, *F. proliferatum*, did not vary significantly between sorghum crop stages.

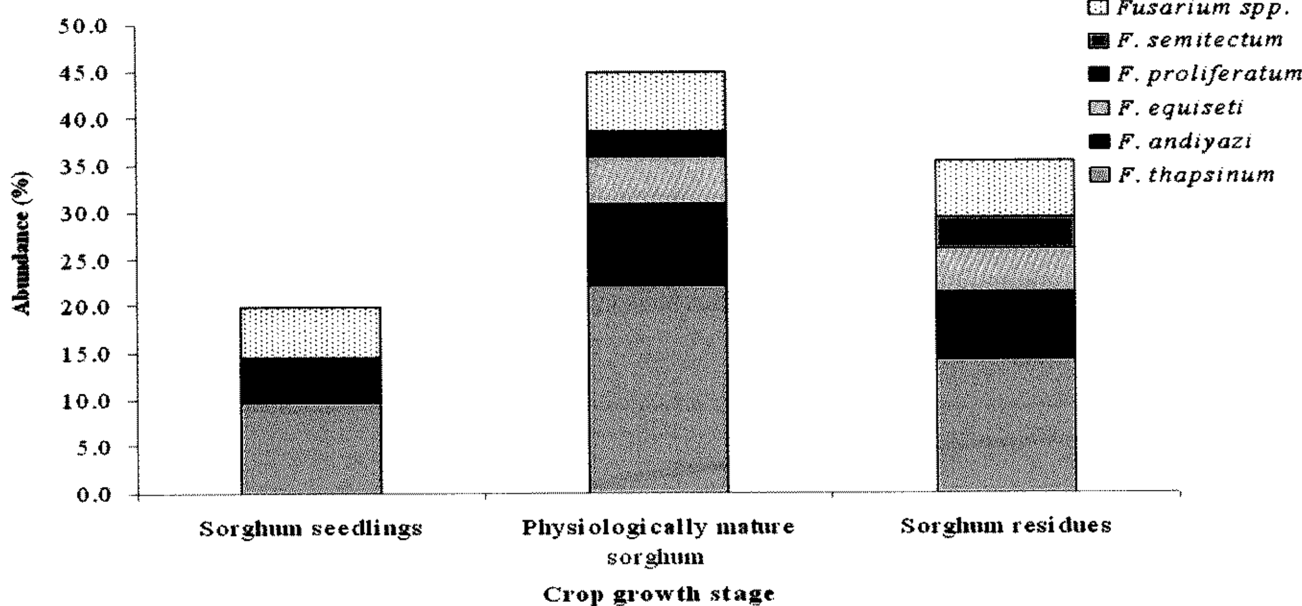


Figure 4-15 Contributions of the most abundant species to the composition of the *Fusarium* communities associated with each of the three sorghum crop stages. *Fusarium* species with abundance less then 2% were consolidated into *Fusarium* spp.

Two-way ANOVA indicated that the abundance of the most commonly isolated species (*F. thapsinum*, *F. andiyazi*, *F. equiseti*, *F. proliferatum*, *F. semitectum*) and the remaining *Fusarium* spp., was significantly correlated to the crop stage and the number of *Fusarium* species recovered from each crop stage ($F_{pr.} < 0.001$, df = 10) (Figure 4-15).

The abundance of *F. thapsinum* increased significantly from the seedling stage (9.7%) to physiological maturity (22.1%) and then decreased in sorghum residues (14.3%). The pattern of abundance of *F. thapsinum* might be attributed, to some extent, to the unequal sample size of plant part segments from the three crop stages of sorghum, *viz.* 800 segments from seedlings, 1052 segments from physiologically mature plants and 600 segments from residues (Table 4-8).

Diversity measurements - The least number of *Fusarium* species (11 species) were recovered from the 200 seedlings, whilst 13 species were recovered from sorghum residues. The highest number of species (17 species) were recovered from physiologically mature plants. The species accumulation curves for *Fusarium* communities from seedlings, physiologically mature plants and residues reached an asymptote (Figure 4-16), indicating that it is likely that all species present in each of the three crop stages of sorghum were recovered from at least one plant part.

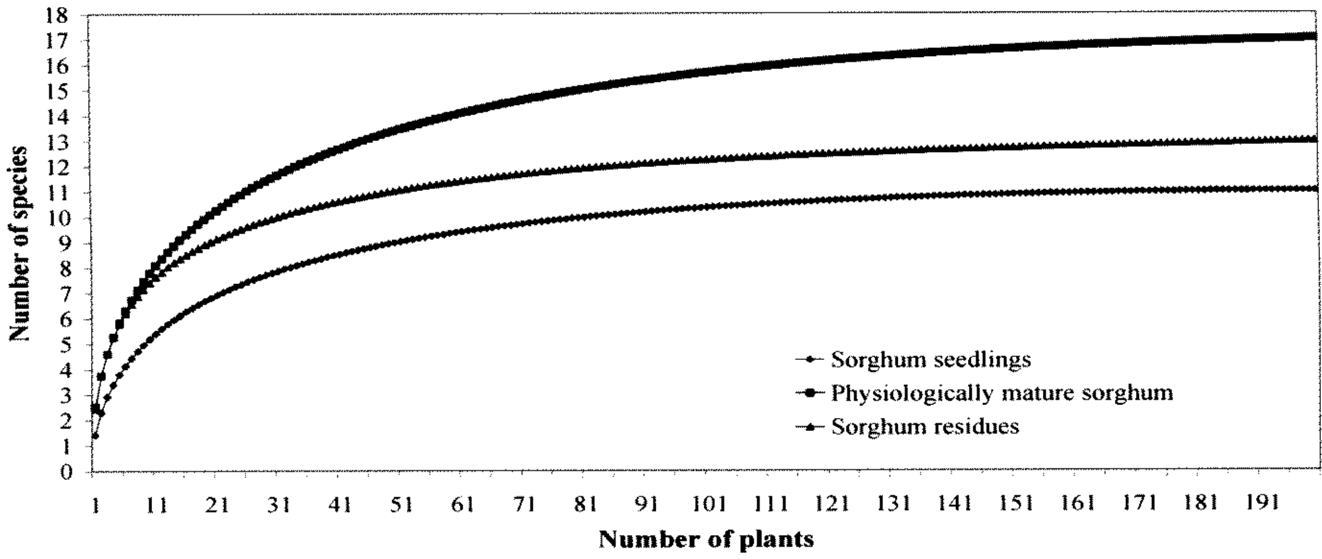


Figure 4-16 Species accumulation curve for *Fusarium* species isolated from grain sorghum in the 2001/2002 growing season at Livingston Farm, Moree

The abundance-based coverage estimators (ACE and ICE) of species richness predicted species richness for *Fusarium* communities associated with seedlings, physiologically mature plants and sorghum residues similar to the observed number of species (Table 4-10).

Values for Simpson's index of diversity ranged from a minimum of 3.74 recorded for the *Fusarium* community from seedlings compared to a maximum of 5.27 for the community from residues (Table 4-10). The evenness index indicates that isolates are

the least evenly distributed between species in the *Fusarium* community from the physiologically mature plants (0.29), and the most evenly distributed in the community from sorghum residues (0.41) (Table 4-4).

Table 4-10 Diversity and richness of the *Fusarium* communities associated with grain sorghum at three sorghum crop stages at Livingston Farm, Moree

Crop stage	Fragments	Species richness	Estimators of species richness		Simpson's	Evenness
			ACE	ICE	index	index
Seedling	800	11	11.0 ± 0.0	11.0 ± 0.0	3.74±0.02	0.34
Physiological maturity	1052	17	17.42 ± 0.13	17.41 ± 0.13	4.97±0.01	0.29
Residue	600	13	13.43 ± 0.09	13.41 ± 0.0	5.27±0.02	0.41

Similarity of the communities - The similarity of *Fusarium* communities from seedlings, physiologically mature plants and residues was estimated based on the number of shared species and species abundance.

Ten species were common to the *Fusarium* communities associated with seedlings, physiologically mature plants and residues. All of the *Fusarium* species isolated from seedlings (11 species) and sorghum residues (13 species) were also recovered from physiologically mature plants. The Chao shared species estimation $V_{(est)}$ was similar to the observed number of shared *Fusarium* species. A slightly higher number of shared species (14.15 species) was estimated between the *Fusarium* communities from physiologically mature plants and residues (Table 4-11).

Table 4-11 Similarity of the *Fusarium* communities based on the number of shared species recovered from different sorghum crop stages

Crop stage	Fragments	Species richness	Shared observed species (†Chao shared estimate $V_{(est)}$)	
			Seedling	Physiological maturity
Seedling	800	11		
Physiological maturity	1052	17	11 (11)	
Residue	600	13	10 (10)	13 (14.15)

†Chao shared estimate $V_{(est)}$ is given in the brackets

Multivariate analyses, nMDS ordination and ANOSIM tests, revealed differences between the *Fusarium* communities from seedlings, physiologically mature plants and residues. The nMDS ordination showed two distinct clusters of *Fusarium* communities: Group I, representing the community from seedlings and Group II, representing the communities from physiologically mature plants and residues (Figure 4-17). Within Group 2, the *Fusarium* community from residues was more coherent than the community from the physiologically mature plants (Figure 4-17). The grouping of communities is supported by a stress value of 0.07, indicating a good ordination of communities with no prospect of a misleading interpretation (Clarke and Warwick 2001). The Group I and Group II clusters are 59% similar.

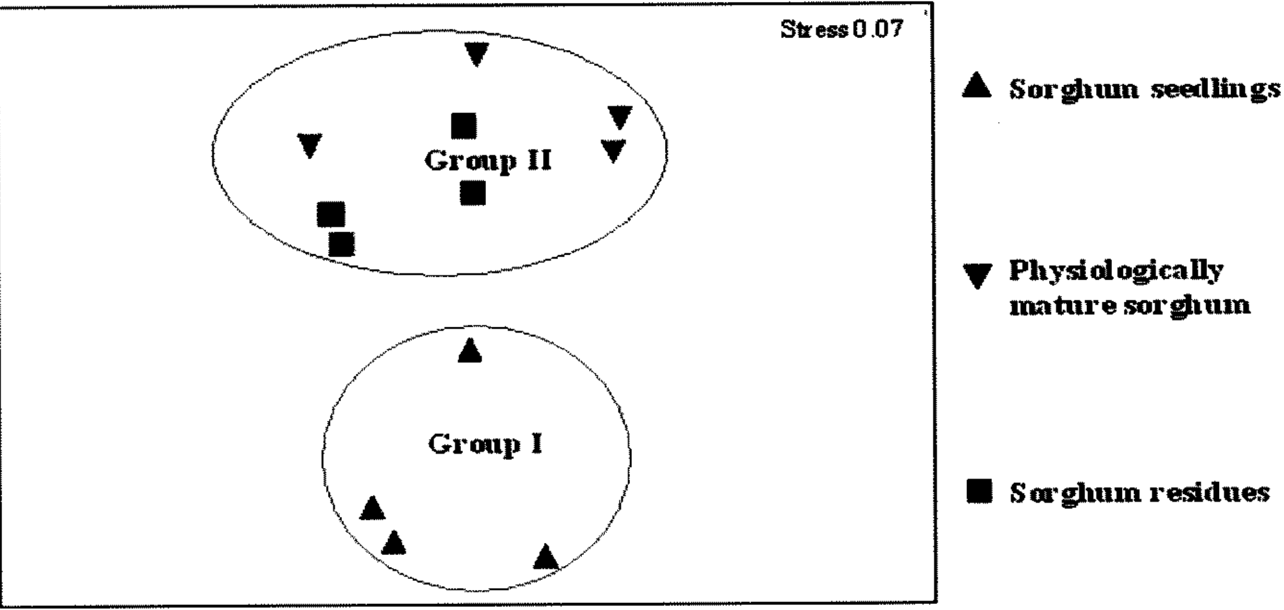


Figure 4-17 Two-dimensional nMDS ordination of the *Fusarium* communities from three sorghum crop stages based on Bray-Curtis similarity coefficients. Each community is comprised of isolates from four replicate plots. Clusters from group-averaged clustering from Bray-Curtis similarities are superimposed at similarity levels of 75% (green line) and 69% (orange line)

Analysis of similarities (ANOSIM) confirmed the pattern in the nMDS ordination, indicating that there are significant differences between the *Fusarium* communities associated with different sorghum crop stages (R = 0.711, P = 0.001) (Appendix 3.4). Pairwise R values revealed differences between the *Fusarium* communities from seedlings and physiologically mature plants (R = 0.927, P = 0.029) and from seedlings and residues (R = 0.906, P = 0.029). However, there was no significant difference between the communities associated with physiologically mature plants and residues (R = 0.385, P = 0.009).

4.4 Discussion

Of the 17 *Fusarium* species recovered during the current study, *F. thapsinum* was the most abundant species being most frequently isolated from all three crop stages [seedling (49.3%), physiological maturity (48.7%) and residues (40.3%)]. Therefore, the hypothesis that *F. thapsinum* is the dominant species associated with grain sorghum irrespective of the crop stage is accepted.

Fusarium thapsinum was also the most abundant species associated with all plant parts in each of the sorghum crop stages, except in the stalks of the sorghum residues where it was the second most abundant species. Therefore, the hypothesis that *F. thapsinum* is the dominant species associated with grain sorghum irrespective of the plant part is largely supported.

The data on abundance indicates that *F. thapsinum* is the main species associated with Fusarium stalk and root rot of grain sorghum at this region. This finding is in line with previous findings on *Fusarium* associations with sorghum seed samples in the USA (Leslie and Plattner 1991), Tanzania (Mansuetus *et al.* 1997) and South Korea (Lim *et al.* 2001). Unfortunately the *F. thapsinum* abundance data from the present studies cannot be compared with similar studies on sorghum roots and stalks due to the changes in taxonomy.

Young and mature, sorghum underground and aboveground tissue were all suitable substrates for colonization by *F. thapsinum*. The decrease in the abundance of *F. thapsinum* (9.9%) observed in sorghum stalk residues and the subsequent prevalence of *F. andiyazi* (12.4%) might be attributed to a decline in temperature and increase in rainfall in the autumn period following harvest. The lower temperatures recorded for May (average 22°C), may favour colonisation by *F. andiyazi* rather than *F. thapsinum*. Such temperature effects have been reported for the interaction between *F.*

graminearum and *F. moniliforme sensu lato* in sorghum stalks (Reed *et al.* 1983), and maize ears (Reid *et al.* 1999; Reid *et al.* 2002). It is also possible that the desiccation of stalk tissue following the first frost (April/May) affected the viability and recovery of *F. thapsinum* more than *F. andiyazi*.

Fusarium andiyazi was the second most abundant species from all crop stages and all plant parts at each crop stage. This result indicates that *F. andiyazi* alone, or in synergistic interaction with *F. thapsinum*, has an important role in the sorghum root and stalk rot disease complex. There are two key features of the activity of *F. andiyazi*. It was abundant in the mesocotyls (9.4%) and symptomatic root tissue (9.7%) of seedlings, and then progressively colonized the stalks of physiologically mature plants and residues. The first finding might be attributed to seed infection, since the recovery of *F. andiyazi* from sorghum grain can be as high as 97% (Marley *et al.* 2004). Sorghum residues on the soil surface are also a source of splash or air dispersed inoculum of both *F. andiyazi* and *F. thapsinum* (Claflin 2000).

The abundance of *F. andiyazi* in the stalks of physiologically mature plants, and its prevalence in the residues of stalks, might indicate that *F. andiyazi* is more associated with Fusarium stalk rot than root rot. Tesso *et al.* (2005) demonstrated no significant differences in virulence between *F. andiyazi*, *F. proliferatum* and *F. thapsinum* in evaluation trials of sorghum hybrids for stalk rot resistance. Further studies are needed to clarify the pathogenic ability of *F. andiyazi* and its role in stalk rot of sorghum under different environmental conditions.

Fusarium thapsinum and *F. andiyazi* comprised the core of the *Fusarium* communities associated with seedlings, physiologically mature plants and sorghum residues. However, the analysis of similarities (ANOSIM) indicated differences in the composition of the *Fusarium* communities associated with sorghum at the three crop

stages (R = 0.711, P = 0.001). Therefore, the hypothesis that the diversity and abundance of *Fusarium* species associated with grain sorghum are similar in different crop stages is rejected.

The *Fusarium* community associated with sorghum seedlings was clearly different from the *Fusarium* communities from physiologically mature plants and residues. This is consistent with reports of low recovery of *Fusarium* species during the vegetative growth of sorghum plants, and their gradual increase towards crop maturity (Reed *et al.* 1983; Trimboli and Burgess 1985). Furthermore, environmental conditions may favour colonization, particularly by saprophytic *Fusarium* species during the bloom and hard dough stages of growth (Claflin 2000), increasing the abundance and distribution of species within the plant, as observed in the current study. The abundance of *Fusarium* species in the 2001/2002 growing season increased from 19.7% in sorghum seedlings to 35.4% in sorghum residues despite more intensive isolations being made from seedlings compared to residues. Although there was some overlap in species composition across crop stages, some species, such as *F. oxysporum*, colonized younger tissue, whereas *F. chlamydosporum* and *F. semitectum* colonized mainly senescent tissue, a similar pattern to that reported in a previous study (Reed *et al.* 1983). Colonization of the mesocotyls and roots of seedlings by *F. oxysporum* is consistent with the accepted biology of this species as a primarily soilborne species, a common coloniser of root surfaces, or a weak invader of the root cortex of non-host plants (Burgess *et al.* 1994; Kistler 2001).

Fusarium thapsinum and *F. andiyazi* also comprised the core of the *Fusarium* communities from all plant parts at each stage of crop growth. However, multivariate analyses and Chao shared species estimation $V_{(est)}$ indicated that the composition of the *Fusarium* communities varied between plant parts at each crop stage. Therefore,

the hypothesis that the diversity and abundance of *Fusarium* species associated with grain sorghum are similar in various plant parts at each crop stage is rejected.

The association of *Fusarium* species with asymptomatic root tissue at both the seedling and physiological maturity stage showed dissimilarities between *Fusarium* communities associated with various plant parts. The association of *Fusarium* species with asymptomatic sorghum stalks has been documented, although no details about the nature of the association were given (Leslie *et al.* 1990; Leslie 2000; Reed *et al.* 1983; Trimboli and Burgess 1985). The association of *Fusarium* species with asymptomatic sorghum roots reported in the current study have not been documented previously, and they may indicate the presence of systemic infection of sorghum as seen for maize seedlings systemically infected with *F. verticillioides* (Oren *et al.* 2003). Furthermore, the abundance data of *F. thapsinum* and *F. andiyazi* in seedlings indicated a pattern of colonization, *viz.* mesocotyls and primary roots as the first developed parts of plants, followed by crowns and crown roots. This corresponds to the pattern of colonization reported for *F. verticillioides* in maize seedlings grown in infested soil (Oren *et al.* 2003).

The relative abundance of *F. andiyazi*, *F. equiseti*, *F. semitectum* and *F. chlamydosporum* in sorghum residues allowed for differentiation of the *Fusarium* communities associated with the roots, crowns and stalks of sorghum residues. Little information is available on the biology and ecology of *F. andiyazi*, but the other three species are considered to be secondary colonizers or saprophytes, colonizing senescent plant tissue (Burgess *et al.* 1994; Leslie 2000). Therefore, increased abundance of *F. equiseti* in the stalks of physiologically mature sorghum and the higher abundance of all three saprophytic species in sorghum residues are consistent with the classification of these species as secondary colonizers. The recovery of these

species in the present study is in line with earlier reports on their colonization ability on sorghum roots, stalks and grain in other sorghum-growing regions (Leslie 2000; Saubois *et al.* 1999).

The distribution of *Fusarium* species in plant parts of physiologically mature sorghum revealed that the crown of the sorghum plant was a specific ecological niche, with the highest diversity and abundance of *Fusarium* species recovered from this part. However, high diversity and abundance of *Fusarium* species in crowns (30.9%) did not result in differentiation of this *Fusarium* community from the most similar and less abundant *Fusarium* community associated with symptomatic root tissue (18.7%). The high diversity and abundance of *Fusarium* species in the crown tissue is attributed to the crown as a transition zone between underground and aboveground plant parts, being suitably placed for colonization by soil or residue borne *Fusarium* species. Furthermore, the abundance of *Fusarium* species in the crowns might reflect the mode of infection, with these species speculated to enter the stalk at or near ground level (Claflin 2000).

The crown of sorghum plants at all crop stages was a favoured site for colonization by *F. proliferatum*. The abundance of *F. proliferatum* was slightly higher in the crowns (> 2.6%) than in the stalks (< 2%) of physiologically mature plants and sorghum residues. This might be correlated to the greater abundance of *F. proliferatum* in the mesocotyls and crowns of sorghum seedlings, indicating a possible systemic infection due to seed infestation with *F. proliferatum*. Underground parts such as bulbs of onion (*Allium cepa*) (du Toit and Inglis 2003), roots of orchids (*Cymbidium* spp.) (Benyon *et al.* 2001), roots and crowns of asparagus (Elmer 2001; Vujanovic *et al.* 2006) and crowns of ornamental palms (Armengol *et al.* 2005) are common substrates colonized by *F. proliferatum*. However, the pathogenic nature and distribution of *F.*

proliferatum in sorghum plants is poorly understood although it is the third abundant "chain-producing" species associated with sorghum (Leslie and Marasas 2002). Data on the abundance of *F. proliferatum* from seedlings, physiologically mature plants and sorghum residues, in the current study is in line with the results of previous studies on sorghum seed samples in the USA (Leslie and Plattner 1991), Tanzania (Mansuetus *et al.* 1997) and South Korea (Lim *et al.* 2001).

Intensive sampling during the growing season and simultaneous isolation from various plant parts has allowed an insight into the abundance, distribution and importance of two predominant *Fusarium* species, *F. thapsinum* and *F. andiyazi*, associated with symptomatic and asymptomatic sorghum tissue. The complex relationships between the *Fusarium* communities associated with grain sorghum were also elucidated in the current study, indicating that plant part, tissue maturity and environmental factors influence the abundance of these two species. Further studies on the comparative abundance of these two species in sorghum grain are needed.

Morphological identification of *Fusarium* isolates revealed considerable variation in morphological and physiological characters for *F. thapsinum*, *F. andiyazi* and *F. proliferatum*. However, the characterisation of these species using biological and molecular methods confirmed their identity (*see* Chapter 6).

Chapter 5

Fusarium Species Associated with Grain Sorghum from Two Agroclimatic Areas in the Northern Grain Belt of Eastern Australia

5.1 Introduction

The geographic distribution, diversity and abundance of *Fusarium* species associated with plants and soil are affected by climate (Burgess *et al.* 1988; Burgess and Summerell 1992; Gargouri *et al.* 2001; Rossi *et al.* 1995; Sangalang *et al.* 1995; Smiley and Patterson 1996; Summerell *et al.* 1993; van Leur and Bailey 2000). Some *Fusarium* species are widely distributed, occurring in different climatic regions whereas other *Fusarium* species are restricted to particular climatic regions. *Fusarium longipes* and *F. beomiforme*, for example, are restricted to tropical and subtropical regions (Burgess and Summerell 1992; Nelson *et al.* 1987; Sangalang *et al.* 1995; Summerell *et al.* 1993). In contrast *F. avenaceum* and *F. acuminatum* are restricted to temperate regions (Burgess *et al.* 1988; Gerlach and Nirenberg 1982). Species such as *F. equiseti*, *F. oxysporum*, *F. solani* and *F. semitectum* appear to be cosmopolitan in distribution. However, populations of these cosmopolitan species need to be analysed using molecular techniques to determine if they include cryptic species, each adapted to different climatic regions (Summerell *et al.* 2003).

Climate also affects the relative importance of *Fusarium* pathogens associated with particular diseases. *Fusarium graminearum*, *F. culmorum*, *F. poae* and *F. avenaceum*, for example, are the major pathogens causing Fusarium head blight (FHB) of small grain cereals worldwide (Parry *et al.* 1995). *Fusarium graminearum* is dominant in

warmer regions of the world, including parts of the USA, Canada, Australia and Central Europe (Obst *et al.* 2002; Parry *et al.* 1995; Waalwijk *et al.* 2003). In contrast, *F. culmorum* is dominant in the cooler, maritime regions in UK (Parry *et al.* 1995) and northern Europe, whilst *F. avenaceum* and *F. poae* are dominant in the colder regions of Scandinavia, Hungary and other mid-European countries (Lukanowski and Sadowski 2002). *Fusarium culmorum* is also one of the major pathogens causing foot and stem rot of wheat across maritime eastern England (Pettitt *et al.* 2003) and humid, sub-humid and semi-arid regions of Tunisia (Gargouri *et al.* 2001). In contrast, *F. pseudograminearum* is the most common pathogen associated with crown rot of wheat throughout the eastern Australian wheat belt, with *F. culmorum* only common in the cooler regions of Victoria and South Australia (Backhouse and Burgess 2002; Backhouse *et al.* 2004).

A survey of 93 fields under maize, sorghum and soybean in the central and eastern United States revealed that *F. acuminatum*, *F. graminearum* and *F. proliferatum* were more frequently recovered from tissue, debris and soil in the northern states whereas *F. chlamydosporum*, *F. compactum*, *F. moniliforme sensu lato*, *F. oxysporum* and *F. semitectum* were more frequently recovered in the southern central and eastern states of the USA (Leslie *et al.* 1990).

Presumably the spectrum of fungal species associated with grain sorghum is also affected by climatic conditions. Grain mould of sorghum results from the colonization of the developing grain by several fungi, including *F. thapsinum*, *Curvularia lunata* and *Alternaria alternata*, and is associated with warm, humid climatic conditions during caryopsis development (Waniska *et al.* 2001) or maturation of grain (Garud *et al.* 2000). Prom *et al.* (2003) studied the effects of *F. thapsinum*, *C. lunata* and a mixture of the two species on grain mould severity (GMS) and seed germination in

eight sorghum cultivars under field conditions. They observed significantly higher levels of GMS caused by either a single pathogen or mixture of these pathogens in 2001 compared to the same period in 2000, a finding attributed to the wetter conditions in 2001. In comparison with *C. lunata* and a mixture of *F. thapsinum* and *C. lunata*, *F. thapsinum* significantly increased GMS level in four cultivars of sorghum in 2000, but only in one cultivar in 2001. It was speculated that the susceptibility of cultivars to *F. thapsinum* in 2000 was affected by climatic factors. Similarly, Marley *et al.* (2004) studied *Fusarium* species associated with freshly harvested sorghum seeds and suggested that detailed studies on the *Fusarium* species associated with sorghum from various ecological zones in Nigeria would provide valuable data on the aetiology of seed mould in relation to climatic factors.

There have been no comparative studies on the *Fusarium* species associated with grain sorghum stalks in different climatic areas. It is an opportune time for such a study given that a number of new species associated with sorghum have been described in the past decade. Furthermore, the results of an intensive studies at one site, reported in the previous chapter, show that two recently differentiated species, *F. thapsinum* and *F. andiyazi*, are present in Australian sorghum, being the dominant species recovered from that site.

The study reported in this chapter was designed to test the hypothesis that the diversity of *Fusarium* species and the dominant species associated with grain sorghum stalks are similar in two agroclimatic areas of the northern grain belt of eastern Australia.

5.2 Materials and Methods

5.2.1 Climatic data and field sampling

Sorghum stalks were collected from dryland crops from each of two agroclimatic areas in the northern summer dominant rainfall region of the eastern grain belt of Australia in 2003. This region is characterized by an average annual rainfall of 500 to 700 mm with annual rainfall being quite variable in amount and distribution. The two agroclimatic areas were designated Goondiwindi and Quirindi after the nearest major towns for which climatic data was available (Figure 5-1A).

The Goondiwindi area is hotter than the Quirindi area, but the average summer rainfall for Goondiwindi is higher than for Quirindi (Figure 5-1B, C). Long-term data indicates that the average rainfall during the sorghum-growing season in Goondiwindi (September-January) is lower (319.3 mm) than in Quirindi (November-April) (387.4 mm) (*Australian Government Bureau of Meteorology*). As Goondiwindi is hotter, evapotranspiration rates are higher and crops are more prone to stress in this area (Burgess, *personal communication*).

The planting dates for sorghum in each area are variable, being dependant on available soil moisture. In the Quirindi area in 2002, sorghum was planted from mid-November to mid-December, depending on the site, whereas in the Goondiwindi area in 2002, sorghum was planted in September of 2002. Details of the location of each sampling site in the Goondiwindi and Quirindi area, the sorghum variety, date of sowing and approximate date of physiological maturity, sample size and crop rotation history (from 1998 till 2002/2003) are given in Table 5-1.

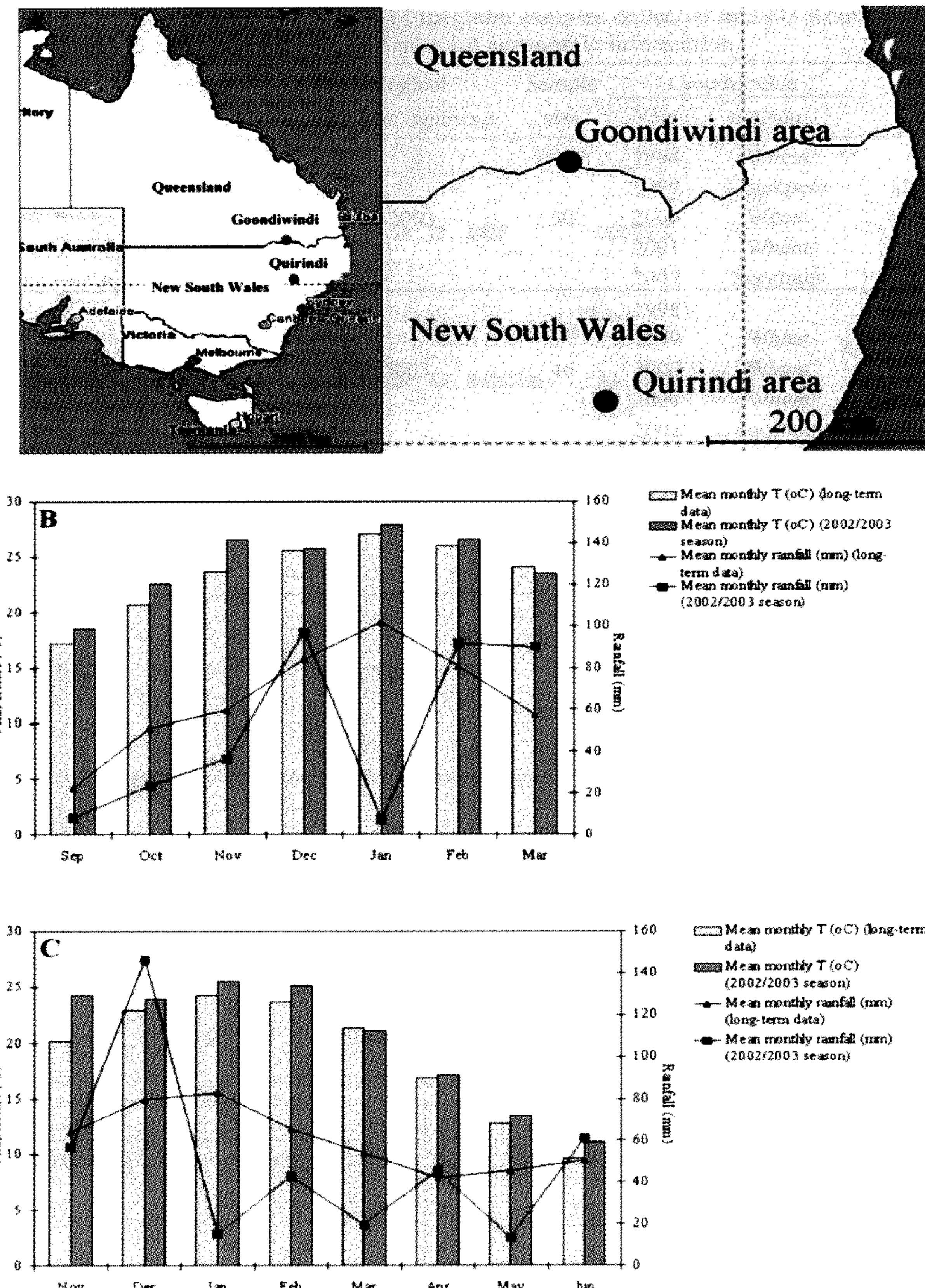


Figure 5-1 Map of eastern Australia showing the location of two agroclimatic areas, Goondiwindi and Quirindi, from which grain sorghum stalks were collected in 2003 (A) and comparison of long-term and 2002/2003 season climatic data from Goondiwindi (B) and Quirindi (C) (*Australian Government Bureau of Meteorology*)

Table 5-1 Location of sites and details of sorghum samples collected in 2003 from two areas, Goondiwindi - G and Quirindi – Q, and relevant agronomic information

Site	Variety†	Sowing date	Physiological maturity date (approx.)	Sample size	Crop rotation		Latitude/ Longitude
					Year	Crop	
G_1	MR-Buster	23/09/2	11/01/2003	50	1998	Wheat	S28°05.259' E150°5.499'
					1999	Chickpeas	
					2000	Wheat	
					2001	Wheat	
					2002	Sorghum	
G_2	MR-Buster	16/09/2	4/01/2003	46	1998	-	S28°31.499' E150°35.169'
					1999	Wheat	
					2000	Wheat	
					2001	Wheat	
					2002	Sorghum	
G_3	Maxi	9/09/20	28/12/2002	50	1998	Wheat	S28°3'53.60" E150°1'37.45"
					1999	Chickpeas	
					2000	Wheat	
					2001	Wheat	
					2002	Sorghum	
Q_1	MR-Buster	17/11/2	25/03/2003	46	1998	Wheat	S31°29'05.0" E150°31'34.2"
					1999	Wheat	
					2000	Cow Peas	
					2001	Sorghum	
					2002	Sorghum	
					2003	Sorghum	
Q_2	MR-Buster	8/12/20	7/04/2003	50	1998	Wheat	S31°18'48.38" E150°11'10.1"
					1999	Wheat	
					2000	Faba Beans	
					2001	Wheat	
					2002	Faba Beans	
					2003	Sorghum	
Q_3	MR-Buster	19/12/2	15/04/2003	46	1998	Faba Beans	S31°30'09.9" E150°12'13.0"
					1999	Wheat	
					2000	-	
					2001	Sorghum	
					2002	Wheat	
					2003	Sorghum	

†All sorghum grain varieties were produced by Pacific Seeds Pty Ltd

At each site, 46 or 50 sorghum stalks were collected on an ad hoc basis from a radial pattern within the site, 100 m long and 50 m wide. Stalks were not collected within 25 m of the edge of the crop (Figure 5-2). All crops were sampled approximately seven to nine weeks from the beginning of physiological maturity stage of sorghum.

Samples were transported to the laboratory under cool dry conditions and stored under similar conditions prior to isolation studies.

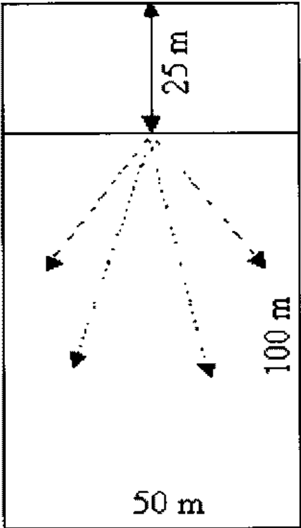


Figure 5-2 Spatial sampling strategy for collection of sorghum stalks from the Goondiwindi and Quirindi areas in 2003

5.2.2 Isolation, purification and identification of isolates

Leaves were removed and sorghum stalks were surface sterilized with 70% ethanol. One tetragonal segment (20 x 10 x 15 mm) including cortical and inner tissue was removed from the fourth node of each stalk, soaked in a 1.2 % solution of NaOCl for 30 s and rinsed twice in sterile distilled water for 2 min. Air-dried segments were then plated on PPA. Colonies developing from each segment were subcultured and purified as described in Chapter 4.

Morphological identification and sexual compatibility tests were performed as described in Chapter 4. Four morphologically identified species, namely *F. thapsinum* (110 isolates), *F. verticillioides* (three isolates), *F. proliferatum* (ten isolates) and *F. subglutinans* (three isolates), were all assessed via sexual compatibility studies with tester strains of *G. thapsina* (MP F), *G. moniliformis* (MP A), *G. intermedia* (MP D)

and *G. subglutinans* (MP E), respectively. Morphologically delineated isolates of *F. andiyazi* (117 isolates) were included in sexual compatibility studies with tester strains of *G. thapsina* and *G. moniliformis* to exclude the possibility of morphological misidentification of these isolates.

Molecular identification based on phylogenetic analysis of a portion of the translation elongation factor 1-α (TEF) gene sequences was performed on 11 randomly selected isolates of *F. andiyazi*, five from Goondiwindi and six from Quirindi.

5.2.3 Data analysis

The abundance, expressed either as counts of isolates of each species or percentages, diversity and similarity of the *Fusarium* communities from the two agroclimatic areas were analysed using the same statistical methods as for comparison of *Fusarium* communities associated with different plant parts and crop stages (*see* Chapter 4). In addition, species contributions to similarity (SIMPER), as a multivariate analysis, were performed to estimate the species contributions to average similarity within each agroclimatic area and the average dissimilarity between agroclimatic areas.

5.3 Results

The majority of sorghum stalks collected were affected by stalk rot. Stalk rot symptoms included soft, shredded internal tissue that was brown to reddish brown in colour and had a powdery appearance (Fig. 5-3A). The remaining stalks were hard, with no visible change to the structure of the internal tissue (Fig. 5-3B).

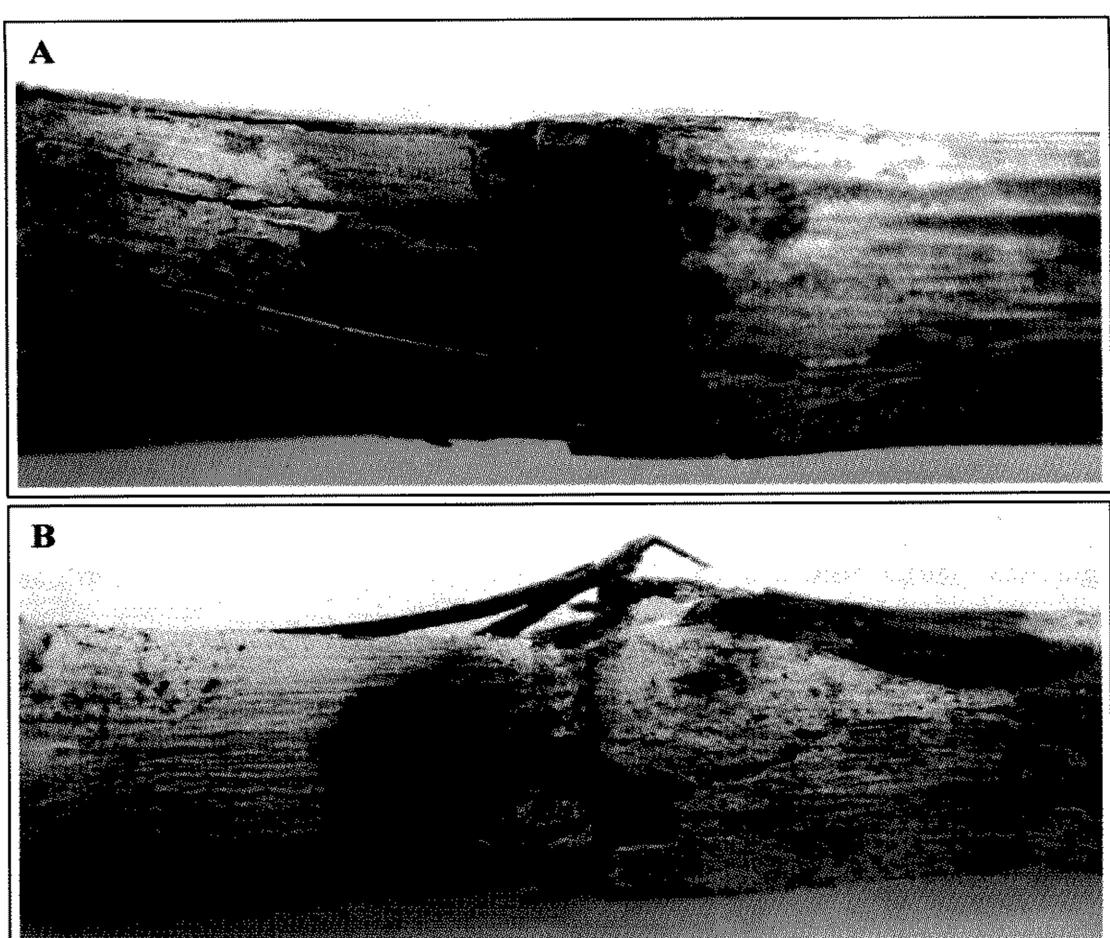


Figure 5-3 Sorghum stalk from which *F. thapsinum* and *F. andiyazi* were isolated (A). Sorghum stalk from which *F. andiyazi* was isolated (B)

In the Goondiwindi area, moisture stress occurred during physiological maturity in the 2002/2003 growing season. The mean monthly rainfall in January 2003 (15.4mm) was significantly lower than the historic mean monthly rainfall (82.6mm) (Figure 5-1). The mean monthly temperature during flowering and post-flowering stages was higher than 25°C, indicating that sorghum plants were prone to heat stress. In contrast, in the Quirindi area, moisture stress occurred both before and after flowering, but not during physiological maturity in the same season (Figure 5-1). The mean monthly temperature during flowering and post-flowering stages was lower than 25°C, indicating that sorghum plants were most likely not affected by heat stress.

5.3.1 *Abundance of* **Fusarium** *species*

A total of 182 isolates comprising 10 species were recovered from three sites in the Goondiwindi area (Table 5-2). A total of 142 isolates comprising 10 species were recovered from three sites in the Qurindi area (Table 5-2). The abundance of *Fusarium* species was significantly higher in the Goondiwindi area compared to the Quirindi area ($\chi^2 = 4.95$, df = 1, P = 0.026). The number of sorghum stalks collected from three sites was not significantly different for the two agroclimatic areas (Table 5-2), indicating that the number of sorghum stalks did not cause the significant difference observed in the abundance of *Fusarium* species from the two agroclimatic areas.

Table 5-2 Total number of samples, isolates and species from the Goondiwindi and the Quirindi areas

	Area		χ^2	df	P
	Goondiwindi	Quirindi			
Samples	146	142	0.06	1	0.814
Isolates	182	142	4.95	1	*0.026
Species	10	10	0.00	1	1.000

*significant at P = 0.05

The same number of species were recovered from both agroclimatic areas (Table 5-2), but the spectrum of species varied (Table 5-3). The distribution of isolates among the species was uneven in both the Goondiwindi ($\chi^2 = 86.18$, df = 5, $P < 0.001$) and Quirindi ($\chi^2 = 57.46$, df = 2, $P < 0.001$) areas.

Two species, *F. thapsinum* and *F. andiyazi*, collectively comprised 61.5% and 82.4% of the *Fusarium* communities from the Goondiwindi and Quirindi areas, respectively (Table 5-3). There was significant difference in the abundance (expressed as counts of isolates) of *F. thapsinum* and *F. andiyazi* in the two agroclimatic areas ($\chi^2 = 16.37$, df

= 1, P < 0.001) (Figure 5-4), indicating that the occurrence of these two species was correlated with agroclimatic area.

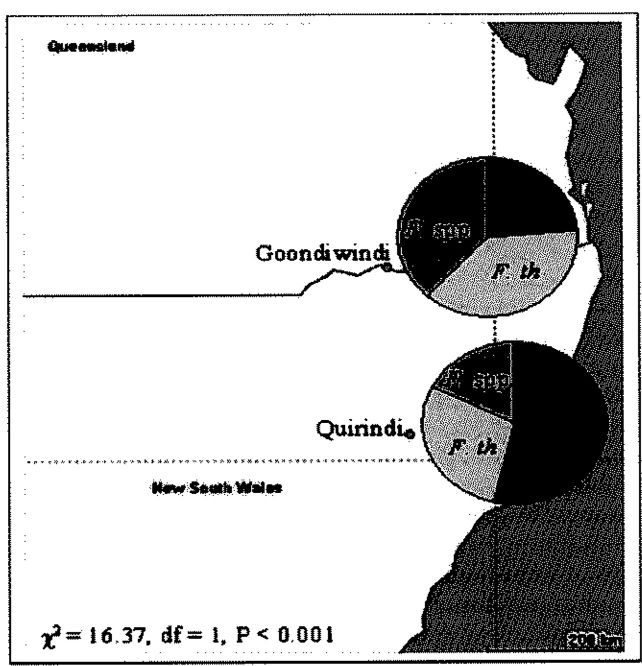


Figure 5-4 Relative proportions of the dominant species *F. andiyazi* (*F. and*), *F. thapsinum* (*F. th*) and other *Fusarium* spp. (*F.* spp.) isolated from the fourth node of grain sorghum in 2003 from two agroclimatic areas in the northern grain belt of eastern Australia

In the Goondiwindi area, *F. thapsinum* (37.9%) was a significantly more abundant species than *F. andiyazi* (23.6%) (χ^2 = 6.09, df = 1, P = 0.014). The species intermediate in abundance, *F. chlamydosporum*, *F. compactum* and *F. nygamai*, comprised 33.3% of the *Fusarium* community. The five remaining species, *F. equiseti*, *F. proliferatum*, *F. verticillioides*, *F. oxysporum* and *F. solani*, were the least abundant species, comprising 5.2% of the *Fusarium* community (Table 5-3).

Table 5-3 Percentage abundance of *Fusarium* species[†] isolated from the fourth node of grain sorghum stalks from the Goondiwindi and Quirindi areas

	Area	
Species	Goondiwindi	Quirindi
F. thapsinum	37.9	28.9
F. andiyazi	23.6	53.5
F. chlamydosporum	13.7	-
F. compactum	10.3	0.7
F. nygamai	9.3	-
F. equiseti	2.8	1.4
F. proliferatum	0.6	7.1
F. verticillioides	0.6	1.4
F. oxysporum	0.6	-
F. solani	0.6	-
F. subglutinans	-	2.1
F. graminearum (G. zeae)	-	2.1
F. scirpi	-	1.4
F. semitectum	-	1.4
Total	100	100

[†]Species are listed in order of abundance in the Goondiwindi area

In the Quirindi area, *F. andiyazi* (53.5%) was significantly more abundant than *F. thapsinum* (28.9%) (χ^2 = 10.63, df = 1, P = 0.001). *Fusarium proliferatum* was the only species intermediate in abundance (7.1%), with seven less abundant species comprising 10.5% of the *Fusarium* community (Table 5-3).

5.3.2 Community diversity measurements of Fusarium *species*

The total number of species obtained from both the 146 samples in the Goondiwindi area and from the 142 samples in the Quirindi area, was 10. The species accumulation curve for the Quirindi area reached an asymptote, but the curve for the Goondiwindi area did not (Figure 5-5).

The abundance-based coverage estimators (ACE and ICE) predicted species richness in the Goondiwindi area at 24.8 ± 0.0 and 23.14 ± 0.02 species, respectively (Table 5-

4). In the Quirindi area the estimators predicted 10.96 ± 0.41 (ACE) and 10.93 ± 0.4 (ICE) species.

Values for Simpson's index of diversity were higher in Goondiwindi (4.26) than in the Quirindi (2.69) area, indicating that the *Fusarium* community in the Goondiwindi area is more diverse than it is in the Quirindi area (Table 5-4). The higher evenness index value for the Goondiwindi area (0.43) compared to the Quirindi area (0.27) indicates that *Fusarium* isolates are more evenly spread between species in the Goondiwindi area (Table 5-4).

Table 5-4 Diversity and richness of *Fusarium* species associated with the fourth node of sorghum in two agroclimatic areas

Area	Samples	Species richness	Estimators of species richness		Simpson's index	Evenness index
			ACE	ICE		
Goondiwindi	146	10	24.58 ± 1.17	22.93 ± 1.1	4.26 ± 0.03	0.43
Quirindi	142	10	10.96 ± 0.41	10.93 ± 0.4	2.69 ± 0.02	0.27

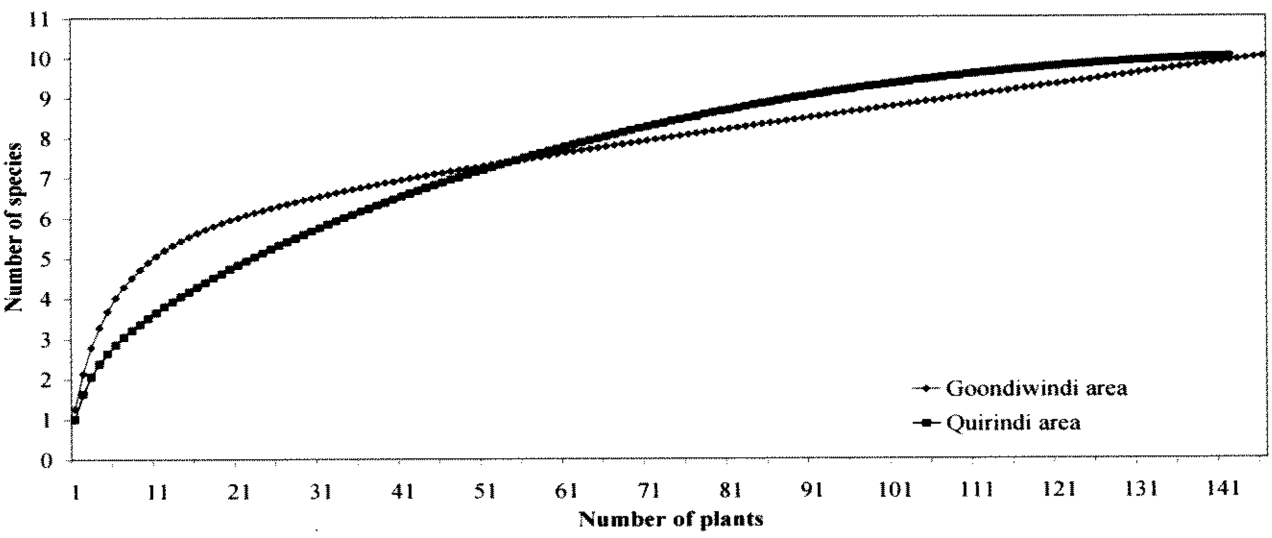


Figure 5-5 Species accumulation curve for the Goondiwindi and Qurindi areas

5.3.3 Similarity of **Fusarium** *communities*

The similarity of the *Fusarium* communities from Goondiwindi and Quirindi was estimated based on the number of shared species, species abundance and contribution of individual species.

A total of 6 species were shared between the communities, namely *F. andiyazi*, *F. proliferatum*, *F. thapsinum*, *F. verticillioides*, *F. compactum* and *F. equiseti*. The Chao shared species estimator $V_{(est)}$ suggested that there were 12.74 unobserved shared species in the two agroclimatic areas.

Multivariate analyses (nMDS ordination and ANOSIM) and SIMPER tests detected differences between the *Fusarium* communities from the two agroclimatic areas. The nMDS ordination showed two distinct *Fusarium* community clusters, which were related to agroclimatic areas (Figure 5-6). The grouping of communities is strongly supported by a stress value of 0.01, indicating an excellent representation with no prospect of misinterpretation (Clarke and Warwick 2001). The similarity of the site communities within the Goondiwindi area is 63.7%, and 65.1% within the Quirindi area based on the Bray-Curtis similarity coefficient. Similarity of the communities between the two agroclimatic areas is 52.9%.

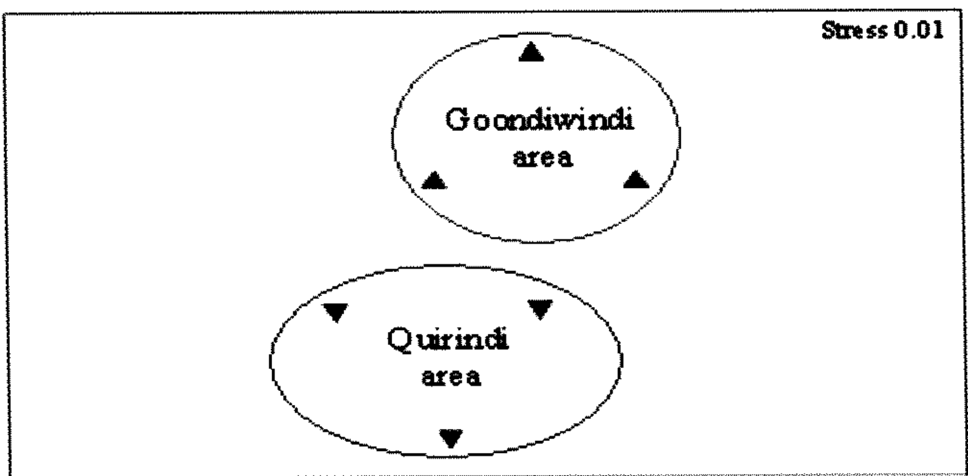


Figure 5-6 Two-dimensional nMDS ordination of communities from six sites based on Bray-Curtis similarity coefficients. Clusters from group-averaged clustering from Bray-Curtis similarities are superimposed at similarity levels of 63.7% (green line) and 65.1% (blue line). Stress value of 0.01

Analysis of similarities (ANOSIM) confirmed the pattern of the nMDS ordination, indicating that communities from two agroclimatic areas are significantly different from one another (global R = 0.815, P = 0.1) (Appendix 4).

SIMPER analysis identified eight *Fusarium* species as discriminatory species giving an average contribution to the total average dissimilarity of 47.1% between communities from the Goondiwindi and Quirindi areas (Table 5-5). The most abundant species, *F. andiyazi* and *F. thapsinum* contributed 10.53 and 10.50 of the average dissimilarity respectively, which is 22.37% and 22.29% of the overall value of 47.1%.

Table 5-5 Discriminating species contributing to the dissimilarity of communities in the Goondiwindi and Quirindi areas

Species	Average contribution	Contribution (%)	Cumulative (%)
F. andiyazi	10.53	22.37	22.37
F. thapsinum	10.50	22.29	44.66
F. chlamydosporum	7.13	15.14	59.81
F. compactum	5.38	11.43	71.24
F. nygamai	5.09	10.80	82.04
F. proliferatum	3.02	6.42	88.47
F. graminearum (G. zeae)	0.99	2.10	90.57

5.4 Discussion

Of the 14 *Fusarium* species recovered during this study, *F. thapsinum* and *F. andiyazi* were an integral component of the communities from the Goondiwindi (61.5%) and Quirindi (82.4%) areas. This study confirms the findings reported in Chapter 4 that *F. thapsinum* and *F. andiyazi* are the dominant *Fusarium* species associated with mature sorghum stalks in this region of the northern grain belt of eastern Australia. The result is also consistent with the recovery of *F. moniliforme sensu lato* from sorghum stalks in a previous study from the same region of the northern grain belt of eastern Australia (Burgess *et al.* 1981; Trimboli and Burgess 1985).

Six species out of 14 recovered in the current study were present in both agroclimatic areas. Abundance and relative proportions of two major species, *F. thapsinum* and *F. andiyazi,* in the hotter and dryer Goondiwindi area (37.9% and 23.6%, respectively) and cooler and wetter Quirindi area (28.9% and 53.5%, respectively) contributed to the dissimilarities in the composition of *Fusarium* communities from these two agroclimatic areas. The exclusive occurrence and abundance of *F. chlamydosporum* and *F. nygamai*, and the abundance of *F. compactum* in the Goondiwindi area, as well as restricted occurrence of *F. graminearum* and abundance of *F. proliferatum* in the

Quirindi area also contributed to the dissimilarities in the composition of two *Fusarium* communities. Consequently, the hypothesis that the diversity of *Fusarium* species and the dominant species associated with grain sorghum stalks are similar in two agroclimatic areas of the northern grain belt of eastern Australia is rejected.

The abundance of *Fusarium* species was significantly higher in the hotter, dryer Goondiwindi area (182 isolates) compared to the cooler, wetter Quirindi area (142 isolates). The slightly unequal sample sizes in the Goondiwindi and Quirindi areas did not affect the abundance of *Fusarium* communities in these two agroclimatic areas ($P = 0.815$). However, the species accumulation curve indicated that the number of sampled stalks was not large enough to ensure the recovery of all species likely to be present in the Goondiwindi area (Figure 5-5). Estimates of species richness for the Goondiwindi area (24 species) would appear to be an overestimation, but are the result of an equal number of species with ≤ 10 isolates and species with > 10 isolates (ACE), as well as the number of species found in ≤ 10 sorghum stalks (ICE). Thus, it is reasonable to expect that more intensive sampling in the Goondiwindi area would reveal more species, although the current sampling size was adequate for determining the predominant species. The sample size would need to be increased significantly if the objective was to recover all *Fusarium* species present in sorghum stalks or to demonstrate presence or absence of particular species for quarantine purposes.

The diversity of *Fusarium* communities, as expressed by Simpson's index of diversity, was higher in the hotter, dryer Goondiwindi area (4.26 ± 0.03) compared to the cooler, wetter Quirindi area (2.69 ± 0.02). In general, isolates were unevenly distributed among species in both agroclimatic areas due to the dominance of *F. thapsinum* and *F. andiyazi* in both communities. However, isolates were more evenly

spread between species in the Goondiwindi area (0.43) than in the Quirindi area (0.27).

The diversity and composition of the *Fusarium* communities in two agroclimatic areas with quite different growing conditions might reflect a long-term influence of available moisture and temperature. In addition, time of sowing/physiological maturity, time of sampling, environmental conditions during the 2002/2003 growing season, cropping system associated with a particular site/area and level of inoculum accumulated over the years in these agroclimatic areas may also influence the *Fusarium* communities. Based on current results, it is not possible to single out any individual factor as responsible for the differences in diversity and composition of the communities associated with sorghum stalks in the Goondiwindi and Quirindi areas. It is speculated, however, that long-term difference in growing season conditions has led to the development of different *Fusarium* communities.

There have been no previous studies on the relative impact of agroclimatic conditions on *F. thapsinum* and *F. andiyazi* distribution. This is possibly a consequence of the complex nature of stalk rot, head blight and grain mould, the challenges of identifying *Fusarium* species, and the fact that *F. andiyazi* was only recently described.

Ryley *et al.* (2002) reported that in southern Queensland in the 1998/1999 growing season, the incidence and severity of stalk rot associated with *F. moniliforme sensu lato* was the worst it had been in 20 years. It was hypothesised that a soil moisture deficit at grain-filling had caused stress, which coupled with root rot and severe infection by rust, contributed to stalk rot.

Results from the Goondiwindi area suggest that *F. thapsinum*, *F. andiyazi* and *F. nygamai*, species probably considered in the past as *F. moniliforme sensu lato*, caused

stalk rot of sorghum under water deficit during physiological maturity in the 2002/2003 growing season. Higher abundance of *Fusarium* species in the Goondiwindi area compared to the Quirindi area also suggests that water deficit and higher average temperature contributed to plant stress at the beginning of the 2002/2003 growing season, and was especially damaging during physiological maturity. Furthermore, the agroclimatic factors favoured colonization of sorghum by *F. compactum* a species that occurs more commonly in hot arid and tropical areas.

Results from the Quirindi area suggest that available moisture at both the beginning of the growing season and the post-flowering stages, and temperatures lower than 25°C made plants less susceptible to stress and colonization by *Fusarium* species. *Fusarium andiyazi* was significantly more abundant than *F. thapsinum* in the Quirindi area ($P = 0.014$), suggesting that cooler, wetter areas favour *F. andiyazi*. This finding is consistent with that of Marley *et al.* (2004) who showed that *F. andiyazi* was the predominant species in sorghum seed samples (97%) in the Katsina area of Nigeria. This area is at an altitude of 516 m, with an average annual rainfall of 500 to 750 mm falling mainly during the growing season, and an annual average temperature of 26°C (http://www.weatherbase.com).

The abundance of *F. andiyazi* and *F. thapsinum* contributed the most to the total average dissimilarity of the communities (SIMPER analysis) in the two agroclimatic areas (47.3%), although *F. chlamydosporum*, *F. compactum*, *F. nygamai*, *F. proliferatum*, *F. graminearum* and *F. subglutinans* also contributed to the dissimilarity (Table 5-5). The distribution of *F. chlamydosporum* and *F. nygamai* was restricted to the hotter, dryer Goondiwindi area, whereas the distribution of *F. proliferatum*, *F. graminearum* and *F. subglutinans* was limited to the cooler, wetter Quirindi area. The recovery of *F. compactum* was higher in the hotter (10.3%) than

the cooler (0.7%) region, a finding consistent with earlier mycogeographic studies (Burgess and Summerell 1992; Sangalang *et al.* 1995; Summerell *et al.* 1993).

Fusarium proliferatum was the third most abundant species recovered from sorghum stalks in the Quirindi area. This finding corresponds with the recovery of *F. proliferatum* from sorghum seeds in the USA (Leslie and Plattner 1991) and Tanzania (Mansuetus *et al.* 1997). Variation in the abundance of *F. proliferatum* in the two agroclimatic areas was consistent with the variation in abundance between three locations in Tanzania, although this difference was not attributed to the agroclimatic conditions (Mansuetus *et al.* 1997).

Fusarium subglutinans was recovered from the cooler, wetter Quirindi area, but not from the hotter, dryer Goondiwindi area. Recently, Burgess *et al.* (2002) also recovered this species from sorghum stalks in the Quirindi area (loc. Spring Ridge) of the Liverpool Plains. Previous Australian studies indicate that this species is more commonly recovered from maize stalks from cooler areas such as Bathurst (Edwards 1935; Francis and Burgess 1975). The low level of recovery of *F. subglutinans* from sorghum stalks in this study is consistent with earlier studies on *Fusarium* species associated with sorghum grain in Tanzania and Argentina (Gonzalez *et al.* 1997; Mansuetus *et al.* 1997; Saubois *et al.* 1999).

The dominant pathogen of maize, *F. verticillioides*, was recovered rarely from sorghum stalks in the Goondiwindi (0.6%) and Quirindi (1.4%) areas, confirming that host preference can be important for species identification, *viz. F. verticillioides* colonizes maize and *F. thapsinum* colonizes sorghum (Leslie and Marasas 2002).

One other pathogen of maize, *F. graminearum*, was recovered rarely from sorghum stalks in the cooler, wetter Quirindi area. This finding may reflect low levels of

inoculum of this fungus at these sites during the survey, as it is a known colonizer of mature sorghum (Burgess *et al.* 2002).

This study has extended understanding of the abundance and distribution of *F. andiyazi* and *F. thapsinum* in the northern grain belt of eastern Australia. The knowledge of species known as maize colonizers, *F. proliferatum*, *F. subglutinans* and *F. verticillioides* is extended in the context of their potential to colonize grain sorghum. Furthermore, the possible risk of *F. graminearum* drift from sorghum to its main hosts, wheat and maize, is confirmed in this study as *F. graminearum* sporadically colonized sorghum.

Additional studies on the distribution of *Fusarium* species associated with sorghum need to be undertaken in other agroclimatic areas of Australia, such as the Emerald region of central-eastern Queensland and the Kununurra region of tropical northern Western Australia. A high diversity of *Fusarium* species associated with grain sorghum can be expected due to the spread of inoculum between crops, as inoculum can be seedborne and consequently seed-transmitted (Claflin and Giorda 2002). Further, sampling in the Goondiwindi and Quirindi areas is justified to confirm the findings reported here. It is possible that the *Fusarium* spp. populations associated with sorghum fluctuate within and between cropping seasons. However, it should be noted that the overall findings for the intensive study of *Fusarium* species associated with grain sorghum (reported in Chapter 4) from the 2001/2002 growing season were consistent with the findings from the 2002/2003 season.

Intensive sampling, isolation and identification need to be optimized in order to identify isolates correctly. The most desirable approach for identification of *Fusarium* isolates will be based on morphological, sexual compatibility and molecular analyses.

However, this stepwise process is time-consuming if large numbers of isolates need to be identified. Consequently, a compromise between the number of samples, isolates and the approach to identification may need to be made depending on available resources. Sexual compatibility tests followed by partial sequencing of the translation elongation factor 1-α (TEF) gene for randomly selected isolates, incompatible with available tester strains, could provide reliable conformation of morphological identification, confirmed for *F. andiyazi* in the current study.

Chapter 6

Characterisation of the Dominant *Fusarium* Species Associated with Grain Sorghum

6.1 Introduction

In this chapter the identity of Australian isolates of *Fusarium andiyazi*, *F. thapsinum*, *F. verticillioides* and *F. proliferatum* from grain sorghum were confirmed based on a polyphasic approach. In this stepwise process, morphological criteria were initially used as diagnostic criteria for putative identification of *F. andiyazi*, *F. thapsinum* and *F. verticillioides*. Subsequently their identification was confirmed using sexual compatibility tests, AFLP analysis and phylogenetic analysis of a portion of the TEF gene sequences.

The identity of *F. proliferatum* was clarified based on a similar approach. However, morphological identification was confirmed only by sexual compatibility tests and phylogenetic analysis of a portion of the TEF gene sequences.

In Australia, *F. andiyazi*, *F. thapsinum* and *F. verticillioides* were previously classified as *F. moniliforme sensu lato*, whereas *F. proliferatum* was recognized as a distinct species.

6.2 Materials and Methods

6.2.1 Isolates examined

A total of 63 isolates of *F. andiyazi*, 64 isolates of *F. thapsinum*, three isolates of *F. verticillioides* and 16 isolates of *F. proliferatum* were included in this study (Appendix 5.1). Isolates were arbitrarily selected to represent the limited variation in morphological characters within each species. Isolates of *F. andiyazi* and *F.*

thapsinum were recovered from different plant parts of grain sorghum (Appendix 5.1) from Livingston Farm, Moree (NSW) in 2002 (Chapter 4). Isolates of *F. verticillioides* and the majority of the isolates of *F. proliferatum* were recovered from sorghum stalks from the Goondiwindi (QLD) and Quirindi (NSW) region in 2003 (Appendix 5.1).

Reference isolates of *F. andiyazi* (five), *F. thapsinum* (39), *F. verticillioides* (two) and *F. proliferatum* (two) were also included in the analysis (Appendix 5.2). Reference isolates of *F. andiyazi* originated from the Republic of South Africa, Ethiopia, the USA (Marasas *et al.*, 2001) and Mexico (Montes-Belmont, *personal communication*). Reference strains of *F. thapsinum* originated from the Republic of South Africa, the USA (Klittich *et al.*, 1997; Prom *et al.*, 2003), Mexico (Montes-Belmont, *personal communication*) and South Korea (Lim *et al.*, 2001). Mating population tester strains, which are internationally accepted standards for sexual compatibility tests, were used as reference strains for *F. verticillioides* and *F. proliferatum*.

6.2.2 Characterisation of **F. andiyazi, F. thapsinum** *and* **F. verticillioides**

6.2.2.1 Morphological characterisation

Isolates were grown on PDA, CLA and SNA under the standard conditions described in Chapter 4. Representative isolates of each species were also grown on SNA at 22°C ± 2°C under continuous near-UV black light blue (BLB) (Phillips TLD 18W/08) for ten to14 days (Nirenberg and O'Donnell 1998). The colony colours based on the Methuen Handbook of Colour (MHC) (Kornerup and Wanscher 1978) were determined in cultures grown in the dark at 25°C for nine days.

The radial growth rates of colonies initiated from single microconidia on PDA in 90 mm Petri plates were measured after 72 h of incubation in the dark at 20°C, 25°C and

30°C. Measurements were made based on three replicate colonies for each isolate at each temperature.

At least 50 clavate/obovoid to elliptical microconidia of four putative isolates and five reference strains (KSU4804, KSU4647, KSU10771, KSU11155, F15910) of *F. andiyazi* were measured. The same number of clavate microconidia were measured in the putative isolates of *F. verticillioides* for comparison with *F. andiyazi* microconidia, using cultures on SNA and CLA under the standard conditions described above.

At least 30 pyriform/citriform microconidia of seven putative isolates and 12 to 30 pyriform/citriform microconidia of four reference strains (KSU4093, MRC 6002, MRC 6120, MRC 6148) of *F. thapsinum* were measured, using cultures on SNA grown in the dark at 25°C, and under BLB at 22 ± 2°C.

Morphological characters of Australian isolates of *F. andiyazi* and *F. thapsinum* isolates were compared with reference strains of these species grown under the same conditions.

6.2.2.2 Molecular characterisation

Molecular characterisation was undertaken using AFLP analysis. Selected reference strains of *F. andiyazi* (four isolates), *F. thapsinum* (ten isolates) and *F. verticillioides* (two isolates) were included in the analyses (Appendix 5.2). Details on the protocol are given in Chapter 3. AFLP profiles were scored for the presence or absence of bands between 100 and 400 bp. Similarity of isolates was calculated based on the DICE coefficient, and UPGMA clustering method was used to generate a dendrogram in NTSYSpc version 2.10q (Applied Biostatistics, Inc., U.S.A.).

6.2.2.3 Biological characterisation

The biological traits (mating type and male/female fertility) of all putative isolates and reference strains of *F. thapsinum* were determined by specific PCR amplification of the two mating type alleles (Kerényi *et al.*, 1999; Steenkamp *et al.*, 2000) and crossings. Crosses were conducted using one of the standard tester strains (with the opposite mating type allele) for MP A (*Gibberella moniliformis*) and MP F (*G. thapsina*) (Klittich and Leslie 1988) as described in Chapter 3.

6.2.2.4 Phylogenetic analysis of *F. andiyazi* isolates

Eighteen *F. andiyazi* isolates, 12 from Moree (NSW), three from Quirindi (NSW) and three from Goondiwindi (QLD) were selected for phylogenetic analysis (Appendix 5.1).

The protocol for amplification of a portion of the TEF gene and purification of amplified products is described in detail in Chapter 3. DNA sequences were obtained using an ABI PRISM® 3700 DNA Analyser (Applied Biosystems Inc., U.S.A). Sequences of both strands from a portion of the TEF gene were assembled and edited using BioEdit Sequence Alignment Editor version 7.0.5 (Hall 2005). Consensus sequences for all isolates were obtained and aligned with GenBank sequences (Appendix 5.3) using the same software. Phylogenetic analyses were performed with PAUP version 4.0b10 (Swofford 1988). Alignment gaps were treated as missing data. The data set was analysed based on four different optimisation criteria: maximum parsimony (MP), weighted parsimony (WP), neighbour-joining (NJ) and maximum likelihood (ML). Maximum parsimony trees were obtained using heuristic searches with random sequence addition of 1000 replicates and a tree-bisection-reconnection branch-swapping algorithm. The transition : transversion ratio estimated from ML (2.36 : 1) was used in the WP analysis by including a step matrix in the input data.

Consistency index (CI) and retention index (RI) were calculated as an estimate of the amount of homoplasy present. The NJ tree was based on the HKY85 model (Hasegawa *et al.* 1985). For ML and NJ analyses, base frequencies were estimated with among-site ratios assumed to be equal. The gamma distribution was also estimated. The trees were viewed using TreeView (Win32) version 1.6.6 (Page 2001). The trees were rooted using *F. oxysporum* and *F. inflexum* as outgroup taxa.

Stability of clades was assessed by 1000 parsimony bootstrap replications with random sequence addition implemented in PAUP.

6.2.3 Characterisation of **F. proliferatum**

6.2.3.1 Morphological examination

Morphological examination of cultures on CLA and SNA, and growth rates were carried out under the conditions described for *F. andiyazi*, *F. thapsinum* and *F. verticillioides*.

6.2.3.2 Biological characterisation

The biological traits (mating type and male/female fertility) of isolates were determined in sexual compatibility tests with standard tester strains of MP D (*G. intermedia*), as described in Chapter 3.

6.2.3.3 Phylogenetic analysis

Five isolates originating from the Goondiwindi, Moree and Quirindi areas were chosen for sequencing of a portion of the TEF gene (Table 6-1). Phylogenetic analysis was performed as described for *F. andiyazi*. The WP analysis was performed based on the transition : transversion ratio estimated from ML (2.28 : 1). The trees were rooted using *F. oxysporum* as the outgroup taxon.

6.3 Results

6.3.1 Characterisation of **F. andiyazi, F. thapsinum** *and* **F. verticillioides**

6.3.1.1 Morphological characterisation

Four morphological characters and pigmentation on PDA accurately differentiated *F. andiyazi*, *F. thapsinum* and *F. verticillioides*. The morphological characters that consistently differentiated the isolates examined were length of chains, shape and size of microconidia, germination of microconidia along the chains and the type of conidiophore (Figure 6-1 & Figure 6-4). All of the prominent morphological characters of these species are listed in Table 6-1.

Table 6-1 The key morphological and physiological characters of isolates of *F. andiyazi*, *F. verticillioides*, *F. thapsinum* from sorghum in the northern grain belt of eastern Australia

Characters	*F. andiyazi*	*F. verticillioides*	*F. thapsinum*
Profuse mycelial growth on PDA	-	+	+
Veins in substrate[a]			
Fine	+	-	-
Robust, distinct	-	+	+
Pigmentation in reverse on PDA after 9 days			
Orange white to pale orange	+	-	-
Lilac, dull violet	-	+	+
Radial growth rate (mm / 72h)			
20°C	14.3 ± 0.4	13.1 ± 1.5	10.0 ± 1.3
25°C	31.1 ± 1.2	32.5 ± 1.5	25.8 ± 1.7
30°C	37.9 ± 1.9	38.6 ± 1.5	28.0 ± 3.1
Pseudochlamydospores[b]/cell wall thickening[c]	(+)	+	-
Chains			
Long to medium (> 15)	+	+	-
Short to medium (< 15)	-	-	+
Shape of microconidia			
clavate	+	+	-
obovoid to elliptical	+	-	+
pyriform/citriform	-	-	+
Size of microconidia (μm)			
Clavate/obovoid to elliptical			
SNA 25°C	13.3 ± 3.6 x 3.4 ± 0.6	9.9 ± 2.3 x 3.2 ± 0.5	*
CLA 25°C light/25°C dark	14.4 ± 4.3 x 3.4 ± 0.6	9.1 ± 2.0 x 3.0 ± 0.5	*
SNA 22°C n-UV BLB	16.0 ± 5.1 x 3.9 ± 0.8	10.8 ± 2.9 x 3.4 ± 0.6	*
Pyriform			
SNA 25°C			10.9 ± 1.8 x 5.9 ± 1.3
SNA 22°C n-UV BLB			12.4 ± 2.2 x 6.4 ± 1.4
Germination of microconidia	(+)	-	+
Conidiogenous cells			
Monophyalidic	+	+	+
Polyphialidic	-	-	(+)

[a]Prominent lines spreading from the centre of the actively growing culture

[b, c]Characters not clearly defined

+ = character observed; - = character not observed; (+) = character only occasionally observed

* = obovoid to elliptical microconidia not measured

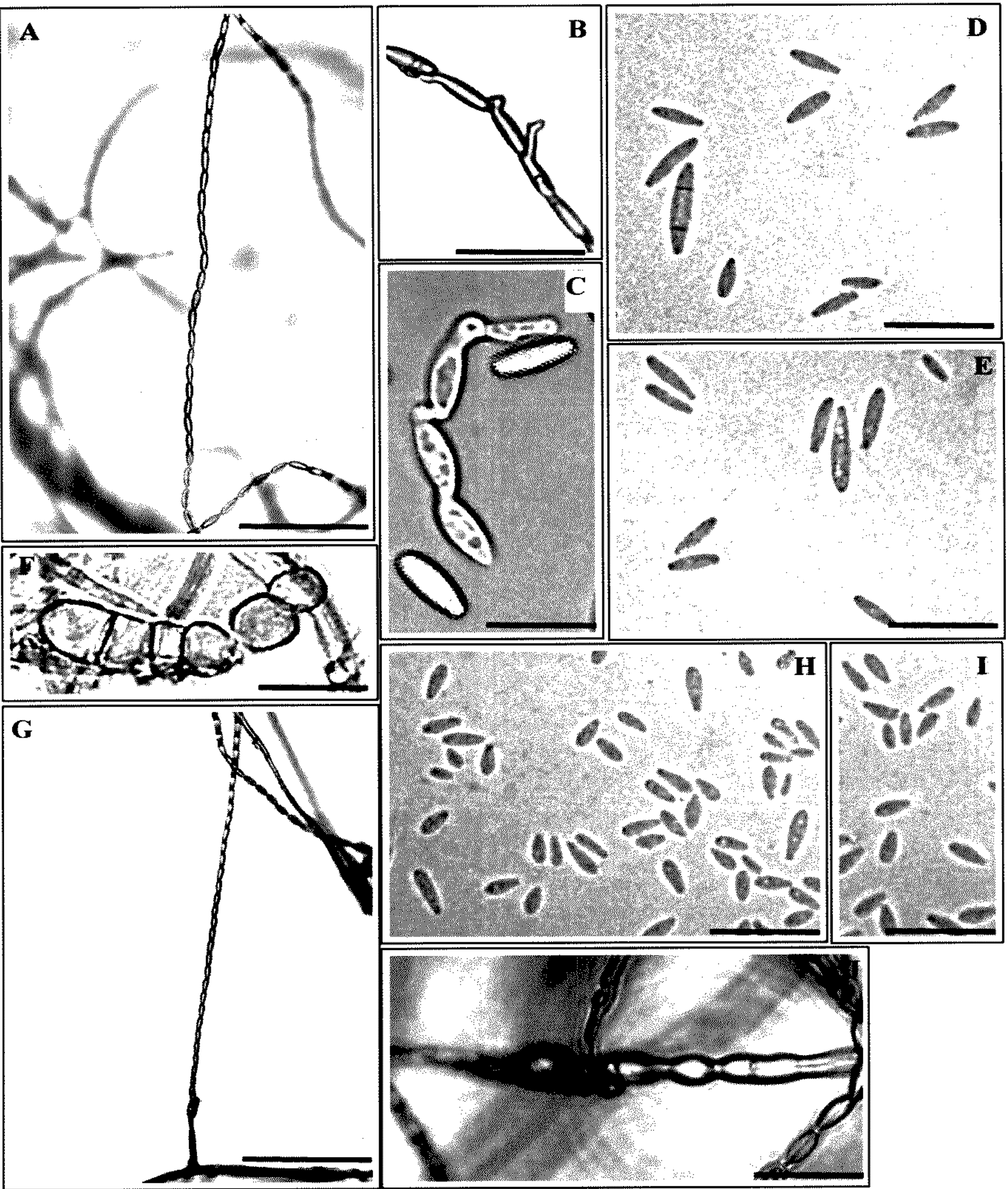


Figure 6-1 Comparison of morphological characters of *F. andiyazi* (A, B, C, D, E, F) and *F. verticillioides* (G, H, I, J). Germination of microconidia in *F. andiyazi* (B, C), but not in *F. verticillioides* (G). Size of microconidia in *F. andiyazi* (D, E) and *F. verticillioides* (H, I). "Pseudochlamydospores" in *F. andiyazi* (F) and "thickening" of hyphal cells (J) in *F. verticillioides*. Scale bars: A, G, J = 100μm; B = 50μm and C-E, H-I = 25μm

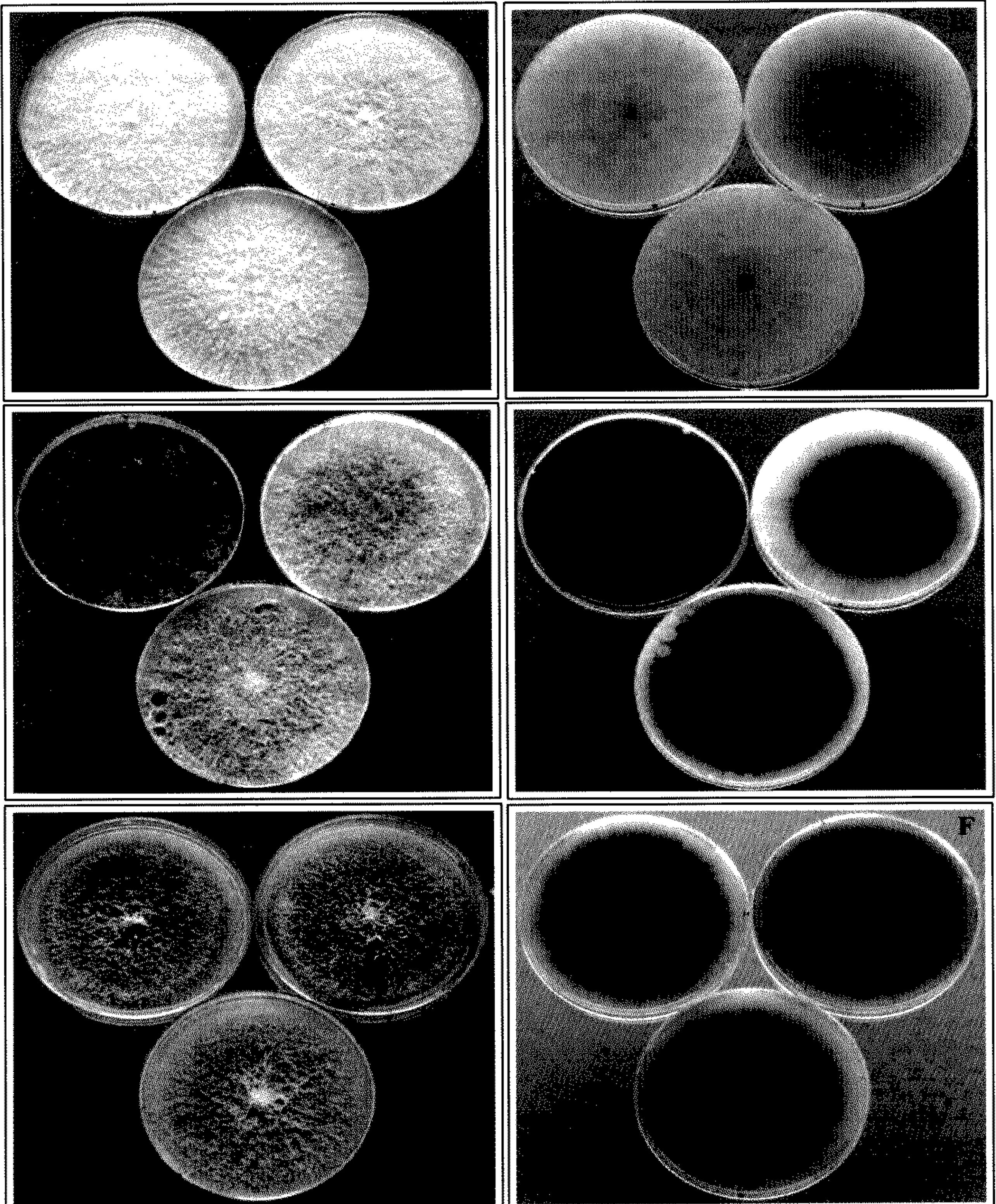


Figure 6-2 Colony morphology of nine day old cultures of putative isolates of *F. andiyazi* (A, B), *F. verticillioides* (C, D) and *F. thapsinum* (E, F) at 25°C in the dark. Upper (A, C, E) and reverse (B, D, F) pigmentation

F. andiyazi and F. verticillioides - The characters shared by *F. andiyazi* and *F. verticillioides* were long linear chains with more then 15 clavate/obovoid to elliptical microconidia, long, simply branched conidiophores with verticillate monophialides, and "thickening" of hyphal cells in the aerial mycelium that resemble "pseudochlamydospores" (Figure 6-1). These were the most morphologically similar species of the four considered in this chapter.

The size of microconidia, the presence of up to 2 septa, and 3 septa (F18940) (under BLB conditions) and clavate microconidia differentiated *F. andiyazi* from *F. verticillioides* (Table 6-1; Figure 6-1D, E, H, I). Two-septate clavate microconidia were rarely present in *F. verticillioides* isolates grown in the dark at either 25°C (2165) or under BLB (2032). Microconidia of *F. andiyazi* were longer than microconidia of *F. verticillioides* under all three light/temperature conditions (Table 6-1).

Germination of microconidia along chains was another character occasionally observed in *F. andiyazi* but not *F. verticillioides* isolates (Figure 6-1B, C). This character was observed in *F. andiyazi* mostly on SNA cultures grown in the dark at 25°C, and under BLB at 22 ± 2°C.

Although radial growth rates were similar between them, *F. andiyazi* and *F. verticillioides* differed in colony morphology and pigmentation on PDA (Table 6-1). Aerial mycelia of *F. andiyazi* isolates was fine, loosely floccose, pale orange (MHC 6A3) to pale violet (MHC 17A3). Pigmentation on the reverse side was pale orange (MHC 6A3), with a pale to dull violet circle (MHC 17A3-MHC 17A4) at the periphery of colonies with radial substrate veins of the same colour (Figure 6-2A, B).

The aerial mycelium of *F. verticillioides* isolates was abundant, woolly, lilac (MHC 15B3) to greyish violet (MHC 17B3). Robust veins were present in the substrate. Pigmentation on the reverse side was either greyish rose (MHC 11B6) or more often dull violet (MHC 17D4) (Figure 6-2C, D).

The morphological and physiological characters of the putative *F. andiyazi* isolates were similar to the reference strains from the Republic of South Africa (KSU4804, KSU10771), Ethiopia (KSU4647), the USA (KSU11155) and Mexico (F15910). Prominent clavate microconidia were present in all isolates, but were less frequent in the Mexican isolate, that had predominantly abundant obovoid to elliptical microconidia. Hence, the size range of clavate/obovoid to elliptical microconidia of the putative *F. andiyazi* isolates was least comparable to the Mexican reference strain (Figure 6-3).

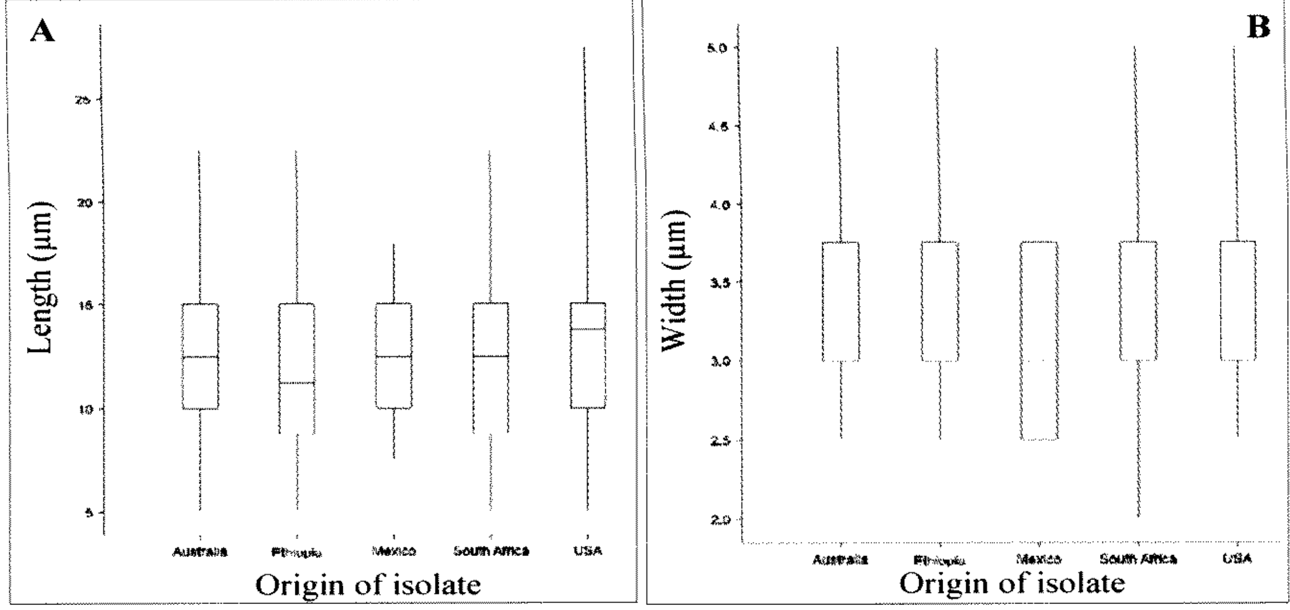


Figure 6-3 Comparison of length (A) and width (B) ranges of clavate/obovoid to elliptical microconidia of *F. andiyazi* isolates and reference strains on SNA at 25°C. Reference strains are from the Republic of South Africa (KSU4804, KSU10771), Ethiopia (KSU4647), the USA (KSU11155) and Mexico (F15910). The size of 50% of the microconidia is within boxes whilst the line in the box indicates the symmetry of the data

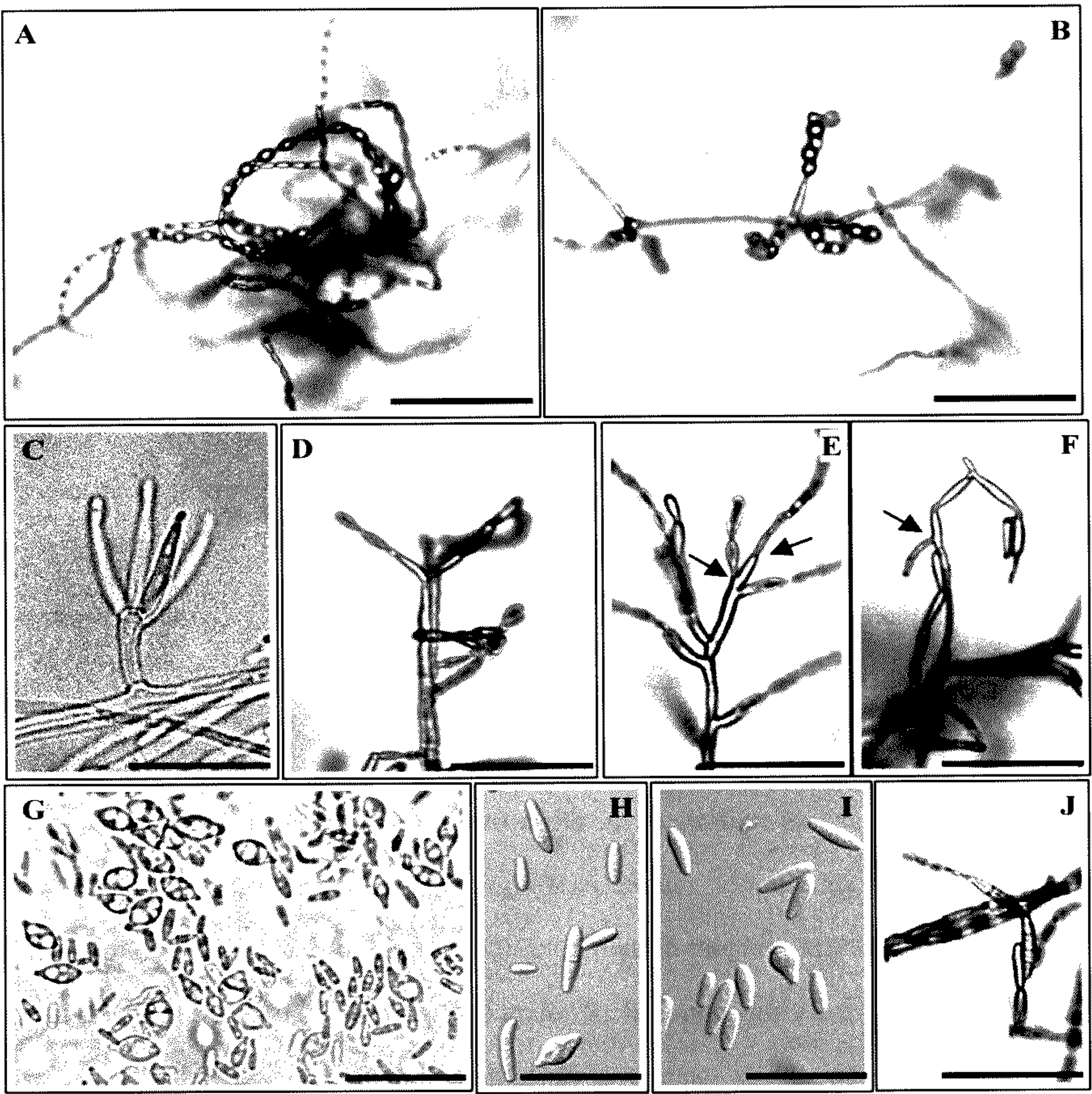


Figure 6-4 Morphology of *F. thapsinum*. Chains of pyriform/citriform microconidia formed on SNA in the dark at 25°C (A) and on CLA at 25°C light/20°C dark (B). Conidiophore with verticillate phialides (C) and polyphialides (D, E). Germination of microconidia (F, J). Pyriform/citriform microconidia (G). Obovoid to elliptical microconidia and citriform microconidium (H). Obovoid to elliptical microconidia with pyriform microconidium (I). Scale bars: A-F, J = 100μm and G-I = 25μm

F. thapsinum – A number of characters distinguished *F. thapsinum* from both *F. andiyazi* and *F. verticillioides*. The main characters were: presence of pyriform/citriform microconidia with 0-1 septa, short to medium linear chains with less then 15 microconidia which often germinated along chains, especially on CLA at 25°C light/20°C dark with a 12h photoperiod, and bulbous conidiophores (Figure 6-4). Conidiogenous cells were mostly monophialidic and occasionally polyphialidic (Figure 6-4D, E).

Pyriform/citriform microconidia were commonly produced on SNA in the dark at 25°C and under BLB at 22 ± 2°C (Figure 6-4A, G-I), but rarely on CLA with a 12h photoperiod at 25°C light/20°C dark (Figure 6-4B). This type of microconidium was more abundant in isolates with yellowish pigmentation than in the isolates with violet pigmentation on PDA.

The size of pyriform/citriform microconidia in Australian isolates of *F. thapsinum* was significantly influenced by light/temperature conditions (Fpr. < 0.001). Pyriform/citriform microconidia in cultures grown in the dark at 25°C were 7.5 – 17.5 x 3.8 – 10 μm (av. 10.9 x 5.9 μm) and under BLB conditions were 7.5 – 20 x 3 – 12.5 μm (av. 12.4 x 6.4 μm). Mean length, but not mean width, of pyriform/citriform microconidia of putative isolates was significantly lower than that of microconidia of the *F. thapsinum* reference isolates grown under the same conditions (Fpr. < 0.001) (Figure 6-5A, B). There was also a significant difference in mean length between Australian isolates (*i.e.* F18946 and F18947) and between reference isolates of *F. thapsinum* [*i.e.* MRC 6002 and all other reference strains (MRC 6120, MRC 6148 and KSU4093)] (Figure 6-5A). A significant difference in mean width was only found between reference isolates (*i.e.* MRC 6002 and all other reference and Australian isolates) (Figure 6-5B).

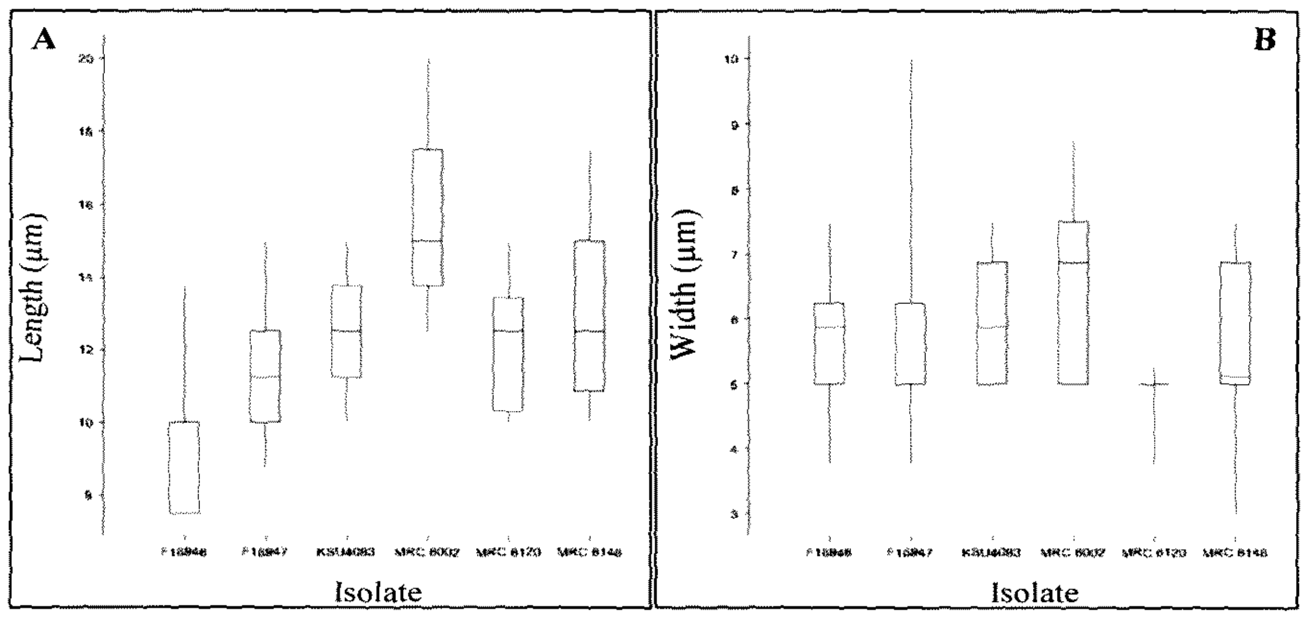


Figure 6-5 Comparison of ranges of length (A) and width (B) of pyriform/citriform microconidia of the putative isolates (F18946 and F18947) and reference strains (KSU4093, MRC 6002, MRC 6120 and MRC 6148) of *F. thapsinum* grown on SNA in the dark at 25°C. The size of 50% of the microconidia is within boxes whilst the line in the box indicates the symmetry of the data

Germination of microconidia along chains was a distinct diagnostic character for delineating isolates of *F. thapsinum* (Figure 6-4F, J), with germinated microconidia bearing new microconidia singularly or in short chains (Figure 6-4J). In spite of the sporadic presence of germinated microconidia in *F. andiyazi* isolates, the formation of new microconidia was not observed.

F. thapsinum differed significantly from *F. andiyazi* and *F. verticillioides* in colony morphology and in radial growth rate at 20°C, 25°C and 30°C (Table 6-1). Aerial mycelia were woolly, semi-woolly to powdery woolly, white, pale orange (MHC 6A3) to pale violet (MHC 15A3). Dotted and/or entire veins, flesh (MHC 6B3) to dull violet (MHC 16D3) developed in the substrate. Pigment on the reverse side was

orange white (MHC 6A2), with flesh (MHC 6B3) or dull violet (MHC 16D3) colour in the centre (Figure 6-1E, F).

Apart from mean length of pyriform/citriform microconidia of the putative isolates of *F. thapsinum*, which were significantly different to the microconidia of the reference strains, other morphological and physiological characters of the putative isolates were similar to the majority of the reference strains examined.

6.3.1.2 Molecular characterisation

Cluster analysis of AFLP banding patterns distinguished three main groups (I, II and III), representing the three recognized species (Figure 6-6). These clusters were separated from one another at a DICE similarity level of 25%. Group I comprised isolates of *F. andiyazi* with an overall similarity of 66%, whereas group II consisted of isolates of *F. thapsinum* with a similarity of 58%. The clustering of these two groups was well supported by a bootstrap value of > 99%. Within each of the two groups, the putative isolates were clustered with reference strains of either *F. andiyazi* (four strains) or *F. thapsinum* (ten strains). Both groups were further divided into two subgroups, but the subclustering was not supported by bootstrap values.

Group III included two reference strains of *F. verticillioides* that shared less then 25% similarity with the other two species.

Isolates of *F. andiyazi* were slightly less diverse than those of *F. thapsinum* (Figure 6-6). Out of 63 isolates of *F. andiyazi* examined, 37 isolates shared ≥ 72% similarity with the reference strains of this species. The remaining 26 isolates of *F. andiyazi* shared a similarity level of ≥ 68%. The 64 isolates of *F. thapsinum* examined clustered into two groups. The majority of the isolates (56) shared ≥ 60% similarity with eight of the *F. thapsinum* reference strains. The remaining eight isolates were

more heterogeneous than the majority of isolates, but shared $\geq$ 63% similarity with two of the *F. thapsinum* tester strains.

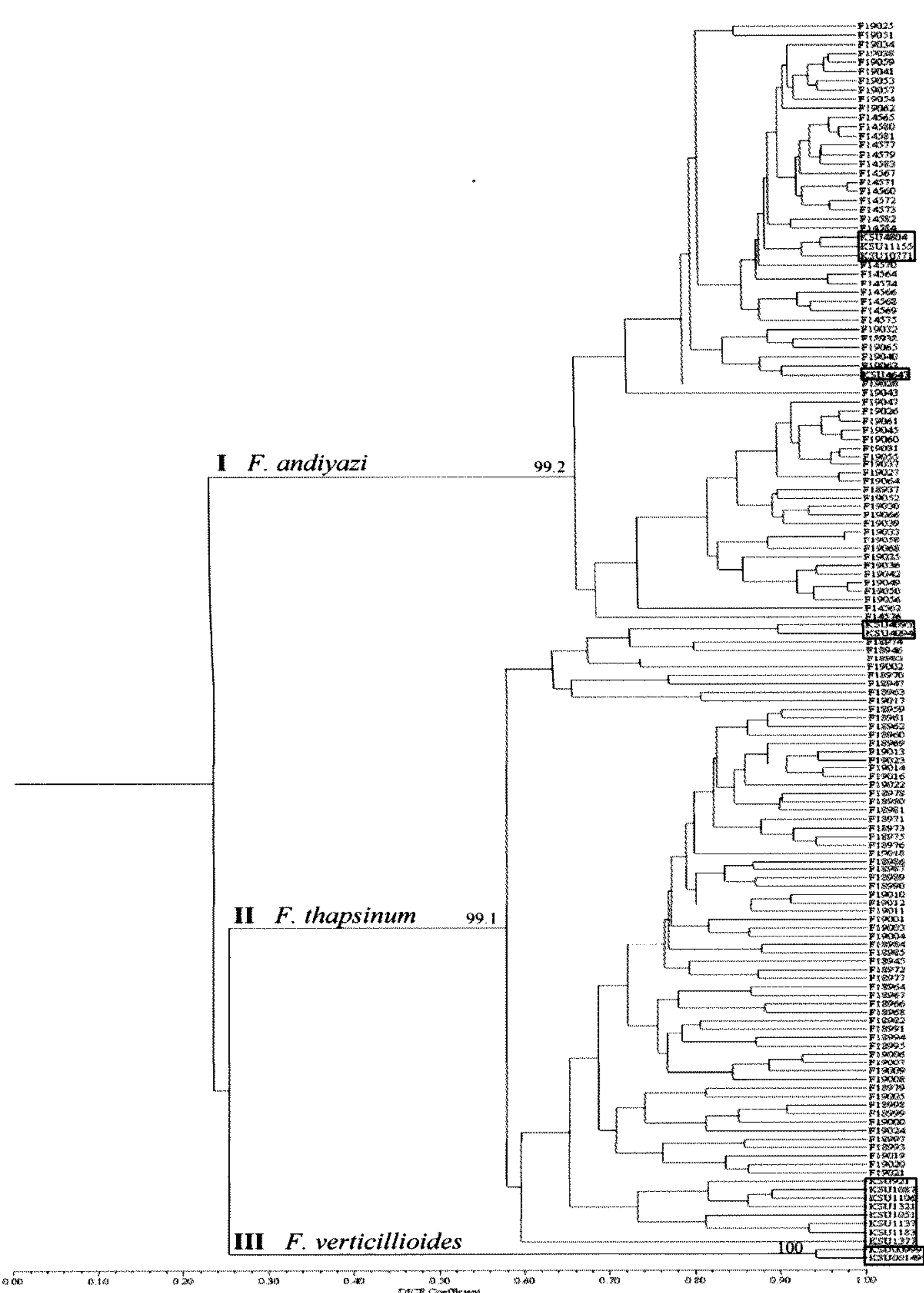


Figure 6-6 Grouping of *F. andiyazi* (63 isolates) and *F. thapsinum* (64 isolates) from Australia with reference strains of *F. andiyazi* (red rectangle), *F. thapsinum* (blue rectangle) and *F. verticillioides* (green rectangle), based on DICE similarity and UPGMA cluster analysis. Bootstrap support is shown for three major branches based on 1000 replications

6.3.1.3 Biological characterisation

Both mating types were observed among the *F. andiyazi* isolates (38 *MAT-1* and 25 *MAT-2*), *F. thapsinum* (34 *MAT-1* and 30 *MAT-2*) and *F. verticillioides* (two *MAT-1* and one *MAT-2*) (Appendix 5.1). Mating type ratios in *F. andiyazi* and *F. thapsinum* were not significantly different to a 1:1 ratio (*F. andiyazi*: $\chi^2 = 2.68$, df = 1, P = 0.101; *F. thapsinum*: $\chi^2 = 0.25$, df = 1, P = 0.617).

Isolates of *F. andiyazi* were infertile as males when crossed with both *F. thapsinum* and *F. verticillioides* standard tester strains. All *F. thapsinum* isolates were fertile as males with one of the *F. thapsinum* tester strains and infertile with *F. verticillioides* testers. Of the 64 *F. thapsinum* isolates tested, only four isolates were female fertile in a reciprocal cross with standard tester strains (F18990, F18998, F18999, F19022).

All three isolates of *F. verticillioides* were male fertile with *F. verticillioides* and infertile with *F. thapsinum* tester strains. Isolates 2165 and 2208 (*MAT-1*) were female fertile in a reciprocal cross with a *F. verticillioides MAT-2* tester strain.

6.3.1.4 Phylogenetic characterisation of *F. andiyazi*

Parsimony analysis of a portion of the TEF gene (649 bp) based on 119 parsimony-informative characters, yielded two most parsimonious trees, 511 steps in length with a CI of 0.75 and a RI of 0.87. The ten Australian isolates and two reference strains of *F. andiyazi* formed a monophyletic group (Figure 6-7). The trees generated based on two optimality criteria ML and NJ also show a clade of *F. andiyazi* isolates separated from all other groups (Figures 6-8, 6-9). No major topological variations were detected among trees obtained using the MP, ML and NJ optimality criteria.

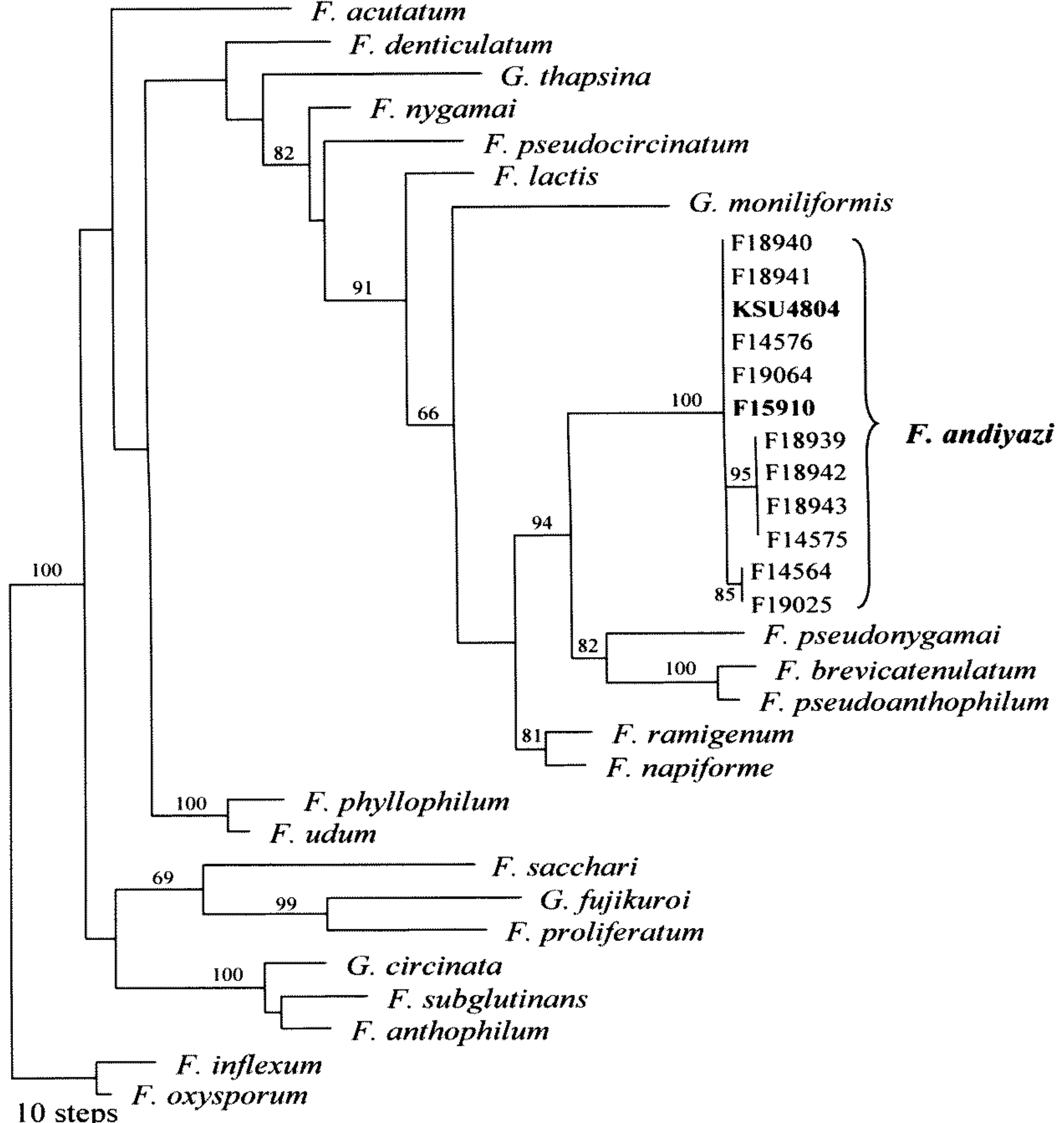


Figure 6-7 One of the two most parsimonious trees generated from the TEF sequence data (649 bp) of *F. andiyazi* isolates and related species. The tree was rooted using *F. oxysporum* and *F. inflexum* as outgroup taxa. Reference strains of *F. andiyazi* are highlighted in red. The length of tree is 511 steps; CI = 0.75, RI = 0.87. Bootstrap values > 50% from 1000 maximum parsimony replicates are indicated above nodes

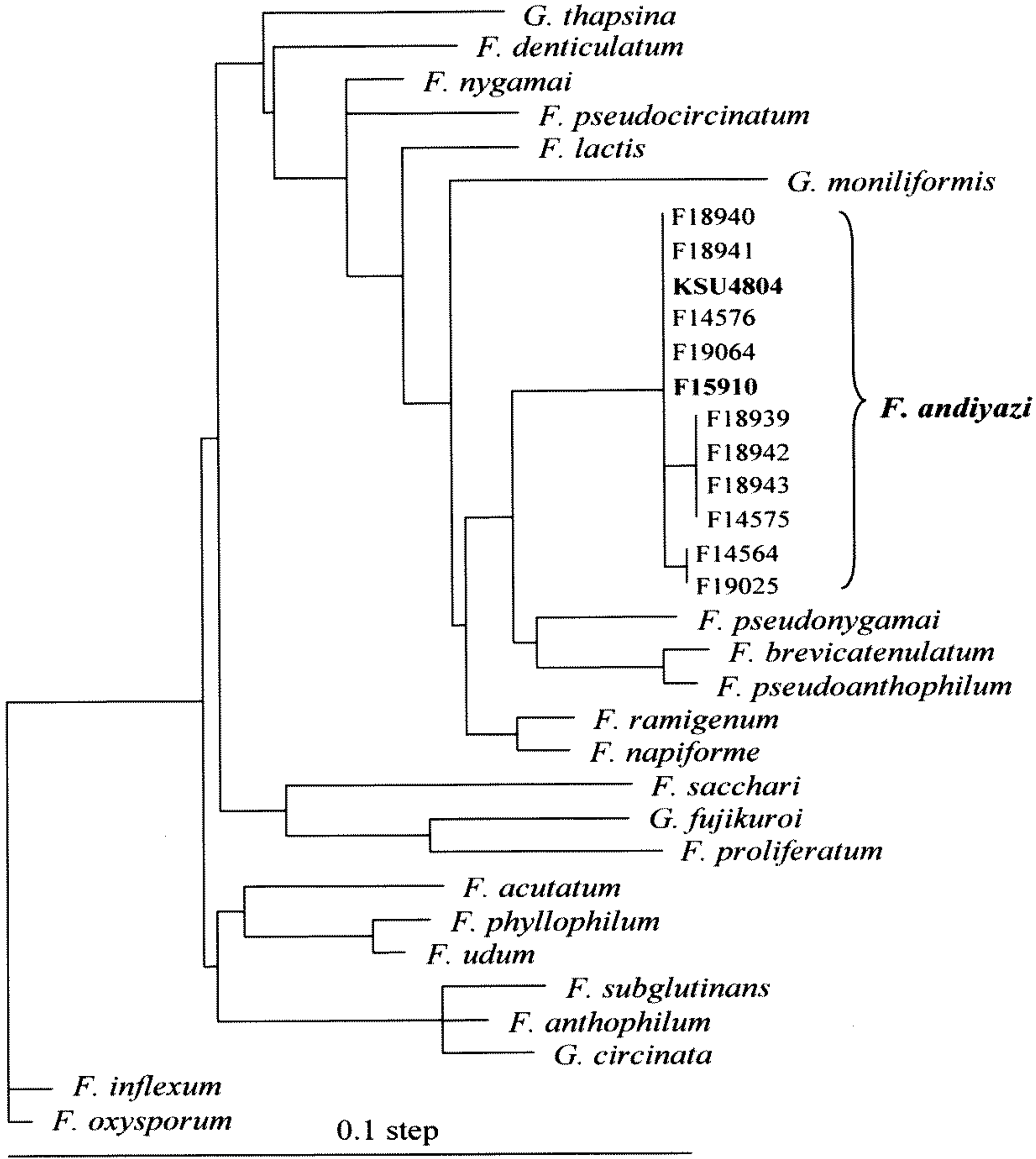


Figure 6-8 The maximum likelihood tree generated from the TEF sequence data of *F. andiyazi* isolates (649 bp) and related species. The tree was rooted using *F. oxysporum* and *F. inflexum* as outgroup taxa. Reference strains of *F. andiyazi* are highlighted in red. The length of tree is 364 steps; CI = 0.73, RI = 0.84

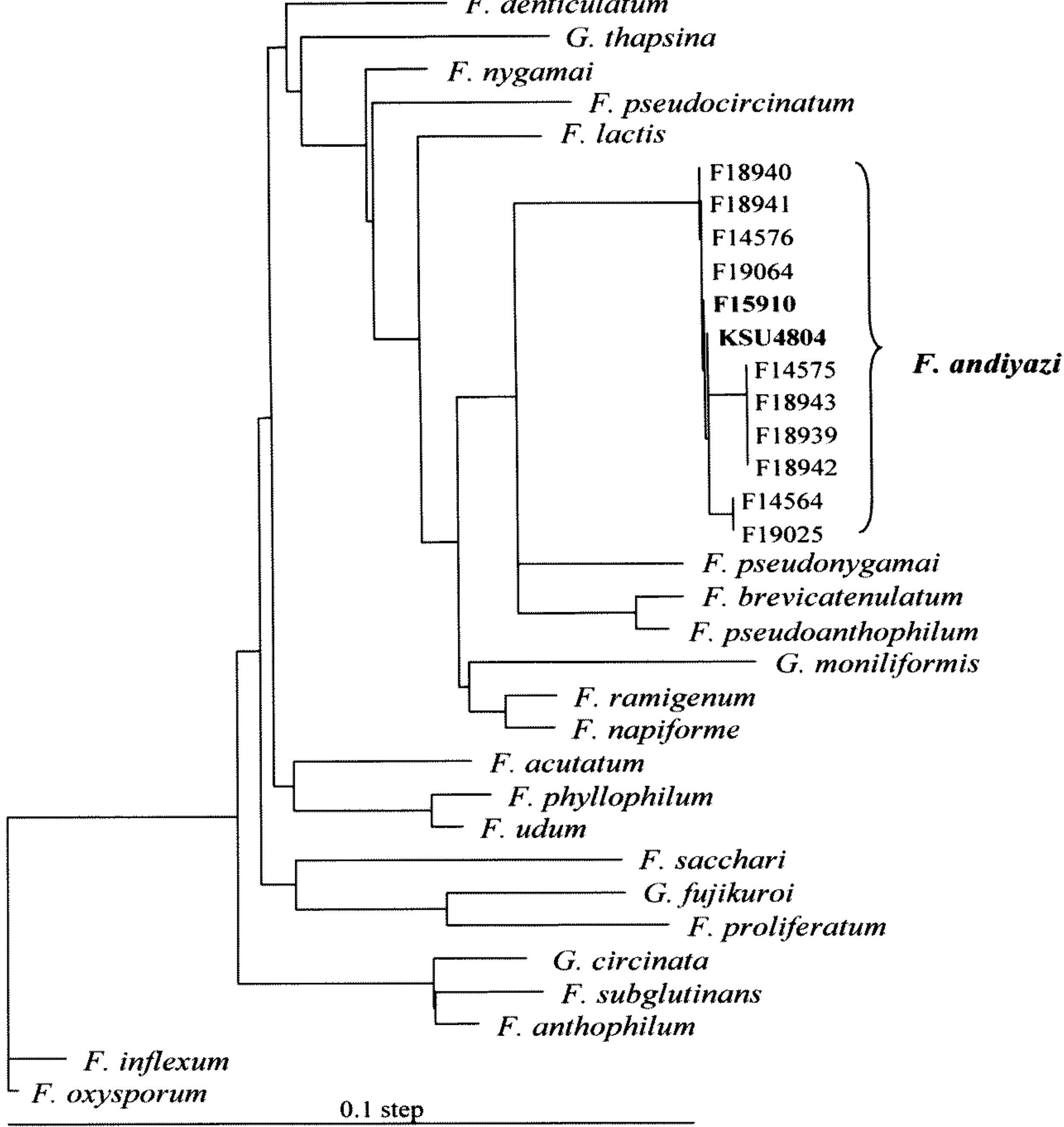


Figure 6-9 The neighbour-joining tree generated from the TEF sequence data of *F. andiyazi* isolates (649 bp) and related species. The tree was rooted using *F. oxysporum* and *F. inflexum* as outgroup taxa. Reference strains of *F. andiyazi* are highlighted in red. The length of tree is 366 steps; CI = 0.72, RI = 0.84

The MP and ML trees were identical in topology within the *F. andiyazi* clade (Figures 6-7, 6-8), whereas the NJ tree demonstrated minor topological variations within this clade (Figure 6-9).

The position of the *F. andiyazi* clade among the other related groups was clearly resolved. *Fusarium andiyazi* is most phylogenetically related to *F. pseudonygamai, F. brevicatenulatum* and *F. pseudoanthophilum.* The monophyly of this clade is demonstrated by the MP (bootstrap value 94%) and ML trees, but not the NJ tree.

6.3.2 Characterisation of F. proliferatum

6.3.2.1 Morphological characterisation

The main morphological characters for identification of *F. proliferatum* were medium to long disordered chains of microconidia, presence of conidiogenous cells with 2-3 openings and pyriform/globose microconidia in chains or false heads (Figure 6-10C, D) especially in cultures on SNA grown in the dark at 25°C. These characters were observed under all three light/temperature conditions.

Colony morphology and pigmentation of *F. proliferatum* differed from that of *F. andiyazi, F. verticillioides* and *F. thapsinum* (Figure 6-10), with a radial growth rate of 13mm/72h at 20°C, 28.8mm/72h at 25°C and 27.8mm/72h at 30°C. Aerial mycelia were abundant, woolly, white, lilac (MHC 15B3) to greyish violet (MHC 17B3), whereas pigmentation on the reverse side was reddish white (MHC 8A2) with greyish magenta centre (MHC 13C3) (Figure 6-10A, B).

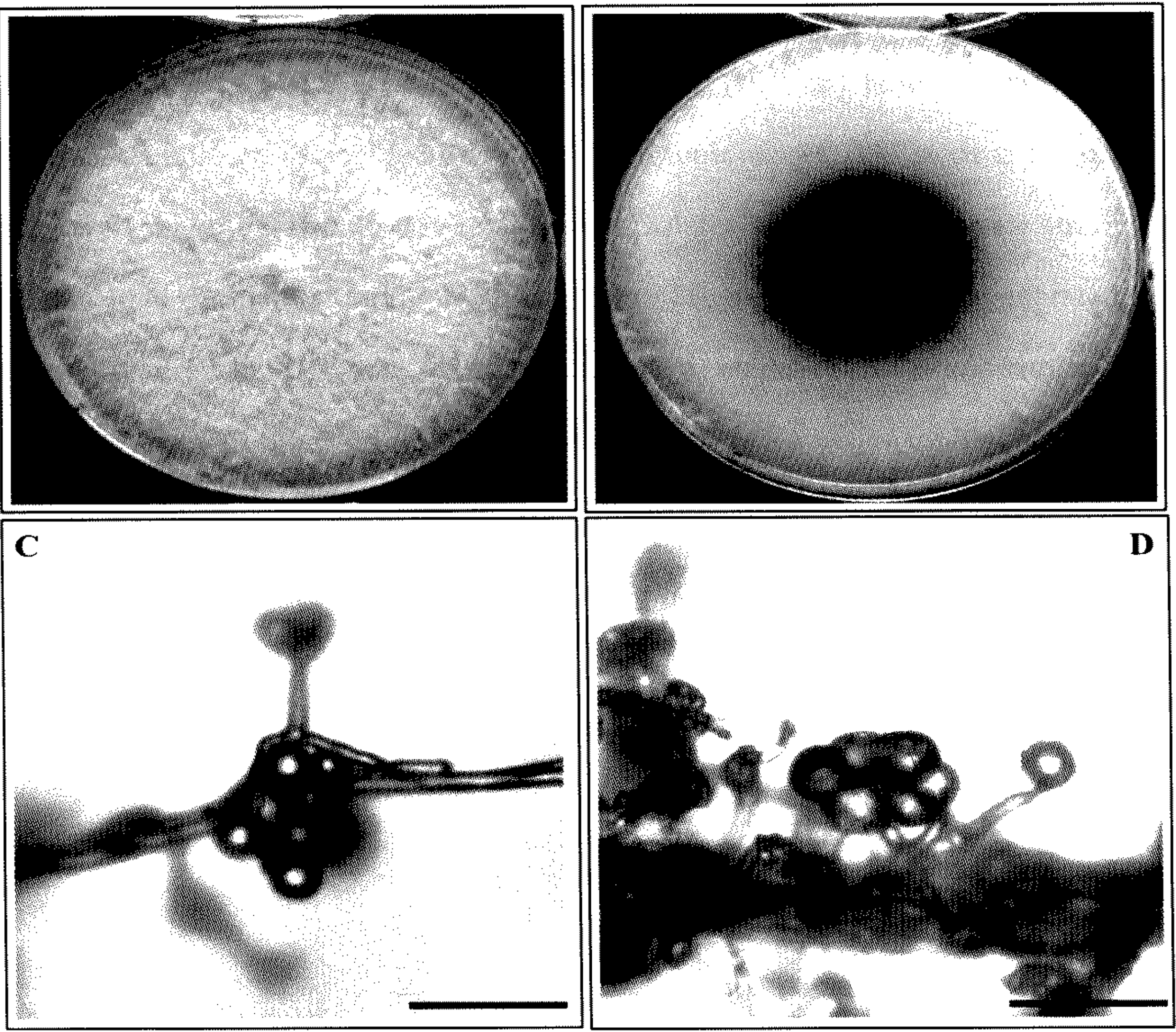


Figure 6-10 Colony morphology of nine day old cultures of *F. proliferatum* (A, B) and the clusters of pyriform microconidia on SNA in the dark at 25°C. Scale bar: C-D = 100µm

6.3.2.2 Biological characterisation

Both mating types were observed among the *F. proliferatum* isolates (five *MAT-1* and 11 *MAT-2*) (Appendix 5.1). The mating type ratio was not significantly different from 1:1 ratio ($\chi^2 = 2.31$, df = 1, P = 0.129). All isolates were male fertile with one of the *F. proliferatum* tester strains, whereas only three isolates (F15598, F15600, F15601) were female fertile in a reciprocal cross with the tester strains.

6.3.2.3 Phylogenetic characterisation

Parsimony analysis of a portion of the TEF gene (639 bp) based on 80 parsimony-informative characters, yielded one most parsimonious tree, 335 steps in length with a CI of 0.81 and a RI of 0.86. Five Australian isolates and seven reference strains of *F. proliferatum* formed a monophyletic group (Figure 6-11). Trees generated using optimality criteria ML and NJ also showed a clade of *F. proliferatum* isolates separated from all other groups (Figures 6-12, 6-13). No major topological variations were detected among trees obtained using the MP, ML and NJ optimality criteria.

The MP and ML trees were identical in topology for the *F. proliferatum* clade (Figures 6-12, 6-13), whereas the NJ tree demonstrated minor topological variations within this clade (Figure 6-14).

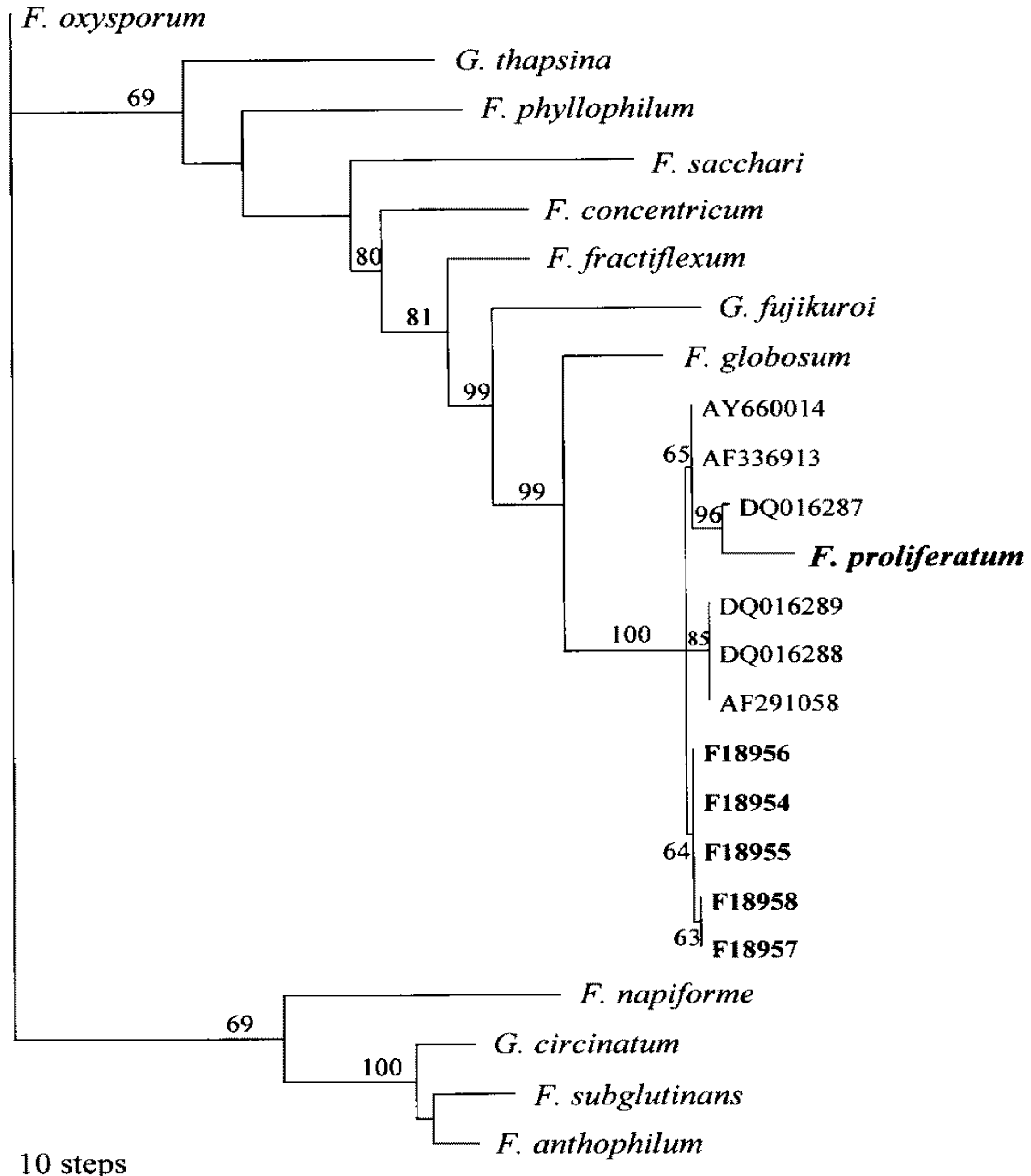


Figure 6-11 Single most parsimonious tree generated from the TEF sequence data of *F. proliferatum* isolates (639 bp) and related species. The tree was rooted using *F. oxysporum* as the outgroup taxon. Australian isolates and reference strain of *F. proliferatum* used by O'Donnell *et al.* (1998a) are highlighted in bold. The length of tree is 335 steps; CI = 0.81, RI = 0.86. Bootstrap values > 50% from 1000 maximum parsimony replicates are indicated above nodes

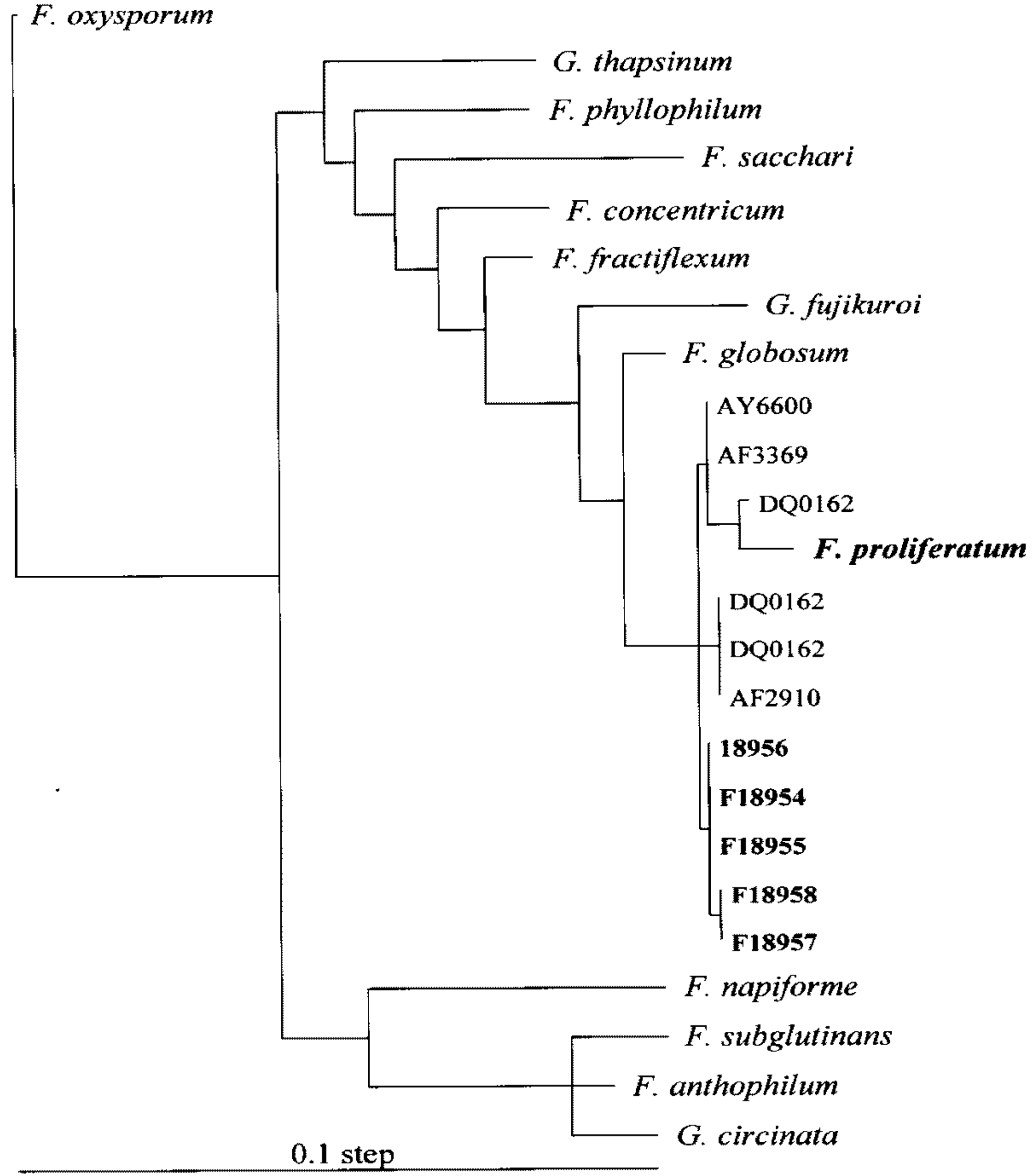


Figure 6-12 The maximum likelihood tree generated from the TEF sequence data of *F. proliferatum* isolates (639 bp) and related species. The tree was rooted using *F. oxysporum* as the outgroup taxon. Australian isolates and reference strain of *F. proliferatum* used by O'Donnell *et al.* (1998a) are highlighted in bold. The length of tree is 239 steps; CI = 0.81, RI = 0.87

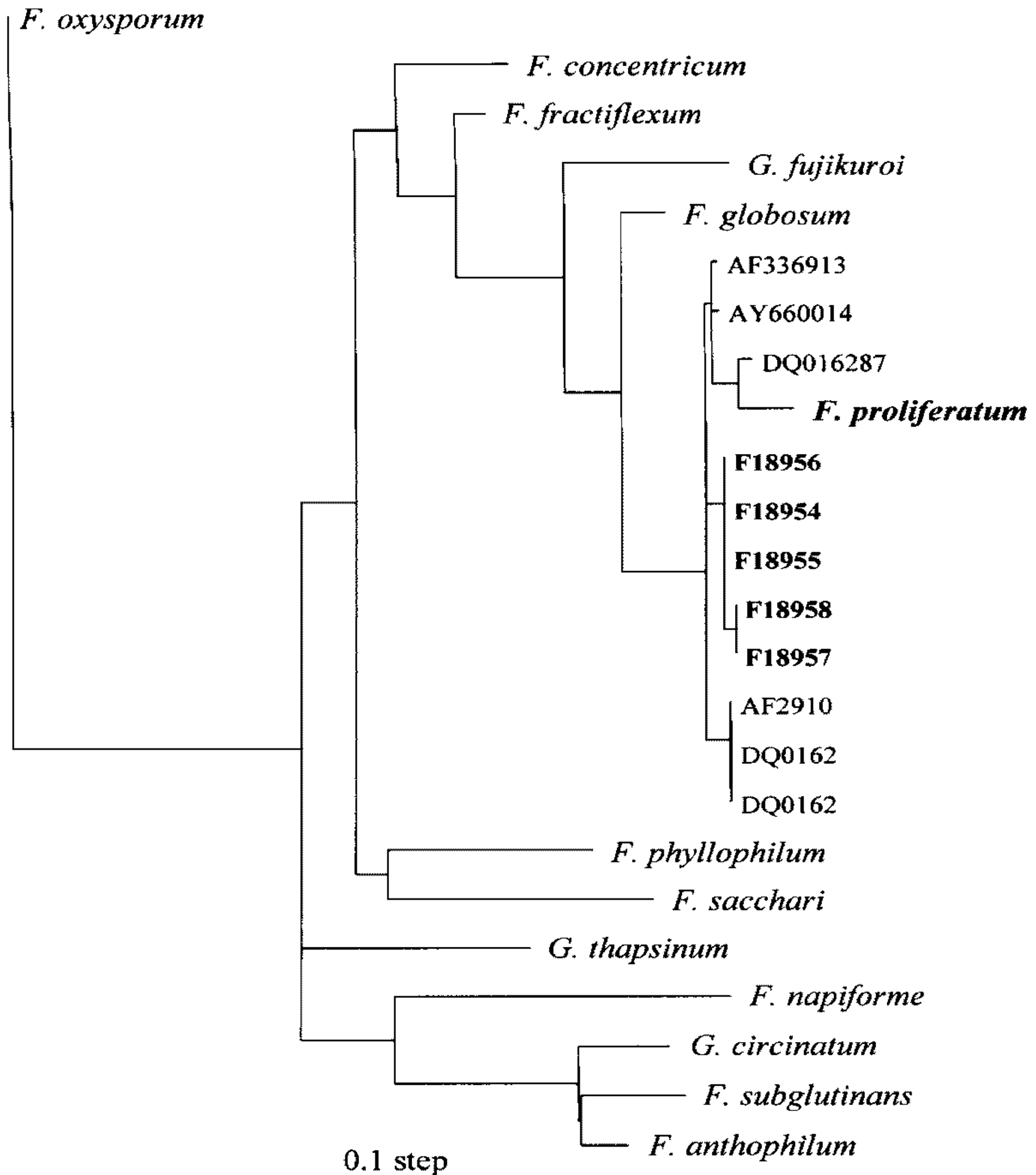


Figure 6-13 The neighbour-joining tree generated from the TEF sequence data of *F. proliferatum* isolates (639 bp) and related species. The tree was rooted using *F. oxysporum* as outgroup taxon. Australian isolates and reference strain of *F. proliferatum* used by O'Donnell *et al.* (1998a) are highlighted in bold. The length of tree is 241 steps; CI = 0.81, RI = 0.86

6.4 Discussion

This study is the first comparative analysis of morphologically similar *Fusarium* species from grain sorghum in Australia. The morphological identifications of *F. proliferatum* and three morphologically similar species, *F. andiyazi*, *F. thapsinum* and *F. verticillioides*, isolated from grain sorghum in Australia were confirmed using sexual compatibility tests, AFLP analysis and/or sequencing of a portion of the TEF gene.

The stepwise identification of isolates confirmed the validity of morphological characters used for the initial groupings of field isolates that resembling *F. moniliforme sensu lato*. The AFLPs separated isolates into three groups, with corresponded to species, in the same manner as morphological characters. The most morphologically similar species, *F. andiyazi* and *F. verticillioides*, were the least genotypically similar. However, isolates of *F. thapsinum* showed almost the same level of similarity to both of these species. The level of genotypic similarity recorded for the Australian isolates of *F. andiyazi*, *F. thapsinum* and *F. verticillioides* is in concert with previous reports of the genotypic similarity of isolates of these species from Africa (Leslie *et al.* 2005b).

The previously proposed level of similarity between conspecific isolates of *Fusarium* (≥ 65%) (Summerell *et al.* 2003) is shown to be relevant for isolates of *F. andiyazi* but not for *F. thapsinum*. The overall similarity of ≥ 58% among studied isolates and reference strains of *F. thapsinum* is supported by biological species limits (based on sexual compatibility tests). All isolates were male fertile with standard tester strains of *F. thapsinum*, which conclusively identified them as *F. thapsinum*.

The lack of standard tester strains for *F. andiyazi* precluded the biological confirmation of identification using sexual compatibility studies. Therefore,

sequencing of a portion of the TEF gene in selected isolates of *F. andiyazi* was used to confirm the AFLP results. Sequence analysis of Australian *F. andiyazi* isolates showed close phylogenetic relatedness to the holotype (KSU4804) and reference strain (F15910) of this species. Furthermore, phylogenetic analysis demonstrated that *F. andiyazi* is not phylogenetically closely related to the morphologically similar, but genotypically different species *F. thapsinum* and *F. verticillioides. Fusarium andiyazi* is phylogenetically most related to *F. brevicatenulatum*, *F. pseudoanthophilum* and *F. pseudonygamai* (bootstrap value 94%), species that share some similar morphological features. Hence, sequence analysis and AFLPs confirmed morphological identification of *F. andiyazi* isolates.

This study demonstrated that morphological features can be used as reliable diagnostic characters for the two common *Fusarium* species from sorghum, namely *F. andiyazi* and *F. thapsinum*. All of the morphological features of *F. andiyazi* reported in the original species description (Marasas *et al.* 2001) were observed in *F. andiyazi* isolates in the current study. However, "pseudochlamydospores" could not be clearly distinguished from round "thickened" hyphal cells present in *F. andiyazi* or *F. verticillioides* isolates, even in the reference strains. The longer, 2-septate clavate microconidia observed in *F. andiyazi* isolates resemble the clavate microconidia found in the isolates of *F. verticillioides* from banana (Hirata *et al.* 2001). However, their size under all conditions studied suggested that septate clavate microconidia were a striking diagnostic character for *F. andiyazi*, together with occasional germination of microconidia in chains on SNA, loosely floccose mycelia and pale orange pigmentation in young cultures on PDA.

The morphological features of the isolates of *F. thapsinum* from grain sorghum in Australia are in agreement with previous studies (Klittich *et al.* 1997; Nirenberg and

O'Donnell 1998). However, an additional, unique diagnostic character for *F. thapsinum*, pyriform/citriform microconidia, was revealed in this study. Germination of microconidia along chains was frequently observed in *F. thapsinum*, bearing new microconidia which gave rise to a 'degenerated' culture appearance under the microscope. However, germination of microconidia was occasionally observed in *F. andiyazi*. Germination of microconidia and the occasionally observed polyphialidic conidiogenous cells may be helpful diagnostic characters along with the reliable presence of pyriform/citriform microconidia.

Apart from *F. thapsinum*, there are at least three additional *Fusarium* species that produce pyriform/napiform microconidia. Two species, *F. napiforme* (Marasas *et al.* 1987) and *F. proliferatum* (Nirenberg 1976) are associated with sorghum and have a similar size range for their pyriform/citriform microconidia to *F. thapsinum*. The third species, *F. pseudoanthophilum* is associated with maize and has a notably smaller size range of pyriform microconidia (Nirenberg *et al.* 1998). Therefore, this character should be considered in combination with a set of other characters in the morphological identification of *F. thapsinum*.

Unlike sorghum, the dominant *Fusarium* species on maize is *F. verticillioides* (Leslie and Marasas 2002). Therefore, the risk of incorrect morphological identification of *F. andiyazi* and *F. verticillioides* isolates from sorghum is low. However, it is still necessary to confirm all morphologically identified isolates with at least two additional tools to ensure accurate identification.

The morphology of *F. proliferatum* does not resemble any of the other three species mentioned above. It is a well-defined morphological species (Nirenberg 1976), but the biology of this species is still incompletely understood. Partial interfertility of *F. proliferatum* (MP D) and *F. fujikuroi* (MP C) has been observed in previous studies

(Leslie *et al.* 2004a). Therefore, the identity of *F. proliferatum* isolates that were fertile with one of the two tester strains of *G. intermedia* in the present study needed to be re-confirmed using molecular phylogenetic analysis of one gene sequence (Figures 6-11, 6-12, 6-13).

The stepwise approach to identification applied in this study has allowed the resolution of isolates commonly associated with grain sorghum that might have previously been identified as *F. moniliforme sensu lato*. Hence, a polyphasic approach to species identification based on conventional and modern tools has allowed accurate and definitive characterisation of the key *Fusarium* species associated with grain sorghum in eastern Australia.

Chapter 7

Population Structure of *Fusarium thapsinum* Associated with Grain Sorghum and Grasses

7.1 Introduction

Fusarium thapsinum causes a spectrum of sorghum diseases worldwide and is mycotoxigenic, producing high levels of moniliformin, moderate levels of fusaric acid and low levels of fumonisins in sorghum (Porter *et al.* 2002). Average grain yield reduction in infected crops is about 5%, but may reach 100% in some locations (Claflin 2000). Variation in the severity and incidence of disease are attributed to the growth stage of the sorghum plant, environmental conditions, and variables such as soil fertility and drainage, cultural practices, insects and other diseases (Claflin and Giorda 2002).

The role of *F. thapsinum* population diversity in Fusarium stalk rot is not known, but it can be assumed that environmental effects and cultivation practices create selection pressure, which in turn affect the pathogens genetic structure, and subsequently the disease incidence (McDonald and Linde 2002). Low levels of genetic diversity observed in a global *F. thapsinum* population indicate that the species is relatively clonal in nature, and vegetatively propagated (Klittich and Leslie 1988; Leslie and Marasas 2002). Low levels of genetic diversity in *F. thapsinum* have been attributed to the limited role of the sexual phase and possible loss of female fertility (Leslie and Klein 1996; Leslie 1999; Leslie and Marasas 2002).

The host range of *F. thapsinum* has been shown to be limited to grain sorghum as the main host, followed by maize, banana and peanut (Klittich *et al.* 1997). Recent studies in Australia have demonstrated sporadic associations of *F. thapsinum* with weed

[*Sorghum halepense* (Lee and Walsh, *personal communication*) and *Echinochloa crus-galli* (Quazi 2005)] and native [*Austrostipa aristiglumis* (Bentley 2002), *Coix gasteenii* (Johansen 2003), *Heteropogon triticeus* (Phan 2004) and *Sorghum interjectum* (Walsh 2006)] grasses in agricultural and non-agricultural ecosystems. Agricultural ecosystems typically have stronger selection for a particular genotype(s) or pathogenically specialized, virulent population(s) compared to non-agricultural ecosystems (Lenne and Ortiz 2002). It is therefore speculated that the level of genetic similarity and diversity of *F. thapsinum* is attributed to the ecosystems of the host. Unlike non-cultivated plants, cultivated plants are genetically, numerically and spatially uniform, and are subject to regular human disturbance. It is possible that native and weed grasses play a role in the incidence and severity of Fusarium stalk rot of cultivated sorghum, as is documented in other pathosystems, *i.e.* grain sorghum – *Cercospora sorghi* (Okori *et al.* 2004), cotton (*Gossypium hirsutum*) – *Fusarium oxysporum* f.sp. *vasinfectum* (Wang *et al.* 2004), potato (*Solanum tuberosum*) – *Phytophthora infestans* (Flier *et al.* 2003).

The global distribution of *F. thapsinum* in diverse ecosystems may suggest a subdivision of populations, their adaptation to these ecosystems and perhaps incipient speciation as a process of a delineating existing species into new ones, especially in a situation of reproductive isolation. Hence, documenting of the current population genetic structure of *F. thapsinum* may reveal evolutionary signals in the population and help to predict the evolutionary potential of this species. Knowledge of population genetic structure and the evolution of a species can also increase understanding of how virulence emerges and spreads among local populations, and may have practical applications in disease control (Bull 1994).

The aim of this study was to characterise *F. thapsinum* populations in Australia through (1) determination of the genetic structure at various spatial levels including assessment of the inbreeding potential, (2) determination of the level of genetic diversity in *F. thapsinum* isolates from agricultural and non-agricultural ecosystems, and (3) determination of the level of genetic diversity of *F. thapsinum* from Australia and four other continents. These comparisons will give an insight into the overall relatedness of *F. thapsinum* populations. In practice, this knowledge may allow the development of more targeted disease management programs for Fusarium stalk rot.

7.2 Materials and Methods

7.2.1 Isolates examined

Isolates of *F. thapsinum* recovered from grain sorghum in Australia (Chapters 4 and 5), weed and native grasses in Australia, and grain sorghum from Mexico, the Republic of South Africa, South Korea and the USA (Texas) were included in this study (Figure 7-1).

Plant part level population comparison – Plant part level comparisons were based on 184 isolates recovered from roots/mesocotyls, crowns and the fourth/sixth node of sorghum stalks (Appendix 6.1). Isolates were recovered from plots F, M and S at Livingston Farm, Moree.

Populations were mainly composed of individual isolates derived from one plant part. Where two or three isolates were recovered from the same plant part, the additional isolates were also included.

Plot level population comparison – Plot level comparisons were conducted on the same set of isolates as the plant part levels of comparison, but were grouped on the basis of the plots from which the isolates were recovered (Appendix 6.1).

Geographic level population comparison - Three geographic *F. thapsinum* populations were obtained from physiologically mature grain sorghum plants collected from Moree, Goondiwindi and Quirindi in the 2002/2003 growing seasons (Figure 7-1). Isolates recovered from sorghum plants from three sites each at Goondiwindi and Quirindi represented the *F. thapsinum* populations from these geographic areas (Chapter 5). The set of isolates from stalks used in the plant part comparisons was also used in this study, representing the Moree population.

The geographic level comparisons were conducted on 203 isolates from stalks (Moree – 76 isolates, Goondiwindi – 78 isolates and Quirindi – 49 isolates). The populations comprised isolates either from the fourth/sixth node (Moree population) (Appendix 6.1) or the fourth node (Goondiwindi and Quirindi populations) (Appendix 6.2).

Ecosystem level isolate comparison - A collection of 51 *F. thapsinum* isolates from grain sorghum, weed and native grasses were included in this study. Of the 51 isolates, 33 were from agricultural ecosystems in NSW and the remaining 18 isolates were from non-agricultural ecosystems in NSW, QLD and the NT (Figure 7-1).

Isolates from agricultural ecosystems comprised isolates from grain sorghum (12 isolates randomly selected from the Moree, Goondiwindi and Quirindi population), *Sorghum halepense* (20 isolates) and *Echinochloa crus-galli* (one isolate). Isolates from non-agricultural ecosystems comprised isolates from the native grasses *Austrostipa aristiglumis* (six isolates), *Coix gasteenii* (nine isolates), *Heteropogon triticeus* (one isolate) and *Sorghum interjectum* (two isolates) (Appendix 6.3).

Isolates from weed (Lee and Walsh, *personal communication*; Quazi 2005) and native grasses (Bentley 2002; Johansen 2003; Phan 2004; Walsh 2006) were collected during intensive ecological studies of *Fusarium* species associated with these grasses.

Country/continent level isolate comparison - A collection of 35 *F. thapsinum* isolates from Australia (12), Mexico (ten isolates), the Republic of South Africa (eight isolates), South Korea (two isolates) and the USA (three isolates) were included in this study. The isolates were obtained from research collections, being originally recovered from grain, root and stalk tissue of grain sorghum (Figure 7-1; Appendix 5.2). Twelve isolates of *F. thapsinum* from the geographic and plot level comparisons in the northern grain belt of eastern Australia were selected as representatives of *F. thapsinum* population from Australia (Appendices 6.1 and 6.2).

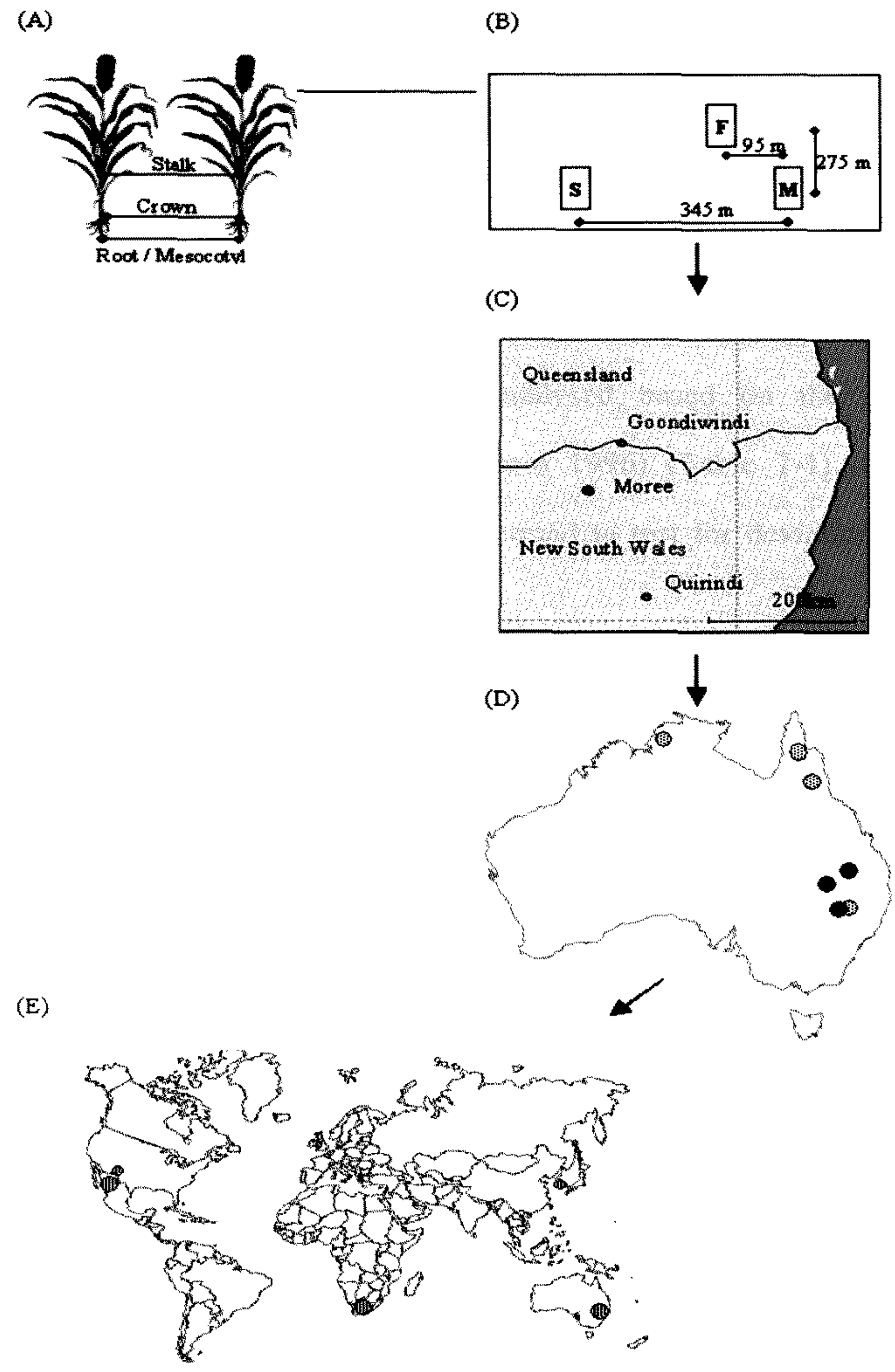


Figure 7-1 Levels of comparisons of *F. thapsinum* populations. Three plant part composite populations from three plots (A). Three plot populations (F, M, S) from Livingston Farm, Moree (B). Three geographic populations from grain sorghum in the northern grain belt of eastern Australia (Moree, Goondiwindi and Quirindi populations) (C). Comparison of *F. thapsinum* isolates from agricultural (black dots) and non-agricultural ecosystems (shaded) in Australia (not to scale) (D). Collection of isolates from five countries - Australia, Mexico, the Republic of South Africa, South Korea and the USA (Texas) (not to scale) (E)

7.2.2 Sexual compatibility tests

The mating type of all isolates was determined using the previously described specific PCR technique (*see* Chapter 3). Isolates of *F. thapsinum* were paired with the opposite mating type standard tester strains of *Gibberella thapsina* (MP F) (KSU4093, KSU4094) in both male and female combinations as described in Chapter 3. All crosses were repeated at least once.

Results of sexual compatibility tests were analysed based on the population parameters for haploid species (Leslie and Klein 1996) (Table 7-1). Chi-square goodness of fit based on ML method was performed to test for deviation from 1:1 mating type ratio in GenStat version 8.1.

Table 7-1 Equations for population parameters of haploid species (Leslie and Klein 1996)

Equation	Definition
Effective population number	
Mating type:	
$N_{e(mt)} = \frac{(4N_m N_f)}{(N_m + N_f)} \times 100$	N_m: number of strains with one mating type allele
	N_f: number of strains with the other mating type allele
Female fertility:	N: total number of strains in the population
$N_{e(f)} = \frac{(4N^2 N_h)}{(N + N_h)^2} \times 100$	N_h: number of hermaphrodite strains in the population
Mean number of mutations per strain	
$e^{-M}\left[M^i / i!\right]$	i: number of mutations in a given class of strains
$e^{-M} = f_{S_o}$ $M = \left\{\frac{\log\left(\frac{1}{f_{So}}\right)}{(\log e)}\right\}$	f_{So}: frequency of hermaphrodites before sexual reproduction (or minimum hermaphrodites)
Length and range in hermaphrodite frequencies for equilibrium cycle	
Observed hermaphrodites	
$f_{S_o} = \frac{N_h}{N}$	
Maximum hermaphrodites	
$h_a = \sqrt{f_{S_o}}$	
Minimum hermaphrodites	
$f_{S_{o(min)}} = \frac{1}{1 - 1 - mf_{S_o}}$	
Time in asexual / sexual generations	
$h_a = f_{S_o}[(1-\mu)\theta]^i$ $i = \frac{\log h_a}{\log\{f_{S_o}[(1-\mu)\theta]\}}$	$(1-\mu)\theta = 0.98, 0.99, 0.999$
	μ: mutation rate from female fertility to female sterility
	θ: selective disadvantage of hermaphrodites during vegetative propagation

7.2.3 AFLP protocol and population genetic analysis

The AFLP fingerprinting protocol was performed as described by Zeller *et al.* (2000) and described in Chapter 3. A primer pair, *Eco*RI+GG and *Mse*I+CT, was used to generate AFLP fingerprints for *F. thapsinum* isolates. AFLP bands were manually scored as either 1 (presence of a band) or 0 (absence of a band) between 84 and 500 bp.

Genetic variation within and between *F. thapsinum* populations, and the presence of gene flow among populations were analysed using the software packages POPGENE version 1.31 (Yeh *et al.* 1999) and TFPGA version 1.3 (Miller 1999). Genetic variation was measured by Nei's gene diversity *(h)* (Nei 1987). Wright's F_{ST}, as estimated by theta (θ), was calculated using the method described by Weir and Cockerham (1984), which allows for unequal sample sizes. The statistical significance of Nei's gene diversity *(h)* was estimated using an unpaired t-test in GenStat version 8.1.

Estimates of gene flow (Nm) among populations were calculated based on $\theta(F_{ST})$ (Slatkin and Barton 1989). The unbiased genetic distances between populations (D) and genetic identity (*I*) (Nei 1987) were calculated in TFPGA. The Pearson correlation analysis between genetic distances and geographic distances were performed in the Analyse-it™ software, Ltd. for Excel.

The genetic similarity between populations was analysed using NTSYSpc version 2.10q (Applied Biostatistics, Inc., Setauket, NY, USA). Genetic similarities for all possible pairs of isolates were calculated using the DICE coefficient and dendrograms were constructed from the similarity coefficient data with UPGMA. Bootstrapping with 1000 replications was performed on the binary data matrix using PAUP version

4.0b10 (Swofford 1998) in order to test the confidence of the branching pattern in dendrograms.

7.3 Results

7.3.1 Structure of three **F. thapsinum** *plant part populations*

7.3.1.1 Genetic structure

Genetic diversity - A total of 110 loci were analysed for the three plant part populations, of which 95 were polymorphic. The number of polymorphic loci for each population ranged from 91 (root/mesocotyl and stalk populations) to 94 (crown population). UPGMA cluster analysis indicated that each isolate examined had a unique haplotype, clustering together within the range of 67-98% DICE similarity (Figure 7-2). The haplotypes did not cluster according to the plant part from which they were isolated. The highest level of similarity (98%) was observed between haplotypes from different plant parts, whereas haplotypes from the same plant part were ≤ 93% similar.

Within populations, gene diversity values *(h)* ranged from 31% (root/mesocotyl and stalk populations) to 33% (crown population) (Table 7-2). No significant differences in gene diversity were recorded between any of the populations.

Table 7-2 The number of polymorphic loci and gene diversity values for *F. thapsinum* plant part populations based on 110 AFLP loci

Population	Number of polymorphic loci	Nei's gene diversity *(h)*
Root/Mesocotyl	91	0.31
Crown	94	0.33
Stalk	91	0.31
Overall	95	0.32

Population differentiation - The coefficient of gene differentiation $\theta(F_{ST})$ was 0.006, indicating a low level of differentiation between the *F. thapsinum* plant part

populations. The F_{ST} value indicated that about 0.6% of the variation was distributed among populations and 99.4% was distributed within populations. Pairwise $\theta(F_{ST})$ values among populations ranged between -0.00 and 0.02 (Table 7-3), indicating low levels of differentiation between plant part populations. Negative pairwise $\theta(F_{ST})$ values indicate that the isolates between the root/mesocotyl and stalk populations are more genetically similar than isolates within these populations (Zhan and McDonald 2005). High genetic similarity was detected between isolates for all plant part populations.

The indirect estimate of gene flow (Nm) based on $\theta(F_{ST})$ was very high (Table 7-3).

Table 7-3 Population pairwise $\theta(F_{ST})$ of *F. thapsinum* plant part populations (below diagonal) and estimate of gene flow among populations (Nm) (above diagonal)

Population	Root/Mesocotyl	Crown	Stalk
Root/Mesocotyl	*	32.40	-[†]
Crown	0.02	*	85.71
Stalk	-0.00	0.01	*

[†]Negative value cannot be used to estimate the amount of gene flow (Zhan and McDonald 2005)

Genetic relatedness among F. thapsinum *populations* - Nei's unbiased genetic distance (D) among populations was very low [0.00 (root/mesocotyl – stalk populations) and 0.01 (root/mesocotyl – crown and crown – stalk populations], indicating that plant part populations were genetically very similar (Table 7-4). Almost identical alleles were shared among the three plant part populations as demonstrated by Nei's unbiased genetic identity (*I*).

Table 7-4 Nei's unbiased genetic distance (D) (below diagonal) and genetic identity (*I*) (above diagonal) for the plant part populations

Population	Root/Mesocotyl	Crown	Stalk
Root/Mesocotyl	*	0.99	1.00
Crown	0.01	*	0.99
Stalk	0.00	0.01	*

7.3.1.2 Sexual compatibility test

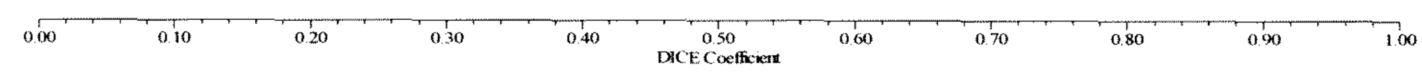
The mating type ratio for the three *F. thapsinum* plant part populations was not significantly different to 1:1. The mating type distribution in both the root/mesocotyl and crown populations was *Ne(mt)* of 99.9%, whilst the mating type distribution in the stalk population was slightly lower, *Ne(mt)* of 95.6% (Table 7-5).

The number of female fertile isolates was low in comparison to male fertile isolates in all three populations. The observed frequency of female fertile isolates ranged from 6.6% (stalk population) to 9.8% (root/mesocotyl population) (Table 7-5). Consequently, effective population size based on female fertility [*Ne(f)*] was low, ranging from 23.2% (stalk population) to 32.6% (root/mesocotyl population) (Table 7-5). However, female fertility was evenly distributed among all plant part populations. The average number of asexual generations per sexual generation was highest in the stalk population (135.4 to 270.8), followed by the crown (122.6 to 245.1) and root/mesocotyl (115.4 to 230.8) populations.

Table 7-5 Biological traits, effective population size parameters, length and range of female fertile frequencies for equilibrium cycles based on the observed frequency of female fertile strains in *F. thapsinum* plant part populations

Parameters		Populations			
		Root/Mesocotyl	Crown	Stalk	Combined
Mating type ratio	*MAT-1* : *MAT-2*	31 : 30	24 : 23	46 : 30	101 : 83
Female fertility	Male : Female	55 : 6	43 : 4	71 : 5	169 : 15
Effective population number	*Ne(mt)*[a]	99.9	99.9	95.6	99.0
	Ne(f)[b]	32.6	28.9	23.2	27.9
	M[c]	2.3	2.5	2.7	2.5
Observed female fertile isolates		0.098	0.085	0.066	0.082
Maximum female fertile isolates		0.314	0.292	0.257	0.286
Number of asexual generations per sexual generation based on maximum female fertile isolates					
	0.98	57.4	60.9	67.3	62.0
Time	**0.99**	**115.4**	**122.6**	**135.4**	**124.7**
	0.999	1159	1231	1360	1253
Minimum female fertile isolates		0.010	0.007	0.004	0.007
Number of asexual generations per sexual generation based on minimum female fertile isolates					
	0.98	114.8	121.9	134.7	124.1
Time	**0.99**	**230.8**	**245.1**	**270.8**	**249.4**
	0.999	2318	2463	2720	2506

[a]*Ne(mt)* - Effective population number based on mating type; [b]*Ne(f)* - Effective population number based on female fertility; [c]M - Mean number of female sterility mutations per strain



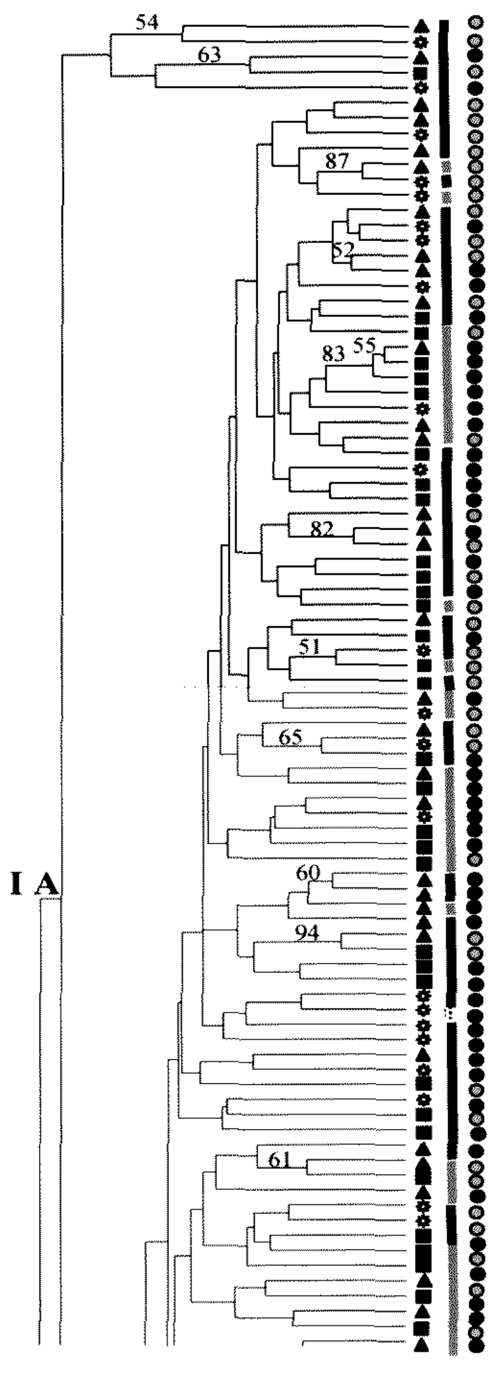


Figure 7-2 UPGMA dendrogram of 184 isolates of *F. thapsinum* showing similarities among isolates of plant part populations (root/mesocotyl - ▲, crown - ☼, stalk - ■), plot populations (F - black line, M - gray line, S - dotted line) and mating type distribution (*MAT-1* – black dot, *MAT-2* – gray dot). Only branches occurring in 50% or more of the bootstrapped dendrograms are shown

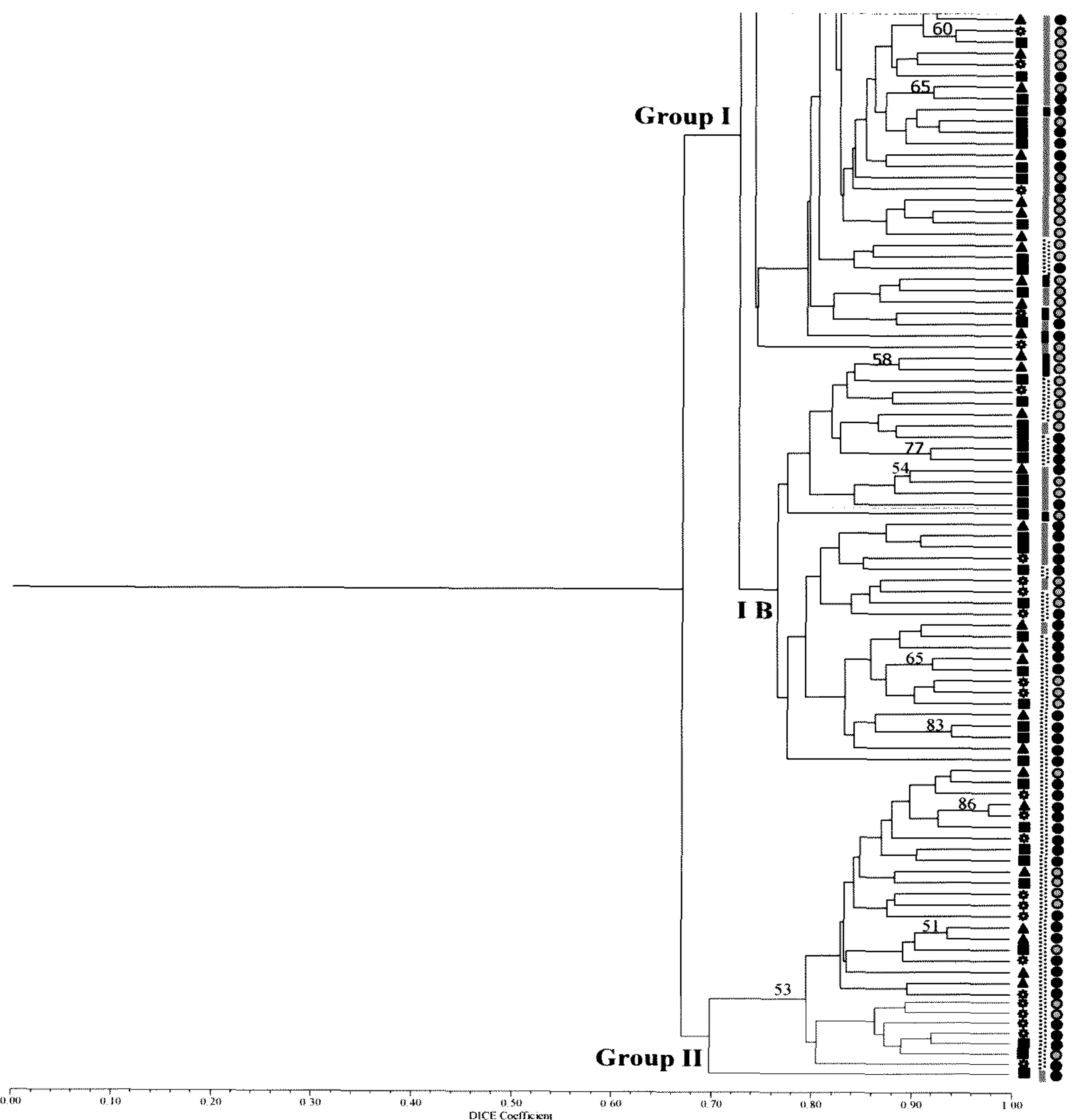


Figure 7-2 (*Continued*)

7.3.2 Structure of three **F. thapsinum** *plot populations*

7.3.2.1 Genetic structure

Genetic diversity - A total of 110 loci were analysed for three plot populations, of which 95 were polymorphic. The number of polymorphic loci for each population ranged from 84 (plot F population) to 89 (plot S population). UPGMA cluster analysis indicated that each isolate examined had a unique haplotype, clustering together within the range of 67-98% DICE similarity (Figure 7-2). Two major groups of haplotypes were observed within the dendrogram (Group I and Group II). Group I consisted of two subgroups that comprised mainly haplotypes from plot populations F and M (sub-group IA) and M and S (sub-group IB). Group II consisted exclusively of plot S haplotypes, with one outlying plot M haplotype. DICE genetic similarity within sub-groups IA and IB was ≥ 76% and 77%, respectively. DICE genetic similarity within Group II was ≥ 70% with similarity of ≥ 80% for plot S haplotypes. However, there was only significant bootstrap support for the clustering of haplotypes within Group II (53%).

Within populations, gene diversity values *(h)* ranged from 25% (plot F and S) to 27% (plot M) (Table 7-6). There were no significant differences in gene diversity between plot populations.

Table 7-6 The number of polymorphic loci and gene diversity values for *F. thapsinum* plot populations based on 110 AFLP loci

Population	Number of polymorphic loci	Nei's gene diversity *(h)*
Plot F	85	0.25
Plot M	84	0.27
Plot S	89	0.25
Overall	95	0.32

Population differentiation - The coefficient of gene differentiation $\theta(F_{ST})$ was 0.25, indicating great differentiation of *F. thapsinum* plot populations. The F_{ST} value indicated that about 25.2% of the variation was distributed among populations and 74.8% was distributed within populations. Pairwise $\theta(F_{ST})$ values among populations ranged from 0.09 to 0.36 (Table 7-7), indicating a great degree of differentiation between the plot S population and the other two populations.

The pairwise indirect estimate of gene flow (Nm) based on $\theta(F_{ST})$ was the highest between the genetically similar populations from plot F and M (Table 7-7). However, the estimate of gene flow between the plot S and F populations (Nm = 0.89) was less than 1, indicating a distinct potential for genetic differentiation to occur between these two populations.

Table 7-7 Population pairwise $\theta(F_{ST})$ of *F. thapsinum* plot populations (below diagonal) and estimate of gene flow among populations (Nm) (above diagonal)

Population	Plot F	Plot M	Plot S
Plot F	*	5.09	0.89
Plot M	0.09	*	1.25
Plot S	0.36	0.25	*

Genetic relatedness among F. thapsinum *populations* - Nei's genetic distance (D) between populations varied from 0.04 for the most genetically similar plot F and M populations, to 0.22 for the least similar plot F and S populations (Table 7-8). Nei's unbiased genetic identity (*I*) demonstrated that most alleles were shared between the plot F and M populations (0.96) (Table 7-8).

Physical distance and Nei's unbiased genetic distance between the plot populations were correlated (R = 0.71), indicating that physical distance between the plot populations might be one of the determinants of genetic distance and might influence gene/genotype flow between the plot S and the other plot populations.

Table 7-8 Nei's unbiased genetic distance (D) (below diagonal) and genetic identity (*I*) (above diagonal) for the plot populations

Population	Plot F	Plot M	Plot S
Plot F	*	0.96	0.81
Plot M	0.04	*	0.85
Plot S	0.22	0.16	*

7.3.2.2 Sexual compatibility

The mating type ratio in the plot F and M populations was not significantly different from 1:1, whereas the ratio in the plot S population was skewed, with a predominance of *MAT-1* isolates (Table 7-9). Even distribution of mating types in the plot F and M populations gave *Ne(mt)* of 100%, whereas *Ne(mt)* in the plot S population was 91.8%.

The number of female fertile isolates was low compared to male fertile isolates in all three populations. The observed frequency of female fertile isolates ranged from 4.8% (plot F population) to 12.1% (plot M population) (Table 7-9). Consequently, effective population size based on female fertility [*Ne(f)*] was relatively low, ranging from 17.6% (plot F population) to 38.6% (plot M population) (Table 7-9). Female fertility was unevenly distributed among three plot populations ($\chi^2 = 8.23$, df = 2, P = 0.02).

The average number of asexual generations per sexual generation was highest in the plot F population (150.7 to 301.3), followed by the plot S (131.3 to 262.6) and plot M (104.9 to 209.9) populations.

Mutation rate at which female fertility is lost was uneven across the populations plots, ranging from 2.1 (plot M) to 3.0 (plot F) mutations per genome.

Table 7-9 Biological traits, effective population size parameters, length and range of female fertile frequencies for equilibrium cycles based on the observed frequency of female fertile strains in *F. thapsinum* plot populations

Parameters		Populations			
		Plot F	Plot M	Plot S	Combined
Mating type ratio	*MAT-1* : *MAT-2*	31 : 31	34 : 32	36 : 20	101 : 83
Female fertility[d]	Male : Female	59 : 3	58 : 8	52 : 4	169 : 15
Effective population number	*Ne(mt)*[a]	100.0	99.9	91.8	99.0
	Ne(f)[b]	17.6	38.6	24.9	27.9
	M[c]	3.0	2.1	2.6	2.5
Observed female fertile isolates		0.048	0.121	0.071	0.082
Maximum female fertile isolates		0.220	0.348	0.267	0.286
Number of asexual generations per sexual generation based on maximum female fertile isolates					
	0.98	75.0	52.2	65.3	62.0
Time	**0.99**	**150.7**	**104.9**	**131.3**	**124.7**
	0.999	1513	1055	1319	1253
Minimum female fertile isolates		0.02	0.015	0.005	0.007
Number of asexual generations per sexual generation based on minimum female fertile isolates					
	0.98	149.9	104.5	130.6	124.1
Time	**0.99**	**301.3**	**209.9**	**262.6**	**249.4**
	0.999	3027	2109	2638	2506

[a]*Ne(mt)* - Effective population number based on mating type; [b]*Ne(f)* - Effective population number based on female fertility; [c]M - Mean number of female sterility mutations per strain
[d]Significantly different at $P < 0.05$; df = 2

7.3.3 Structure of three F. thapsinum *geographic populations*

7.3.3.1 Genetic structure

Genetic diversity - A total of 110 loci were analysed for the three geographic populations, of which 94 were polymorphic. The number of polymorphic loci for each population ranged from 78 (Quirindi population) to 90 (Moree population). UPGMA cluster analysis indicated that each isolate examined had a unique haplotype, clustering together within the range of 69-99% DICE similarity (Figure 7-3). Haplotypes from the Goondiwindi and Quirindi populations were more similar than haplotypes from the Moree population. Thirty-two isolates from the Moree population and one isolate from the Quirindi population were grouped into a cluster that was

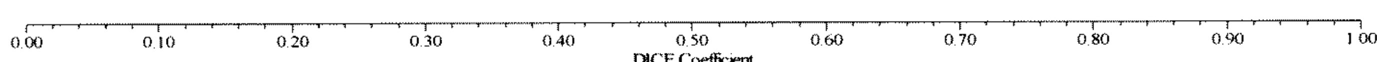
distinct from the cluster made up of the remaining isolates from the Moree, Goondiwindi and Quirindi populations (Figure 7-3). However, there was no bootstrap support for this clustering.

Within populations, gene diversity values *(h)* ranged from approximately 22% (Goondiwindi and Quirindi populations) to 29% (Moree population) (Table 7-3). Significant differences in gene diversity were found between the Moree and Goondiwindi populations (t = -2.33, df = 218, P = 0.02) and the Moree and Quirindi populations (t = -2.53, df = 218, P = 0.01) (Table 7-10).

Table 7-10 The number of polymorphic loci and gene diversity values for *F. thapsinum* geographic populations based on 110 AFLP loci

Population	Number of polymorphic loci	Nei's gene diversity *(h)*
Moree	90	0.29†
Goondiwindi	82	0.23
Quirindi	78	0.22
Combined	94	0.30

†Significantly different from Goondiwindi and Quirindi populations, at $P < 0.05$; df = 218



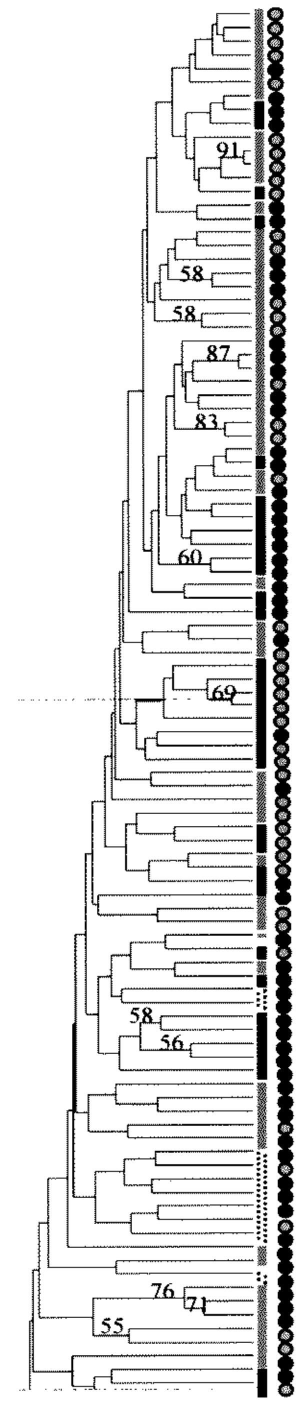


Figure 7-3 UPGMA dendrogram of 203 isolates of *F. thapsinum* showing similarities among isolates of Moree (dotted line), Goondiwindi (gray line) and Quirindi (black line) populations and mating type distribution (*MAT-1*- black dot, *MAT-2* – gray dot). Only branches occurring in 50% or more of the bootstrapped dendrograms are shown

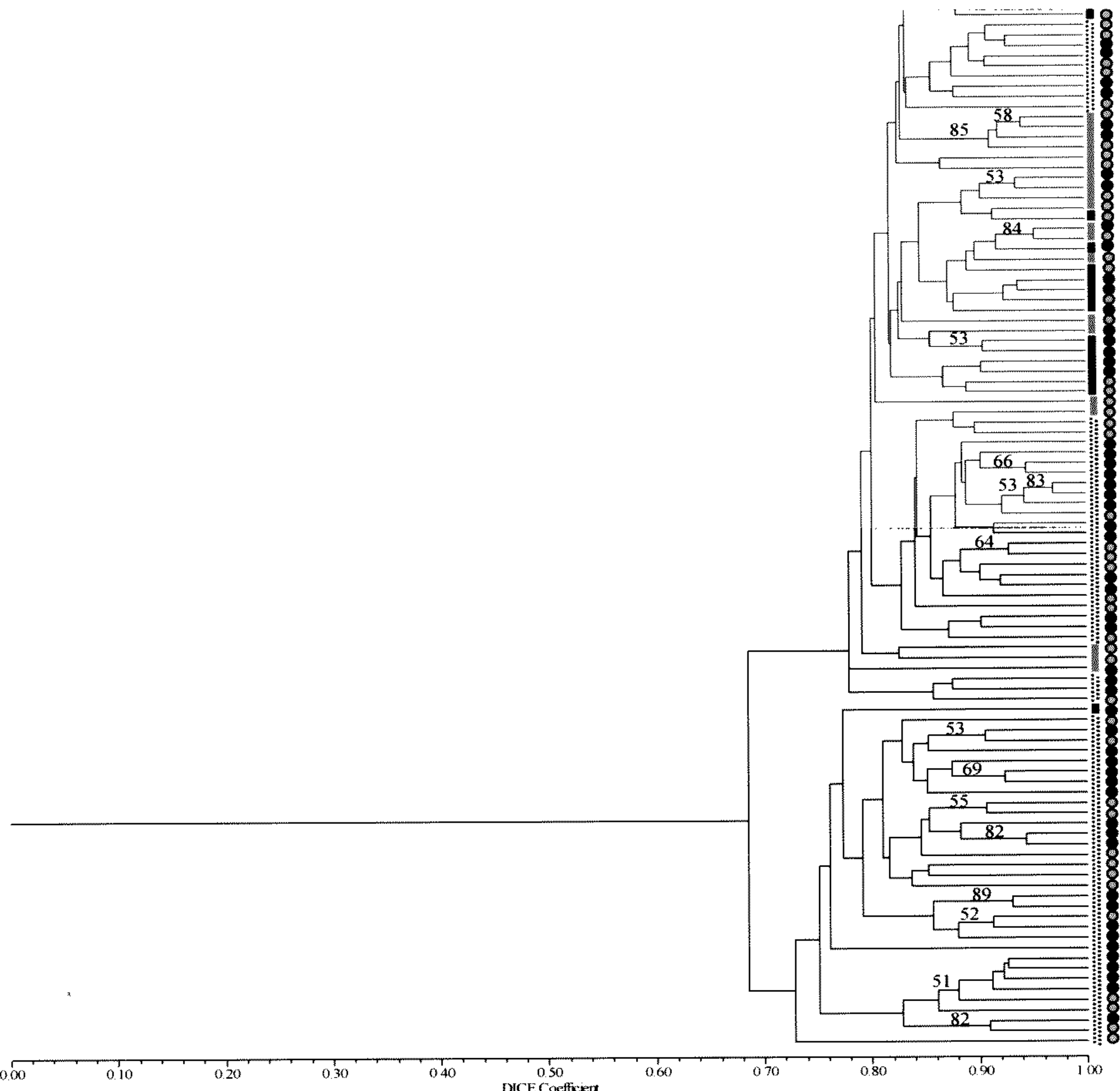


Figure 7-3 (*Continued*)

Population differentiation - The coefficient of gene differentiation $\theta(F_{ST})$ was 0.22, indicating great differentiation of geographic *F. thapsinum* populations. The F_{ST} value indicated that about 22% of the variation was distributed among populations and 78% was distributed within populations. Pairwise $\theta(F_{ST})$ values among populations ranged from 0.06 (Goondiwindi and Quirindi populations) and 0.28 (Goondiwindi and Moree populations) (Table 7-11), indicating a great degree of differentiation between the Moree population and the two other populations.

The pairwise indirect estimate of gene flow (Nm) based on $\theta(F_{ST})$ was highest between the Goondiwindi and Quirindi populations, suggesting extensive gene flow between these populations (Table 7-11). There was also enough gene flow occurring between the Moree and the other two populations ($Nm > 1$) to overcome genetic drift and delay or stop genetic differentiation (Wright 1978).

Table 7-11 Population pairwise $\theta(F_{ST})$ of *F. thapsinum* geographic populations (below diagonal) and estimate of gene flow among populations (Nm) (above diagonal)

Population	Moree	Goondiwindi	Quirindi
Moree	*	1.31	1.53
Goondiwindi	0.28	*	7.53
Quirindi	0.25	0.06	*

Genetic relatedness among F. thapsinum *populations* - Nei's unbiased genetic distance (D) between populations ranged from 0.02 for the most genetically similar Goondiwindi and Quirindi populations, to 0.14 for the least similar Moree and Goondiwindi populations (Table 7-12). Nei's unbiased genetic identity (*I*) demonstrated that most alleles were shared between the Goondiwindi and Quirindi populations (0.98) (Table 7-12).

Geographic distance and Nei's unbiased genetic distance between populations were not correlated ($R = -0.79$), indicating that geographic distance between populations is

not a key determinant of genetic distance, and that geographic proximity is not the main factor determining whether gene/genotype flow will occur between populations.

Table 7-12 Nei's unbiased genetic distance (D) (below diagonal) and genetic identity (*I*) (above diagonal) for the geographic populations

Population	Moree	Goondiwindi	Quirindi
Moree	*	0.87	0.88
Goondiwindi	0.13	*	0.98
Quirindi	0.14	0.02	*

7.3.3.2 Sexual compatibility

The mating type distribution in the Moree, Goondiwindi and Quirindi populations was not statistically different from 1:1. The Goondiwindi population had the most even distribution of mating-types, giving *Ne(mt)* of 99.4%, whereas *Ne(mt)* for the Moree and Quirindi populations was ≤ 95.6% (Table 7-13).

The number of female fertile isolates was low in comparison to male fertile isolates in all three populations. Observed frequency of female fertile isolates ranged from 3.9% (Goondiwindi population) to 6.6% (Moree population) (Table 7-13). Consequently, effective population size based on female fertility [*Ne(f)*] ranged from 14.3% (Goondiwindi population) to 23.2% (Moree population) (Table 7-13). However, female fertility was evenly distributed among all *F. thapsinum* populations ($\chi^2 = 2.42$, df = 2, P = 0.30).

The average number of asexual generations per sexual generation was the highest in the Goondiwindi population (162 to 324), followed by the Quirindi (139 to 278) and Moree (135.4 to 270.8) populations.

The mutation rate at which female fertility is lost was similar in all three populations, ranging from 2.7 (Moree) to 3.3 (Goondiwindi).

Table 7-13 Biological traits, effective population size parameters, length and range of female fertile frequencies for equilibrium cycles based on the observed frequency of female fertile strains in *F. thapsinum* geographic populations

Parameters		Populations			
		Moree	Goondiwindi	Quirindi	Combined
Mating type ratio	*MAT-1* : *MAT-2*	46 : 30	36 : 42	31 : 18	113 : 90
Female fertility	Male : Female	71 : 5	75 : 3	46 : 3	192 : 11
Effective population number	*Ne(mt)*[a]	95.6	99.4	92.9	98.7
	Ne(f)[b]	23.2	14.3	21.8	19.5
	M[c]	2.7	3.3	2.8	2.9
Observed female fertile isolates		0.066	0.039	0.061	0.054
Maximum female fertile isolates		0.257	0.196	0.247	0.233
Number of asexual generations per sexual generation based on maximum female fertile isolates					
	0.98	67.3	80.6	69.1	72.2
Time	**0.99**	**135.4**	**162**	**139**	**145**
	0.999	1360	1628	1396	1456.9
Minimum female fertile isolates		0.004	0.002	0.004	0.003
Number of asexual generations per sexual generation based on minimum female fertile isolates					
	0.98	134.7	161.3	138.3	144.3
Time	**0.99**	**270.8**	**324**	**278**	**290.1**
	0.999	2720	3256	2792	2913.9

[a]*Ne(mt)* - Effective population number based on mating type; [b]*Ne(f)* - Effective population number based on female fertility; [c]M - Mean number of female sterility mutations per strain

7.3.4 Comparison of F. thapsinum *isolates from agricultural/non-agricultural ecosystems and different hosts*

All isolates of *F. thapsinum* clustered together within the range of 76 to 99% DICE similarity (Figure 7-4). Isolates did not cluster according to the ecosystems from which they were derived or from their host of origin. The highest similarity was among *F. thapsinum* isolates from the weed grass *S. halepense* ($\geq$ 98%).

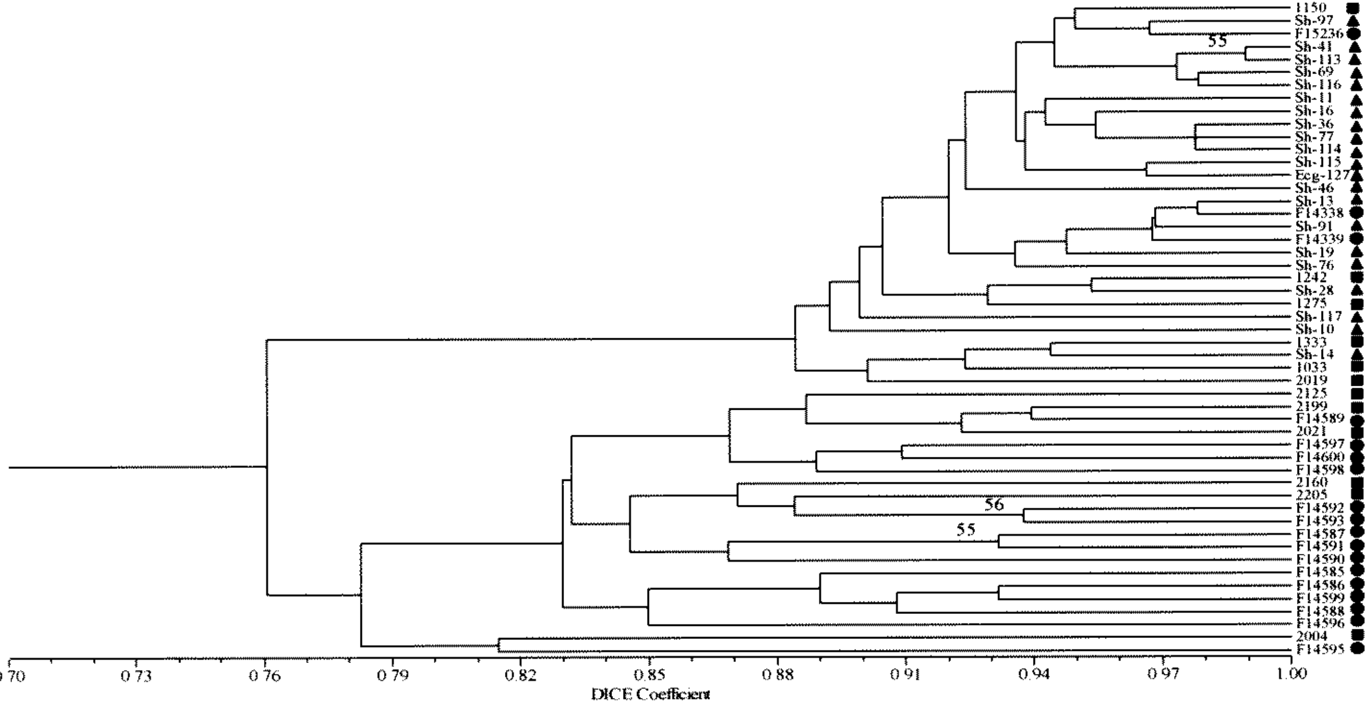


Figure 7-4 UPGMA dendrogram of 51 *F. thapsinum* isolates from grain sorghum (■), native (●) and weed (▲) grasses. Only branches occurring in 50% or more of the bootstrapped dendrograms are shown

Both mating types were present in the agricultural and non-agricultural ecosystems, in weed grass *S. halepense* and two of the native grasses *A. aristiglumis* and *C. gasteenii*. Only a single mating type was recovered from the other grasses from which only small numbers of *F. thapsinum* isolates were recovered (Table 7-14). All isolates were male fertile but only one isolate from the agricultural ecosystem and weed grass *S. halepense* and one from the non-agricultural ecosystem and native grass *C. gasteenii*

were female fertile, indicating that there is no reproductive isolation among isolates from different ecosystems or hosts.

Due to the small sample size effective population size parameters were not calculated for collections of isolates from different grass hosts and ecosystems.

Table 7-14 Mating types and male/female ratios of *F. thapsinum* isolates from different hosts and locations

Host		Location	Ratio		Total
			MAT-1 : *MAT-2*	Male : Female	
Agricultural ecosystem					
Sorghum bicolor	Grain sorghum	NSW/QLD	6 : 6	12 : 0	12
Sorghum halepense	Weed grass	NSW	14 : 6	19 : 1	20
Echinochloa crus-galli	Weed grass	NSW	1 : 0	1 : 0	1
Non-Agricultural ecosystem					
Austrostipa aristiglumis	Native grass	NSW	1 : 5	6 : 0	6
Coix gasteenii	Native grass	QLD	6 : 3	8 : 1	9
Heteropogon triticeus	Native grass	QLD	0 : 1	1 : 0	1
Sorghum interjectum	Native grass	NT	0 : 2	2 : 0	2
Total			28 : 23	49 : 2	51

7.3.5 Comparison of **F. thapsinum** *isolates from different countries/continents*

Twelve isolates of *F. thapsinum* from the geographic and plot level comparisons were selected on the basis of their clustering pattern in order to represent the *F. thapsinum* population from the northern grain belt of eastern Australia. These isolates of *F. thapsinum* together with isolates from Mexico (ten isolates), the Republic of South Africa (eight isolates), South Korea (two isolates) and the USA (three isolates) shared a DICE haplotype similarity within the range of 74-98% (Figure 7-5).

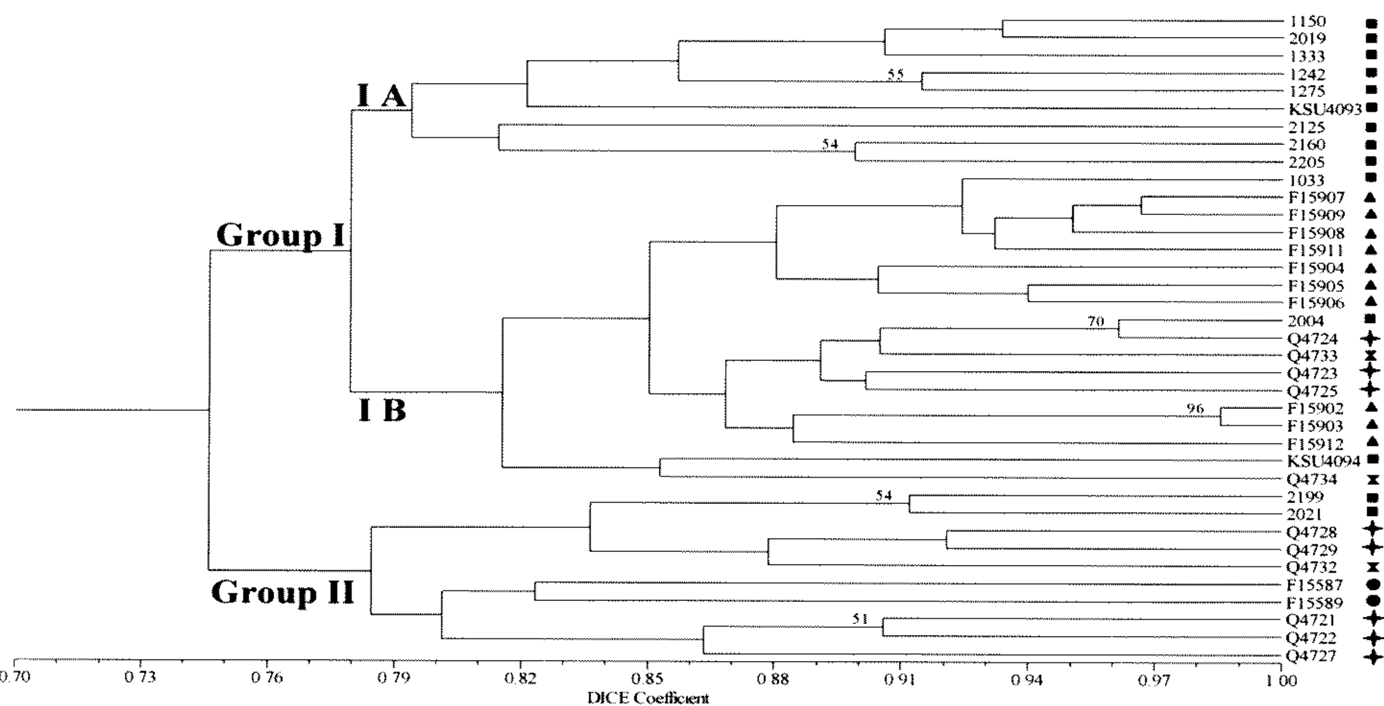


Figure 7-5 UPGMA dendrogram of 37 F. thapsinum isolates from Australia (■), Mexico (▲), the Republic of South Africa (✚), South Korea (●) and the USA (⧗). Isolates clustered with F. thapsinum tester strains, KSU4093 and KSU4094 (red rectangle). Only branches occurring in 50% or more of the bootstrapped dendrograms are shown

Two major groups of *F. thapsinum* haplotypes were observed within the dendrogram (Group I and Group II). Group I consisted of two subgroups that comprised the majority of *F. thapsinum* haplotypes from Australia (eight isolates) (sub-group IA) and intermixed haplotypes from Mexico (ten isolates), the Republic of South Africa

(three isolates), the USA (two isolates) and Australia (two isolates) (sub-group IB). Group II consisted of the remainder of the *F. thapsinum* haplotypes from Australia (two isolates), the Republic of South Africa (five isolates), the USA (one isolate) and two haplotypes from South Korea (Figure 7-5). Both groups shared almost the same level of DICE similarity ($\geq$ 78%). Haplotypes of *F. thapsinum* from Australia (sub-group IA), followed by the haplotypes from the other countries in Group II, showed the lowest level of similarity (78% to 92%).

Only haplotypes of *F. thapsinum* from Mexico were exclusively grouped together (sub-cluster IB), having the highest similarity between isolates ($\geq$ 98%). However, clustering of isolates according to country of origin was not supported by significant bootstrap values.

Of the 35 *F. thapsinum* isolates examined and, excluding standard tester strains, only one isolate (from Mexico) was female fertile (Figure 7-15) indicating that female fertility at country/continent level of comparison is very low.

Table 7-15 Mating types and male/female ratios of *F. thapsinum* isolates from different countries

	Ratio		
Country of origin	*MAT-1* : *MAT-2*	Male : Female	Total
Australia	6 : 6	12 : 0	12
Mexico	4 : 6	9 : 1	10
Republic of South Africa	3 : 5	8 : 0[a]	8
South Korea	0 : 2	2 : 0	2
USA	2 : 1	3 : 0	3
Standard tester strains	1 : 1	0 : 2	2

[a]Male/female fertility ratio determined by Klittich *et al.* (1997)

7.4 Discussion

There is a high level of genetic diversity in *F. thapsinum* populations at various spatial levels (plant part, plot and geographic location) in the northern grain belt of eastern Australia. A high level of genetic diversity was also determined in a collection of *F. thapsinum* isolates from both agricultural/non-agricultural ecosystems and different countries/continents. Similar levels of genetic diversity have previously been recorded for *F. proliferatum* (Laday *et al.* 2004), *F. verticillioides* (Chulze *et al.* 2000; Moretti *et al.* 2004) and other *Fusarium* species recovered from the same or different host species and countries.

The high level of genetic diversity in *F. thapsinum* populations at all levels of comparison could be attributed to one or more factors including gene/genotype flow, recombination, mutation, natural selection and genetic drift (Zhan and McDonald 2005). Sexual reproduction in all *F. thapsinum* populations is a relatively rare event (*av.* 134 ± 268 asexual generations per sexual generation), but in combination with other factors, might be frequent enough to generate the high levels in genetic diversity of populations as detected in this study.

Plant part *F. thapsinum* population comparisons indicated that plant part populations are continuous as a result of extensive gene/genotype flow. Extensive gene flow in these populations might be attributed to the even distribution of female fertility among populations. The completely intermixed *F. thapsinum* haplotypes from different plant parts and the very low level of population genetic differentiation demonstrate that *F. thapsinum* haplotypes do not have plant part/tissue or disease specificity, *viz.* Fusarium root rot or stalk rot. These results are in agreement with molecular data for another pathogen, as *Sclerotinia sclerotiorum*, which causes disease symptoms on more than one canola (*Brassica* napus) plant part (Phillips *et al.* 2002).

Plot and geographic level *F. thapsinum* population comparisons indicated a significant level of gene/genotype flow between most, but not all, *F. thapsinum* populations in the northern grain belt of eastern Australia. These results are to some extent in disagreement with spatial studies on other *Fusarium* species populations, *eg.* *Gibberella zeae* (*F. graminearum*), which had significant genetic exchange among field populations separated by hundreds of kilometres (Zeller *et al.* 2004). The relatively low rate of sexual reproduction and relatively high level of genetic uniformity in the *F. thapsinum* populations indicate that sexual dispersal is limited in plots and geographically separated areas. Hence, asexual reproduction and dispersal via seed, soil, air are proposed as the driving force in movement of these genetically uniform individuals.

The plot S population has the potential to eventually diverge by genetic drift (Nm = 0.89). This result is similar to the population subdivision observed for *F. graminearum* in the USA due to limited gene/genotype flow (Nm = 0.5) that is correlated to subpopulation chemotype (Gale *et al.* 2003). It is likely that isolates from the plot S population partially contribute to the overall hindrance of gene/genotype flow between the Moree and other geographic populations since the Moree population of *F. thapsinum* comprises stalk isolates. Therefore, relatively low gene flow between the *F. thapsinum* Moree population and either the Goondiwindi or the Quirindi populations might be attributed to biological traits of strains, *eg.* pathogenicity and chemoprofile, in the Moree population but not in the other geographic populations.

Several factors influence gene flow between populations and the reproductive cohesiveness of populations, *viz.* loss of sex, movement of infected host material, deployment of host genetic material and fungicides (Kohn 2005). In the present study, loss of sex was not observed as the inbreeding effective population number (*Ne(f)*)

ranged from 17.6% to 38.6% in plots and 14.3% to 23.2% in geographic populations. Lower *Ne(f)* in the present study than in the global population of *F. thapsinum* (32.1%) (Leslie and Klein 1996) is unlikely to contribute to the restricted gene flow among either plot or geographic populations. However, uneven distribution of female fertility among plot populations, but not among geographic populations, may contribute to a delay in gene/genotype flow.

High levels of genetic diversity for plot and geographic populations indicate that sexual reproduction may play a role in these populations and might be more frequent under field than laboratory conditions. Similar inconsistency between high levels of genetic diversity and low level of female fertility have been previously reported for combined field population of *F. verticillioides* (Chulze *et al.* 2000), the species with three times more frequent sexual reproduction than *F. thapsinum* (Leslie and Klein 1996; Leslie and Marasas 2002). Loss of sex, if occurring, would lead to a reduction of genetic diversity meaning that the present results would be in agreement with the reduced diversity detected by vegetative compatibility tests, and clonality of the global *F. thapsinum* population (Leslie and Klein 1996; Leslie and Marasas 2002). In summary, the factors that to some extent hinder gene flow between Moree and the other two geographic populations as well as between the plot S and the other plot populations are not obvious.

Geographic distance was correlated to genetic distance between the plot S population and the other plot populations (R = 0.71). These results do not support the hypothesis that any genetic differentiation within field populations must occur either on a small scale, *eg*. intensive samples of different 0.25 m^2 areas from the same field, or on a larger scale, *eg*. continental/country level (Chulze *et al.* 2000). However, geographic distance was not correlated to genetic distance between the Moree and other

geographic populations (R = -0.79), indicating that the Moree population is not in the early stages of possible speciation due to geographic isolation. Furthermore, partly intermixed haplotypes from the Moree population and the other two geographic populations supported moderate genetic separation of the Moree population. It is likely that the Moree population is in the early stages of ecological adaptation to the sorghum crop/cropping system in Moree. In order to confirm this, the Moree population would need to be sampled over several years to estimate its sexual potential as ecological adaptation precedes or follows reproductive isolation leading to speciation (Kohn 2005).

Plant part, plot and geographic population comparisons demonstrated that the *F. thapsinum* population from the northern grain belt of eastern Australia is highly diverse despite apparently low levels of sexual reproduction. The population of *F. thapsinum* is probably going through early stages of ecological adaptation, as demonstrated by the Moree population in both the plot and geographic level comparisons. The cropping system at Livingston Farm, Moree is characterised by 20 years of continuous sorghum production, reduced tillage practices and residues retained on the soil surface, and these may have caused the proposed ecological adaptation of this population. These results suggest that a broad selection of *F. thapsinum* isolates need to be used to effectively screen germplasm for resistance to Fusarium stalk and root rot. Isolates need to originate from different plots and locations, but not necessarily from different plant parts.

High levels of genetic diversity in *F. thapsinum* were further confirmed in the *F. thapsinum* collections from both agricultural/non-agricultural ecosystems and different countries/continents. Sexual recombination is not the only possible explanation for high levels of genetic diversity. Populations age can also influence

genetic diversity. An older population can harbour more diversity than a recently colonized habitat if the young population was founded by a few colonizers (Urena-Padilla *et al.* 2002).

Results from the present study suggest that *F. thapsinum* isolates from agricultural and non-agricultural ecosystems can be representatives of an older population and that long-distance gene/genotype flow between these ecosystems has been extensive. The artificial agricultural ecosystem with uniform cultivated plants has not resulted in haplotype or host related differentiation compared to the non-agricultural ecosystem with less uniform non-cultivated plants.

The lack of grouping of *F. thapsinum* haplotypes from different cultivated, weed and native grass hosts suggests that adaptation to, and possible speciation on, these hosts is not an ongoing process.

The range of genetic diversity of the 51 isolates from seven hosts, and the lack of clustering according to ecosystem/host is not sufficient evidence for lack of ecosystem/host adaptation or speciation. A larger sample size from each host and ecosystem is needed in order to comprehensively analyse the genetic structure of *F. thapsinum* populations. The larger sample size would also allow for a more objective estimate of sexual reproduction according to ecosystem and host, and its contribution to the genetic diversity of the *F. thapsinum* populations.

Unlike the other economically important grain sorghum pathogens that show high levels of genetic differentiation based on continent/country of origin, *viz. Claviceps africana* (Komolong *et al.* 2002; Tooley *et al.* 2002) and *Sporisorium reilianum* (Naidoo and Torres-Montalvo 2002), differentiation of *F. thapsinum* haplotypes at the country/continent level was not observed. The grouping of eight of the 12 Australian

isolates into one cluster might indicate adaptation to the specific ecological niche in the sorghum-growing region of Australia. However, the range of genetic diversity and the lack of bootstrap support indicate an absence of country/continent association.

These results suggest that movement of the pathogen from other countries to Australia, or vice versa, probable via sorghum seed trade is most likely an ongoing process as *F. thapsinum* is seedborne (Claflin 2000; Claflin and Giorda 2002; Montes-Belmont *et al.* 2003). Furthermore, intensive intercontinental transfer of the perennial weed grass *S. halepense* (Lenne and Ortiz 2002) could have also contributed to the movement of *F. thapsinum* inoculum, and the introduction of new genetic diversity to the Australian *F. thapsinum* population. Therefore, novel haplotypes of *F. thapsinum* with previously unknown combinations of genes can be expected in the Australian *F. thapsinum* population. The creation of novel *F. thapsinum* haplotypes could also arise from the outbreeding of *S. halepense*, and the occurrence of natural hybrids between *S. halepense*, grain sorghum and other cultivated and non-cultivated sorghums (Malaguti 2002).

In conclusion, the Australian *F. thapsinum* population has not been moving towards a clonal population despite apparently rare sexual reproduction in comparison to the Tanzanian (Mansuetus *et al.* 1997), South Korean (Lim *et al.* 2001) and global *F. thapsinum* population (Leslie and Klein 1996; Leslie and Marasas 2002). The Australian *F. thapsinum* population might be undergoing early stage ecological adaptation, indicating that further monitoring of the Moree population is required. The high level diversity in *F. thapsinum* population should be considered in the formulation of disease control strategies, when making changes to the cropping system and for controlling the spread and distribution of *S. halepense*.

Chapter 8

General Discussion

The intensive study of *Fusarium* species associated with sorghum at various growth stages at Livingston Farm, Moree revealed that *F. thapsinum* and *F. andiyazi* were the dominant species associated with root, crown and stalk tissue. These species were also found to be the dominant species in a comparative study of species associated with senescent stalks from the Goondiwindi and Quirindi areas. Furthermore, *F. thapsinum* was more commonly isolated from the hotter and drier Moree and Goondiwindi areas. In contrast, *F. andiyazi* was isolated more commonly from sorghum stalks in Quirindi, a slightly cooler and wetter area. Therefore, the hypotheses that *F. thapsinum* is the dominant species associated with Fusarium stalk rot in the Goondiwindi, Moree and Quirindi areas, and that *F. andiyazi* is not associated with grain sorghum in Australia, are rejected.

The co-dominance of *F. thapsinum* and *F. andiyazi* in sorghum stalks indicates that both species may play a role in Fusarium stalk and root rot of sorghum. Pathogenicity tests using *F. thapsinum* and *F. andiyazi* are required to assess virulence and pathogenicity, as both species have been reported to cause similar levels of stalk rot in evaluation trials for stalk rot resistance in sorghum genotypes (Tesso *et al.* 2005). The greater prevalence of *F. thapsinum* in mature stalks at Livingston Farm, Moree and in senescent stalks from the Goondiwindi area suggests that *F. thapsinum* may be a better colonizer and competitor than *F. andiyazi* in hotter and drier conditions as previously hypothesised for *F. thapsinum* associated with grain mould of sorghum (Prom *et al.* 2003). The greater prevalence of *F. andiyazi* in senescent stalks at Livingston Farm, Moree and in senescent stalks from the Quirindi area suggests that

F. andiyazi may be a better colonizer and competitor than *F. thapsinum* in slightly cooler and wetter conditions.

These results imply that screening of a wide range and frequency of *Fusarium* species in different geographic and agroclimatic areas is required, in order to evaluate the relative importance of *F. thapsinum, F. andiyazi* and other *Fusarium* species in development of Fusarium stalk rot under a variety of environmental conditions. It can be expected that the biology of *F. thapsinum* and *F. andiyazi* are different, and the complex nature of Fusarium stalk and root rot can only be controlled by breeding for resistance to both species.

Both *F. thapsinum* and *F. andiyazi* were also co-dominant in sorghum seedlings from Livingston Farm, Moree, suggesting that either seed inoculum or inoculum surviving in sorghum residues might be the primary infection source (Claflin 2000; Claflin and Giorda 2002; Mansuetus *et al.* 1999; Marley *et al.* 2004). The abundance of these species in mesocotyls and symptomatic root tissue may indicate that infection developed progressively from the seeds and spread into the crowns, as reported for the systemic colonization of maize seedlings by *F. verticillioides* (Oren *et al.* 2003). This is the first report of the co-occurrence of *F. thapsinum* and *F. andiyazi* in sorghum seedlings, indicating that both species might play an important role in sorghum seedling diseases under suitable environmental conditions. Although the virulence and pathogenicity of *F. andiyazi* on sorghum seedlings has been studied under laboratory conditions (Leslie *et al.* 2005b), further studies are needed under greenhouse and field conditions. Pathogenicity tests performed under greenhouse conditions, similar to those of Trimboli and Burgess (1983) with *F. moniliforme sensu lato* and Jardine and Leslie (1992) with *F. moniliforme* MP F on grain sorghum, will allow *F. andiyazi* to

gradually colonize sorghum during its life cycle leading to the manifestation of root and/or stalk rot symptoms.

The results of the intensive study at Livingston Farm, Moree support earlier findings that *F. thapsinum* and *F. andiyazi* can be present as endophytes in sorghum tissue without causing symptoms of diseases. Asymptomatic association of *F. thapsinum* and *F. andiyazi* with sorghum has practical implications due to the potential threat from mycotoxin contamination, especially if grain sorghum is used as fodder. Furthermore, these fungi can infect plants early in the season. The relative importance of inoculum in seed compared to inoculum in residues both in and on the surface soil in the colonization of plants remains to be seen. Experimental work in this area is difficult as most planting seed is contaminated with one or both pathogenic species (Leslie *et al.* 2005b; Leslie 2000).

The spectra of *Fusarium* species associated with grain sorghum in the Goondiwindi, Moree and Quirindi areas overlapped. Of the 19 species recovered from these three areas, only five species were common among the areas, suggesting that there is a high level of diversity of *Fusarium* species associated with grain sorghum in the northern grain belt of eastern Australia. The diversity of *Fusarium* species recovered in the present study was greater than that recorded in an earlier study in the USA (Leslie *et al.* 1990; Reed *et al.* 1983). Apart from the co-dominant species *F. thapsinum* and *F. andiyazi*, three other species, namely *F. proliferatum*, *F. compactum* and *F. equiseti* were recovered from all of the areas studied. However, of these three species only *F. proliferatum* is known to cause stalk rot of certain grain sorghum genotypes (Tesso *et al.* 2004), whilst the other two species are mainly secondary colonizers (Burgess *et al.* 1996; Trimboli and Burgess 1985). Despite the distribution of *F. proliferatum* in the studied areas in the northern grain belt of eastern Australia, the abundance of this

species varied. *Fusarium proliferatum* was significantly less abundant (P = 0.003) in the hotter and drier Goondiwindi area (0.6%) than in the cooler and wetter Quirindi area (7.1%). This finding is in agreement with the observation of Logrieco *et al.* (2002) that *F. proliferatum* tends to be more common in cooler, wetter areas of northern Europe. However, *F. proliferatum* appears to be a ubiquitous species in Australia, occurring over a wider range of climatic regions. It occurs, for example, in association with roots of cabbage palms (*Livistona mariae*) in the arid region of central Australia (Neumann *et al.* 2004), stalks of tallgrass *Heteropogon triticeus* in the tropical savannah zone of north east Queensland (Phan 2004) and stalks of native *Sorghum* species in the wet-dry monsoonal region of the Northern Territory (Walsh 2006).

The range of mycotoxins produced by *F. proliferatum* characterises this species as a potential risk in various cropping systems (Logrieco *et al.* 2002). Although *F. proliferatum* was the third most abundant "chain-producing" species recovered in the current study, the relatively low abundance of this species means that it is not characterised as a probable threat to Australian grain sorghum production.

In general, the *Fusarium* community associated with physiologically mature sorghum from Livingston Farm, Moree was more similar, based on the number of shared species and abundance of the co-dominant species *F. thapsinum* and *F. andiyazi*, to the *Fusarium* community from the Goondiwindi area than to the community from the Quirindi area. This pattern might be expected due to the geographic proximity and climatic similarity between the Livingston Farm, Moree and Goondiwindi areas. For example, the recovery of *F. nygamai* from these two areas is consistent with the more frequent recovery of this species in warm temperate, subtropical and semi-arid areas in Australia (Burgess and Trimboli 1986).

Some *Fusarium* species were exclusively recovered from sorghum in the Quirindi area or at Livingston Farm, Moree, whilst *F. verticillioides* was recovered from the Quirindi and Goondiwindi areas but not from Moree. *Fusarium subglutinans* was only isolated in the cooler and wetter Quirindi area, indicating that climate might be one of the factors influencing the distribution of *F. subglutinans*. *Fusarium subglutinans* was first reported in Australian maize on the cooler central slopes of New South Wales (Edwards 1935). It should be noted that only a small area of maize is grown at Livingston Farm, Moree and it has not being grown in the field where this study was undertaken. In contrast, maize is common in the Qurindi area where sorghum samples were collected. Thus, it is likely that *Fusarium* species associated with maize would be more common in the Quirindi area. Failure to isolate *F. verticillioides* from sorghum at Livingston Farm, Moree is attributed to the intensive cropping of sorghum in this system for 20 years, resulting in at least partially specialised *Fusarium* pathogen populations. The low recovery of *F. verticillioides* from sorghum plants in the present study is in contrast to the relatively high recovery of *F. verticillioides* from sorghum seeds in Tanzania (Mansuetus *et al.* 1997). Mansuetus *et al.* (1997) reported a *F. verticillioides* : *F. thapsinum* ratio close to 1:2 based on sexual compatibility tests, which corresponds more to the *F. andiyazi* : *F. thapsinum* ratio observed in the current study. The discrepancy between the ratio of *F. verticillioides* and *F. thapsinum* in the present study and a similar study in Tanzania can be attributed to the substrate, its origin, climatic conditions and cropping history. Other species recovered only at Livingston Farm, Moree in low abundance were mainly secondary colonizers of sorghum.

The low relative abundance of *F. subglutinans*, *F. verticillioides* and other *Fusarium* species in the present study indicates that these species are unlikely play an important

role in the disease process. However, some of these species might compete with pathogenic isolates in plants (Leslie 2000) and therefore, their relative abundance remains relevant.

It should be noted that a larger number of cultures isolated in the current study were identified solely on the basis of morphological criteria. However, large subsets of isolates of *F. andiyazi*, *F. thapsinum* and *F. proliferatum* and all isolates of *F. verticillioides* and *F. subglutinans* were identified by sexual compatibility tests, and confirmed using molecular techniques. It should be emphasised that all cultures were examined rigorously for the key morphological features on two media, both of which promote the formation of conidia, especially microconidia. It is recommended that further studies on *Fusarium* communities associated with grain sorghum should be based on a wider collection of isolates in space and time, putatively identified using morphological features, with final characterisation of a subset of each species based on one or more alternative techniques.

The dominant species in the Goondiwindi and Moree areas, and the second most abundant species in the Quirindi area, *F. thapsinum*, had a high level of genetic diversity, which is in contrast to the low genetic diversity observed in a global *F. thapsinum* population (Klittich and Leslie 1988). Different parameters such as Nei's gene diversity *(h)*, the coefficient of gene differentiation $\theta(F_{ST})$, estimate of gene flow (Nm) and Nei's unbiased genetic distance (D) indicate that the Moree *F. thapsinum* population is slightly different from the other populations. A 1:1 ratio of mating types and a low level of female fertile isolates provided evidence for inbreeding in these three populations. The hypothesis that populations of *F. thapsinum* are genetically similar in the Goondiwindi, Moree and Quirindi areas is rejected.

A comparison of randomly selected Australian *F. thapsinum* isolates and a collection of isolates from Mexico, the Republic of South Africa, South Korea and the USA showed a high level of genotypic diversity, similar to the diversity level detected at all levels of comparison for the Australian *F. thapsinum* populations. Overall genotypic similarity of $\geq$ 74%, mixed clustering of haplotypes from different countries and the reproductive potential of these isolates under laboratory conditions indicated that *F. thapsinum* populations in Australia are part of a worldwide panmictic population of this species. Therefore, the hypothesis that the Australian population of *F. thapsinum* is an integral part of the international panmictic population of this species is accepted.

A relatively high level of genetic differentiation of three *F. thapsinum* populations was detected within a 400 km area of the northern grain belt of eastern Australia. The geographic proximity of the Moree and Goondiwindi populations and relative distance of both populations from the Quirindi population was not positively correlated to the genetic distances between these populations. The Moree population appears to be differentiated from the Goondiwindi and Quirindi populations with an estimated 72% and 75% of the observed genetic variation in the Moree – Goondiwindi populations and the Moree – Quirindi populations respectively shared in a common gene pool. Despite the geographic distance of the Goondiwindi and Quirindi populations, they appear to be admixed, with an estimated 94% of the observed genetic variation of these two populations shared in a common gene pool [$\theta(F_{ST}) = 0.06$]. These results indicate that gene flow has been somehow restricted between Moree and the other two populations, but not between the geographically distant Goondiwindi and Quirindi populations. However, there has been enough gene flow between Moree and the other two populations to negate the effects of genetic drift, which is one of the evolutionary forces impacting population genetic structure

(Zhan and McDonald 2005). Regardless of the observed genetic differentiation of the Moree population from the other two populations, there is evidence for equal levels of sexual reproduction in all three populations. Effective population size parameters based on both mating type and female fertility indicate that sexual reproduction is evenly spread in the northern grain belt of the eastern Australia. Therefore, the Moree population is still considered a part of the overall *F. thapsinum* population.

The overall Australian population of *F. thapsinum* (311 isolates) showed relatively infrequent sexual reproduction (*av.* 1:134 to 1:268 sexual per asexual generations) in comparison to populations from South Korea (1:52 to 1:103) (Lim *et al.* 2001) and Tanzania (1:83 to 1:165) (Mansuetus *et al.* 1997). However, the frequency of sexual reproduction in the Australian population of *F. thapsinum* corresponds more to that of the global population of *F. thapsinum* (1:114 to 1:228) (Leslie and Klein 1996; Leslie and Marasas 2002; Leslie 2002). Discrepancies in the observed sexual reproduction among Australian, South Korean and Tanzanian *F. thapsinum* populations can be attributed to the origin of isolates, including the sorghum genotype (*eg.* local/foreign germplasm), geographic and climatic regions, agrotechnical practices, plant part (*eg.* seed/other plant parts), sampling techniques and additional influences. In view of this, the absence of female fertile isolates of *F. thapsinum* observed from one of three locations in Tanzania (Mansuetus *et al.* 1997) suggests that some of these factors affect sexual reproduction within subpopulations of *F. thapsinum* in Tanzania. Consequently, it can be expected that some of these factors also influence sexual reproduction in *F. thapsinum* populations at the country/continent level.

The apparently low frequency of sexual reproduction in the Australian *F. thapsinum* population indicates that it might be more clonal in nature compared to populations from other countries. However, the genetic diversity of the Australian *F. thapsinum*

population showed that all isolates had unique haplotypes. It is likely that one or more factors other then sexual reproduction, *viz.* gene/genotype flow, mutation, natural selection and/or genetic drift, might have caused the high genetic diversity of Australian *F. thapsinum* population. Therefore, these results are incongruent to the hypothesis of Leslie and Marasas (2002) who proposed that relatively few multi-locus haplotypes dominate the population of *F. thapsinum*, whilst the remaining strains are clones.

Furthermore, a comparison of selected isolates of *F. thapsinum* from Australia (12) and Mexico, the Republic of South Africa, South Korea and the USA, showed a lack of clones within the collections of isolates from other countries and confirmed high levels of diversity in Australian *F. thapsinum* field populations. Therefore, more genetic diversity was revealed in the present studies ($\geq$ 74%) than in previous studies on populations from South Korea (Lim *et al.* 2001) and a global population of *F. thapsinum* (Klittich and Leslie 1988; Leslie and Marasas 2002) that indicated a move towards a clonal population. Mixed clustering of isolates from different countries also suggested that there is no grouping of isolates based on their country of origin, and therefore it is unlikely that the Australian *F. thapsinum* population has evolved along a separate lineage. It is most likely that the Australian population is an integral part of a worldwide panmictic population. High genetic diversity detected in a collection of isolates from various countries and in the overall Australian *F. thapsinum* population is speculated to be the result of movement of the pathogen between countries either directly or via the movement of sorghum planting seed and grain for feedstuffs. Therefore, the introduction of new haplotypes into the Australian population is likely to be an ongoing process.

More detailed analyses of larger and more geographically diverse populations of *F. thapsinum* from Australia and other countries are required to confirm that the Australian *F. thapsinum* population is a part of this panmictic population. Large collections of isolates from Australia, Africa, India, China and South-East Asia that are considered either regions of domestication of cultivated sorghum or regions where the crop has been grown and the pathogen has evolved for a long period would be desirable (Kimber 2000). In addition, isolates from countries in the North and Central Americas with relatively short history of intensive sorghum production could be included in future studies. This would allow for the genetic structure of the populations from different regions to be better determined.

The present study has also shown that in Australia, *F. thapsinum* has not co-evolved separately with the weed species *Sorghum halepense* and *Echinochloa crus-galli*, or the native grasses, *Austrostipa aristiglumis, Coix gasteenii, Heteropogon triticeus* and *Sorghum interjectum*. It is most likely that isolates have drifted from sorghum fields to be associated with weed grasses and to grasslands and woodlands with native grasses. However, it is possible that *F. thapsinum* comprises one epidemiological unit and has long been present in native grasslands in Australia. The present population on sorghum may be derived both from native grasslands haplotypes and haplotypes introduced on sorghum seed and grain from other countries.

In conclusion, the dominant species associated with Fusarium stalk and root rot of sorghum in the northern grain belt of eastern Australia, *F. thapsinum* is genetically diverse. Consequently, it is suggested that sorghum hybrids and breeding material should be tested for resistance to stalk rot at a number of locations since there have been no studies on variation in virulence and aggressiveness of individuals in the overall population. Furthermore, given the common occurrence of *F. andiyazi* in

Australia, further studies are justified on the population genetics of this species and its ability to cause stalk and root rot under controlled conditions.

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Appendices

Appendix 1 .
Media recipes

1.1. PCNB-peptone agar (PPA / Nash-Snyder Medium) (Burgess *et al.* 1994)

Basal medium consists of 2% Agar, 1.5% Difco Peptone, 0.1% KH_2PO_4, 0.05% $MgSO_4x7H_2O$, 0.1% Terrachlor® (contains Pentachloronitrobenzene (PCNB) 75%) in distilled water. The basal medium was autoclaved at 121°C, 104 kPa for 20 min and cooled to 55°C before adding 1 g Streptomycin sulphate and 0.12 g Neomycin sulphate. The antibiotics were dispensed in 10 ml sterile water before added to the basal medium.

1.2. Water agar (WA) (Burgess *et al.* 1994)

WA medium was prepared by adding 2% agar into distilled water and autoclaved at 121°C for 20 min.

1.3. Carnation leaf agar (CLA) (Fisher *et al.* 1982)

4-5 pieces of sterilised carnation leaves were floated on one side of a 6 mm Petri plate right after 2% Water Agar was poured.

1.4. Spezieller Nährstoffarmer agar (SNA) (Nirenberg 1976)

SNA medium consists of 2% Agar, 0.1 % KH_2PO_4, 0.1 % KNO_3, 0.05% $MgSO_4.7H_2O$, 0.05% KCl, 0.02% Glucose, 0.02 % Sucrose. The mixture was dispensed with distilled water and autoclaved for 20 min at 121°C. A piece of sterile filter paper (50 x 100 mm) was placed on a surface of solid medium, close to the edge of a 6 mm Petri plate.

1.5. Potato dextrose agar (PDA) (Burgess *et al.* 1994)

250 g white unpeeled potatoes were washed and diced before boiling until just soft. The boiled potatoes were filtered through cheesecloth. The broth was added with 20 g Agar, 20 g Dextrose and tap water to make up 1 L.

1.6. Carrot agar (CA) (Klittich and Leslie 1988)

Fresh carrots (400 g) were washed, diced into small pieces and autoclaved in 400 ml distilled water for 10 min. The autoclaved carrots and the liquid were pureed until it formed an even mixture which then added with 20 g Agar and 500 ml distilled water and autoclaved for 30 min at 121°C.

1.7. Modified liquid synthetic nutrient-poor mineral agar (Schilling 1996)

SNA liquid medium consists of 0.1% KH_2PO_4, 0.1% KNO_3, 0.05% $MgSO_4x7H_2O$, 0.05% KCl, 0.2% glucose, 0.2% sucrose and 0.1% yeast extract in distilled water. The mixture was autoclaved for 20 min and cool to room temperature before use.

Appendix 2 .
Reagents in molecular procedures

2.1. Cell lysis solution (or DNA extraction buffer)

Component	Initial concentration	Final concentration	Amount
dH_2O	-	-	300 ml
Tris-HCL	1 M (pH = 8.0)	100 mM	60 ml
Na_2EDTA	0. 5 M (pH = 8.0)	20 mM	24 ml
NaCl	-	0.5 M	17.4 g
SDS	-	0.035 M	6 g

Make up to 600 ml with distilled water and autoclave.

2.2. Protein precipitate solution (PPS)

Component	Initial concentration	Final concentration	Amount
K-acetate	-	3 M	58.88 g
Glacial acetic acid	100%	4%	8 ml
dH_2O	-	Make up to 200 ml	

Autoclave.

2.3. Binding matrix solution

Weigh 187.2 g Guanidine thiocyanate ($CH_5N_3.CHNS$) and make up to 264 ml solution (6 M) with distilled water. Add 66 ml non-diluted binding matrix. Autoclave.

2.4. Salt/ethanol wash solution (SEWS)

Component	Initial concentration	Final concentration	Amount
Na-acetate	-	100 mM	8.2 g
Ethanol	Absolute	70%	700 ml
dH_2O	-	-	300 ml

Weigh Na-acetate and dissolve in dH_2O. Autoclave. After the solution cools down, add absolute ethanol.

2.5. TBE buffer

Component	Initial concentration	Final concentration	Amount (for 5 x solution)
Tris base	-	-	54 g
Boric acid	-	-	27.5 g
EDTA	0.5 M (pH = 8.0)	0.01 M	20 ml
dH_2O		Make up to 1000 ml	

Autoclave.

2.6. TE buffer

Component	Initial concentration	Final concentration	Amount (per 10 ml)
Tris-HCl	1 M (pH = 8.0)	10 mM	100 μl
EDTA	0.5 M (pH = 8.0)	1 mM	20 μl
dH_2O	-	Make up to 10 ml	

Autoclave.

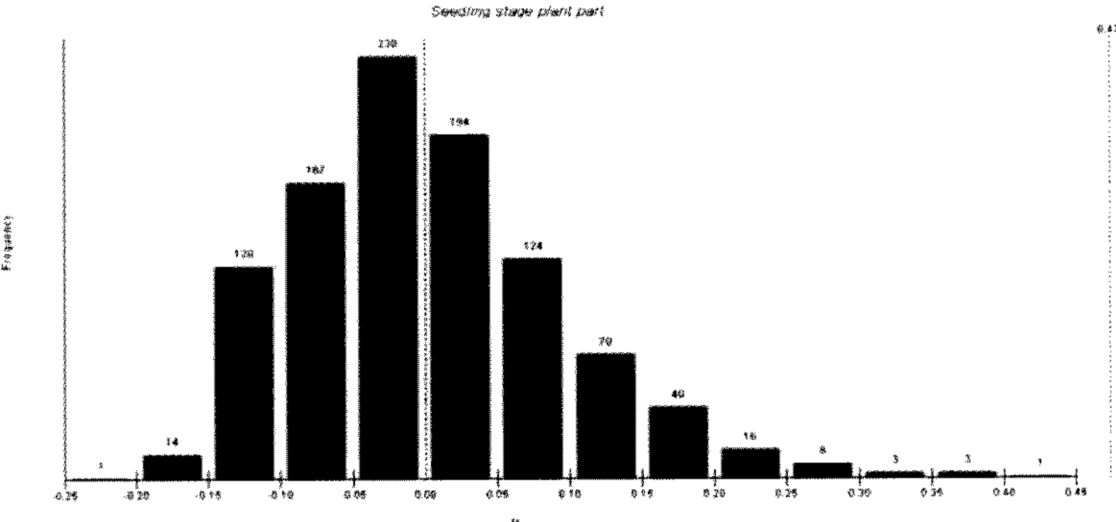
2.7. RNAase

Dissolve 0.082 g of Na-acetate in 100 ml dH_2O and add 1 g of pancreatic RNAase (RNAase A). Heat to 100°C for 15 min. Cool down at room temperature and then adjust the pH by adding 0.1 volume of 1 M Tris-Cl (pH 7.4). One volume of RNAase is ~ 100 ml and 0.1 volume of 1 M Tris-Cl (pH 7.4) is 10 ml.

2.8. 1xTE with RNAase (10 mg/ml)

Appendix 3 .
ANOSIM One-Way Analysis (Chapter 4)

3.1. *Fusarium* communities of plant parts of sorghum seedlings



One-way Analysis

Factor Values

Factor: Plant part
Mesocotyl
Root symptomatic
Root asymptomatic
Crown

Factor Groups

Sample	Plant part
D	Mesocotyl
F	Mesocotyl
M	Mesocotyl
S	Mesocotyl
D	Root symptomatic
F	Root symptomatic
M	Root symptomatic
S	Root symptomatic
D	Root asymptomatic
F	Root asymptomatic
M	Root asymptomatic
S	Root asymptomatic
D	Crown
F	Crown
M	Crown
S	Crown

Global Test

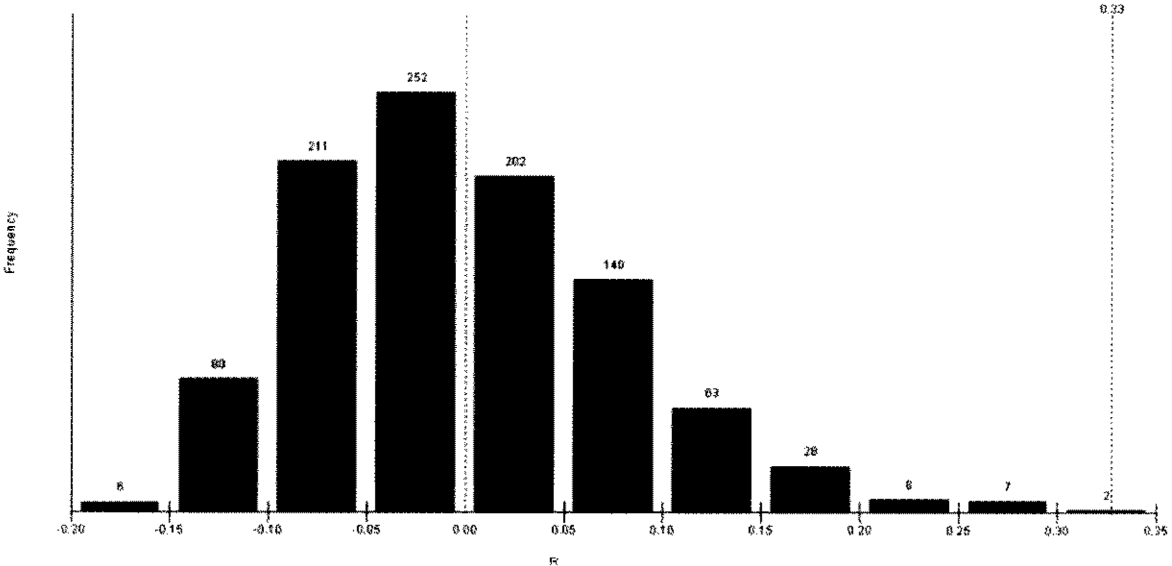
Sample statistic (Global R): 0.474
Significance level of sample statistic: 0.1%
Number of permutations: 999 (Random sample from 2627625)
Number of permuted statistics greater than or equal to Global R: 0

Pairwise Tests

Groups	R Statistic	Significance Level %	Possible Permutations	Actual Permutations	Number >= Observed
Mesocotyl, Root symptomatic	-0.031	45.7	35	35	16
Mesocotyl, Root asymptomatic	0.844	2.9	35	35	1
Mesocotyl, Crown	0.531	2.9	35	35	1
Root symptomatic, Root asymptomatic	0.802	2.9	35	35	1
Root symptomatic, Crown	0.427	2.9	35	35	1
Root asymptomatic, Crown	0.354	5.7	35	35	2

3.2. *Fusarium* communities of plant parts of physiologically mature sorghum

3.3.



Factor Groups

Sample	Plant Parts
D	Root symptomatic
F	Root symptomatic
M	Root symptomatic
S	Root symptomatic
D	Root asymptomatic
F	Root asymptomatic
M	Root asymptomatic
S	Root asymptomatic
D	Crown
F	Crown
M	Crown
S	Crown
D	Stalk 4th node
F	Stalk 4th node
M	Stalk 4th node
S	Stalk 4th node
D	Stalk 6th node
F	Stalk 6th node
M	Stalk 6th node
S	Stalk 6th node

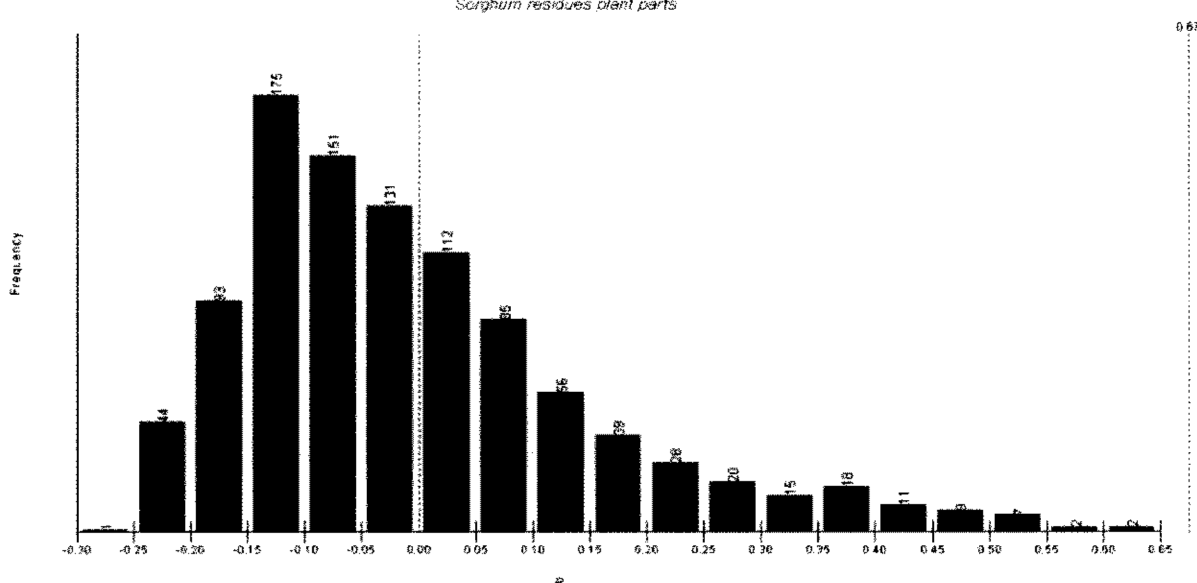
Global Test

Sample statistic (Global R): 0.327
Significance level of sample statistic: 0.3%
Number of permutations: 999 (Random sample from a large number)
Number of permuted statistics greater than or equal to Global R: 2

Pairwise Tests

Groups	R Statistic	Significance Level %	Possible Permutations	Actual Permutations	Number >= Observed
Root symptomatic, Root asymptomatic	0.354	14.3	35	35	5
Root symptomatic, Crown	0.052	31.4	35	35	11
Root symptomatic, Stalk 4th node	0.083	25.7	35	35	9
Root symptomatic, Stalk 6th node	0.219	5.7	35	35	2
Root asymptomatic, Crown	0.896	2.9	35	35	1
Root asymptomatic, Stalk 4th node	0.781	2.9	35	35	1
Root asymptomatic, Stalk 6th node	0.781	2.9	35	35	1
Crown, Stalk 4th node	0.115	14.3	35	35	5
Crown, Stalk 6th node	0.292	5.7	35	35	2
Stalk 4th node, Stalk 6th node	-0.094	68.6	35	35	24

Fusarium communities of plant parts of sorghum residues



Factor Groups

Sample	Plant parts
D	Root
F	Root
M	Root
S	Root
D	Crown
F	Crown
M	Crown
S	Crown
D	Stalk
F	Stalk
M	Stalk
S	Stalk

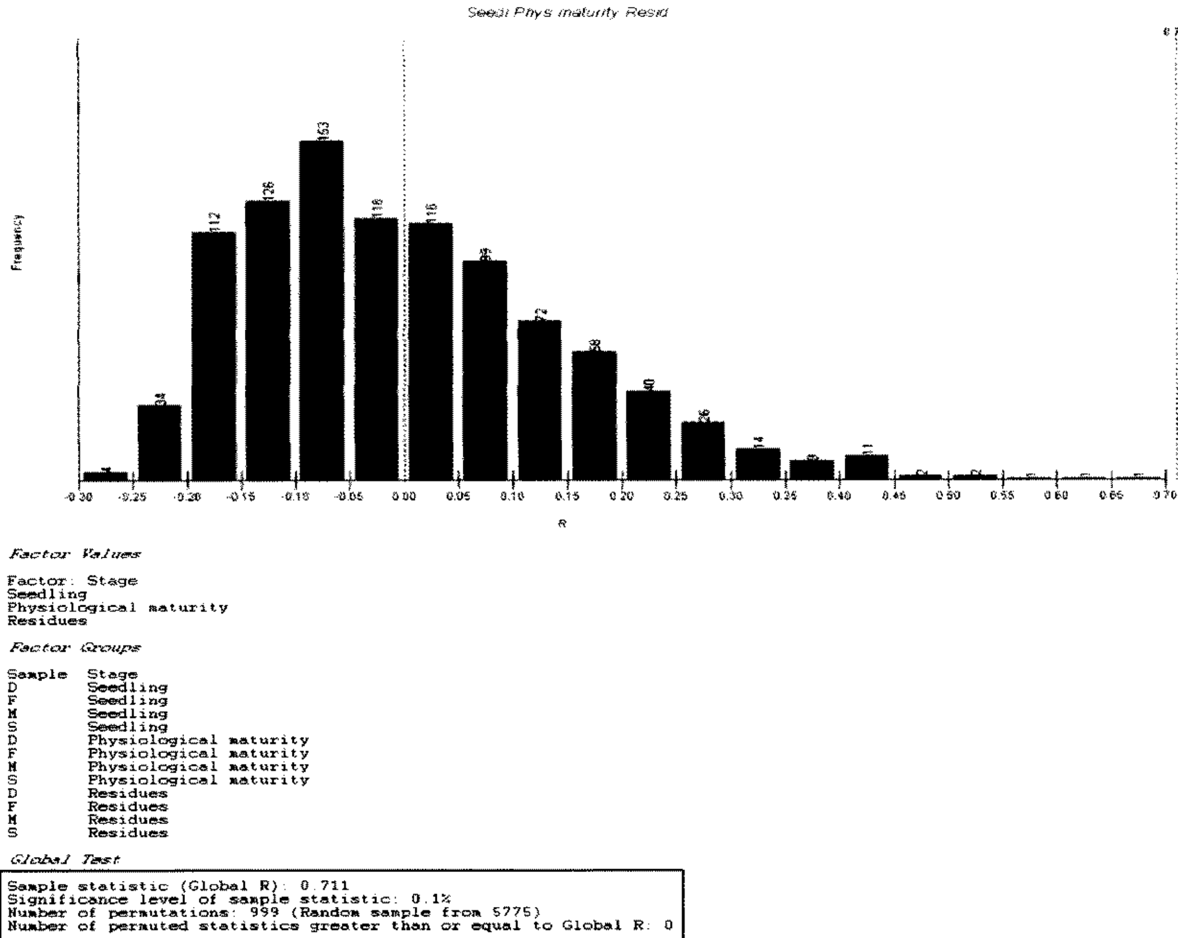
Global Test

Sample statistic (Global R): 0.675
Significance level of sample statistic: 0.1%
Number of permutations: 999 (Random sample from 5775)
Number of permuted statistics greater than or equal to Global R: 0

Pairwise Tests

Groups	R Statistic	Significance Level %	Possible Permutations	Actual Permutations	Number >= Observed
Root, Crown	0.281	8.6	35	35	3
Root, Stalk	1.	2.9	35	35	1
Crown, Stalk	0.656	2.9	35	35	1

3.4. *Fusarium* communities of sorghum seedlings, physiologically mature plants and residues



Factor Values

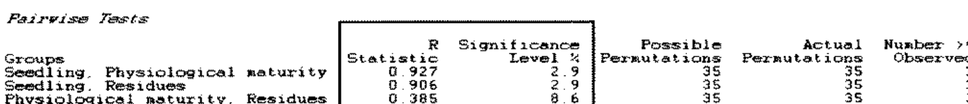
Factor: Stage
Seedling
Physiological maturity
Residues

Factor Groups

Sample	Stage
D	Seedling
F	Seedling
M	Seedling
S	Seedling
D	Physiological maturity
F	Physiological maturity
M	Physiological maturity
S	Physiological maturity
D	Residues
F	Residues
M	Residues
S	Residues

Global Test

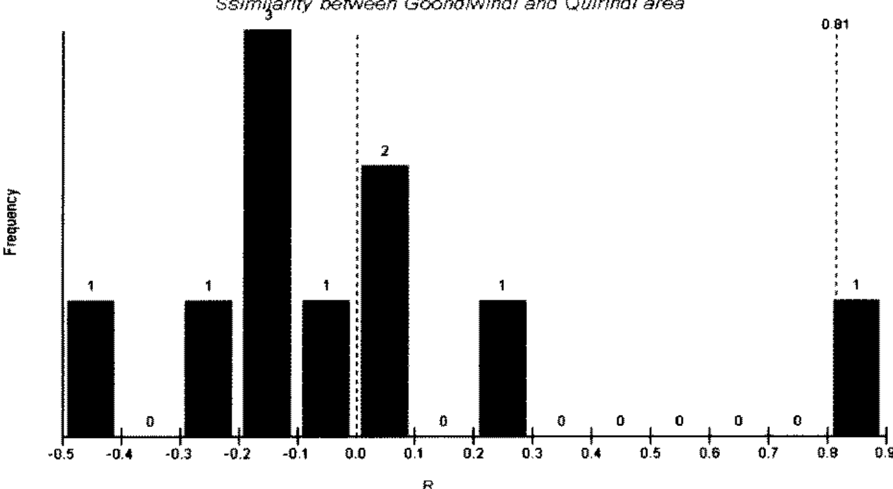
Sample statistic (Global R): 0.711
Significance level of sample statistic: 0.1%
Number of permutations: 999 (Random sample from 5775)
Number of permuted statistics greater than or equal to Global R: 0



Pairwise Tests

Groups	R Statistic	Significance Level %	Possible Permutations	Actual Permutations	Number >= Observed
Seedling, Physiological maturity	0.927	2.9	35	35	1
Seedling, Residues	0.906	2.9	35	35	1
Physiological maturity, Residues	0.385	8.6	35	35	3

Appendix 4 . ANOSIM One-Way Analysis (Chapter 5)



Factor Values

```
Factor: Area
G
Q
```

Factor Groups

```
Sample  Area
G_1     G
G_2     G
G_3     G
Q_1     Q
Q_2     Q
Q_3     Q
```

Global Test

```
Sample statistic (Global R): 0.815
Significance level of sample statistic: 10.%
Number of permutations: 10 (All possible permutations)
Number of permuted statistics greater than or equal to Global R: 1
```

Appendix 5 . List of *F. andiyazi*, *F. thapsinum*, *F. verticillioides* and *F. proliferatum* isolates (Chapter 6)

5.1. The geographic origins, plant part substrates and mating types of the *Fusarium* isolates

Accession no.[a]	Location	Plant part	Mating type
Fusarium andiyazi			
F14560[†]	Moree, NSW	Stalk 4th n.[b]	*MAT-2*
F14565[‡]	Moree, NSW	Stalk 4th n.	*MAT-1*
F14566	Moree, NSW	Crown	*MAT-1*
F14567	Moree, NSW	Crown	*MAT-1*
F14568[•]	Moree, NSW	Root asym.[c]	*MAT-2*
F14569[•]	Moree, NSW	Stalk 4th n.	*MAT-2*
F14570	Moree, NSW	Root sym.	*MAT-2*
F14572*	Moree, NSW	Stalk 6th n.[b]	*MAT-2*
F14573	Moree, NSW	Crown	*MAT-2*
F14574	Moree, NSW	Stalk 4th n.	*MAT-2*
F14577	Moree, NSW	Stalk 4th n.	*MAT-2*
F14579	Moree, NSW	Mesocotyl	*MAT-2*
F14580[■]	Moree, NSW	Stalk 4th n.	*MAT-2*
F14581[■]	Moree, NSW	Stalk 6th n.	*MAT-2*
F14582	Moree, NSW	Stalk 4th n.	*MAT-2*
F14583[□]	Moree, NSW	Stalk 4th n.	*MAT-2*
F14584[□]	Moree, NSW	Stalk 6th n.	*MAT-2*
F18938	Moree, NSW	Crown	*MAT-2*
F19026	Moree, NSW	Stalk 4th n.	*MAT-2*
F19027	Moree, NSW	Crown	*MAT-2*
F19028	Moree, NSW	Stalk 4th n.	*MAT-2*
F19030	Moree, NSW	Root	*MAT-1*
F19031	Moree, NSW	Stalk 4th n.	*MAT-2*
F19032	Moree, NSW	Stalk 4th n.	*MAT-1*
F19033	Moree, NSW	Crown	*MAT-1*
F19034	Moree, NSW	Stalk 4th n.	*MAT-1*
F19036	Moree, NSW	Stalk 4th n.	*MAT-1*
F19037	Moree, NSW	Stalk 4th n.	*MAT-2*
F19038	Moree, NSW	Crown	*MAT-2*
F19039	Moree, NSW	Stalk 4th n.	*MAT-1*
F19041	Moree, NSW	Stalk 4th n.	*MAT-1*
F19042	Moree, NSW	Root	*MAT-1*
F19043	Moree, NSW	Stalk 4th n.	*MAT-1*
F19045	Moree, NSW	Crown	*MAT-1*
F19049	Moree, NSW	Root	*MAT-1*
F19050	Moree, NSW	Stalk 4th n.	*MAT-1*
F19051	Moree, NSW	Crown	*MAT-1*
F19052	Moree, NSW	Crown	*MAT-1*
F19053	Moree, NSW	Stalk 4th n.	*MAT-2*
F19054[◊]	Moree, NSW	Root	*MAT-1*
F19055[◊]	Moree, NSW	Stalk 4th n.	*MAT-2*
F19056	Moree, NSW	Root	*MAT-1*
F19058	Moree, NSW	Crown	*MAT-1*
F19059	Moree, NSW	Crown	*MAT-1*
F19060	Moree, NSW	Crown	*MAT-2*
F19061	Moree, NSW	Stalk 4th n.	*MAT-2*
F19062	Moree, NSW	Crown	*MAT-2*
F19063	Moree, NSW	Stalk 4th n.	*MAT-1*
F19065	Moree, NSW	Root	*MAT-1*
F19066	Moree, NSW	Root	*MAT-1*
F14562[d]	Moree, NSW	Stalk 6th n.	*MAT-1*
F14564[‡]	Moree, NSW	Mesocotyl	*MAT-1*
F14571	Moree, NSW	Crown	*MAT-1*
F14575	Moree, NSW	Crown	*MAT-1*
F14576*	Moree, NSW	Stalk 4th n.	*MAT-2*
F18937	Moree, NSW	Stalk 4th n.	*MAT-1*
F19025	Moree, NSW	Stalk 4th n.	*MAT-1*
F19035	Moree, NSW	Root	*MAT-2*
F19040	Moree, NSW	Root	*MAT-1*
F19057	Moree, NSW	Stalk 4th n.	*MAT-2*
F19064	Moree, NSW	Root	*MAT-2*
F19068	Moree, NSW	Stalk 4th n.	*MAT-1*
F18939	Goondiwindi	Stalk 4th n.	*MAT-1*
F18940	Goondiwindi	Stalk 4th n.	*MAT-1*
F18941	Goondiwindi	Stalk 4th n.	*MAT-1*
F18942	Quirindi	Stalk 4th n.	*MAT-1*
F18943	Quirindi	Stalk 4th n.	*MAT-1*
F18944	Quirindi	Stalk 4th n.	*MAT-1*

(*Continued*)

Accession no.[a]	Location	Plant part	Mating type	Accession no.	Location	Plant part	Mating type
F19047■	Moree, NSW	Stalk 4th n.	*MAT*-2				
Fusarium thapsinum							
F18959	Moree, NSW	Crown	*MAT-2*	F18991	Moree, NSW	Stalk 4th n.	*MAT-1*
F18960	Moree, NSW	Root	*MAT-1*	F18947	Moree, NSW	Root	*MAT-1*
F18961	Moree, NSW	Root	*MAT-2*	F18993	Moree, NSW	Root	*MAT-2*
F18962	Moree, NSW	Crown	*MAT-1*	F18994	Moree, NSW	Crown	*MAT-1*
F18963	Moree, NSW	Crown	*MAT-2*	F18995	Moree, NSW	Crown	*MAT-2*
F18964	Moree, NSW	Root	*MAT-1*	F18997	Moree, NSW	Stalk 4th n.	*MAT-2*
F18945	Moree, NSW	Root	*MAT-1*	F18998	Moree, NSW	Root	*MAT-2*
F18966	Moree, NSW	Root	*MAT-1*	F18999	Moree, NSW	Stalk 4th n.	*MAT-2*
F18967	Moree, NSW	Stalk 4th n.	*MAT-2*	F19000	Moree, NSW	Crown	*MAT-2*
F18968	Moree, NSW	Stalk 4th n.	*MAT-1*	F19001	Moree, NSW	Stalk 4th n.	*MAT-2*
F18969†	Moree, NSW	Root	*MAT-2*	F19002	Moree, NSW	Crown	*MAT-2*
F18970†	Moree, NSW	Crown	*MAT-2*	F19003	Moree, NSW	Root	*MAT-2*
F18971†	Moree, NSW	Crown	*MAT-1*	F19004	Moree, NSW	Stalk 4th n.	*MAT-2*
F18972‡	Moree, NSW	Root	*MAT-1*	F19005	Moree, NSW	Root	*MAT-2*
F18973‡	Moree, NSW	Crown	*MAT-1*	F19006	Moree, NSW	Root	*MAT-2*
F18974	Moree, NSW	Crown	*MAT-2*	F19007	Moree, NSW	Crown	*MAT-1*
F18975	Moree, NSW	Stalk 4th n.	*MAT-2*	F19008	Moree, NSW	Root	*MAT-2*
F18976	Moree, NSW	Stalk 4th n.	*MAT-2*	F19009	Moree, NSW	Stalk 4th n.	*MAT-2*
F18977	Moree, NSW	Crown	*MAT-2*	F19010	Moree, NSW	Stalk 4th n.	*MAT-1*
F18978	Moree, NSW	Crown	*MAT-1*	F19011	Moree, NSW	Root	*MAT-1*
F18979	Moree, NSW	Root	*MAT-1*	F19012	Moree, NSW	Stalk 4th n.	*MAT-1*
F18980	Moree, NSW	Root	*MAT-2*	F19013	Moree, NSW	Root	*MAT-1*
F18981	Moree, NSW	Crown	*MAT-1*	F19014	Moree, NSW	Root	*MAT-2*
F18982	Moree, NSW	Root	*MAT-1*	F19016	Moree, NSW	Stalk 4th n.	*MAT-2*
F18983	Moree, NSW	Crown	*MAT-2*	F19017	Moree, NSW	Root	*MAT-1*
F18984	Moree, NSW	Root	*MAT-1*	F19018	Moree, NSW	Stalk 4th n.	*MAT-1*
F18985	Moree, NSW	Crown	*MAT-1*	F19019	Moree, NSW	Crown	*MAT-1*
F18986	Moree, NSW	Root	*MAT-1*	F19020	Moree, NSW	Crown	*MAT-2*
F18987	Moree, NSW	Stalk 4th n.	*MAT-1*	F19021	Moree, NSW	Root	*MAT-1*
F18946	Moree, NSW	Crown	*MAT-2*	F19022	Moree, NSW	Crown	*MAT-1*
F18989	Moree, NSW	Stalk 4th n.	*MAT-1*	F19023	Moree, NSW	Stalk 4th n.	*MAT-1*
F18990	Moree, NSW	Crown	*MAT-1*	F19024	Moree, NSW	Crown	*MAT-1*
Fusarium verticillioides							
2032	Goondiwindi, QLD	Stalk 4th n.	*MAT-2*	2208	Quirindi, NSW	Stalk 4th n.	*MAT-1*
2165	Quirindi, NSW	Stalk 4th n.	*MAT-1*				
Fusarium proliferatum							
F15598	Quirindi, NSW	Stalk 4th n.	*MAT-2*	F15606	Quirindi, NSW	Stalk 4th n.	*MAT-1*
F15599	Quirindi, NSW	Stalk 4th n.	*MAT-2*	F15607	Quirindi, NSW	Stalk 4th n.	*MAT-1*
F15600	Quirindi, NSW	Stalk 4th n.	*MAT-2*	F15608	Quirindi, NSW	Stalk 4th n.	*MAT-1*
F15601	Quirindi, NSW	Stalk 4th n.	*MAT-2*	F18954	Goondiwindi, QLD	Stalk 4th n.	*MAT-2*
F15602	Quirindi, NSW	Stalk 4th n.	*MAT-2*	F18955	Quirindi, NSW	Stalk 4th n.	*MAT-2*
F15603	Quirindi, NSW	Stalk 4th n.	*MAT-2*	F18956	Moree, NSW	Stalk 4th n.	*MAT-2*
F15604	Quirindi, NSW	Stalk 4th n.	*MAT-1*	F18957	Moree, NSW	Stalk 4th n.	*MAT-2*
F15605	Quirindi, NSW	Stalk 4th n.	*MAT-1*	F18958	Moree, NSW	Stalk 4th n.	*MAT-2*

[a] Fusarium Research Laboratory accession number

[b] Isolates recovered either from the fourth or sixth nodes of stalk

[c] Isolate recovered from asymptomatic root

[d] Accession numbers in bold font indicate isolates used in the phylogenetic analysis

†, ‡, •, ∗, ■, ▫, ■, ◊ †, ‡ Isolates from the same plant but different plant parts

5.2. The geographic origins, plant part substrates and mating types of the reference strains

Strain[a]	Geographic origin	Plant part / Substrate	Mating type	Reference
F. andiyazi				
KSU4804 (Q4151)[b]	Natal, South Africa	Sorghum soil debris	*MAT-2*	Marasas *et al.* 2001
KSU4647 (Q4152)	Ethiopia	Grain	*MAT-2*	Marasas *et al.* 2001
KSU10771 (Q4158)	Natal, South Africa	Sorghum soil debris	*MAT-1*	Marasas *et al.* 2001
KSU11155 (RBG 3355)	Colorado, USA	Grain	*MAT-1*	Marasas *et al.* 2001
F15910	Morelos, Mexico	Grain	*MAT-2*	Montes-Belmont, *pers. com.*
F. thapsinum				
Q4721 (MRC 6001)	Natal, South Africa	Root & Crown	*MAT-2*	Klittich *et al.* 1997
Q4722 (MRC 6002)	Natal, South Africa	Root & Crown	*MAT-2*	Klittich *et al.* 1997
Q4723 (MRC 6003)	Natal, South Africa	Root & Crown	*MAT-2*	Klittich *et al.* 1997
Q4724 (MRC 6004)	Natal, South Africa	Root & Crown	*MAT-2*	Klittich *et al.* 1997
Q4725 (MRC 6005)	Natal, South Africa	Root & Crown	*MAT-2*	Klittich *et al.* 1997
Q4726 (MRC 6120)	Natal, South Africa	Sorghum soil debris	*MAT-1*	Klittich *et al.* 1997
Q4727 (MRC 6121)	Natal, South Africa	Sorghum soil debris	*MAT-1*	Klittich *et al.* 1997
Q4728 (MRC 6148)	Transvaal, South Africa	Grain	*MAT-1*	Klittich *et al.* 1997
Q4729 (MRC 6149)	Transvaal, South Africa	Grain	*MAT-1*	Klittich *et al.* 1997
KSU1051	Kansas, USA	Stalk	*MAT-1*	Klittich *et al.* 1997
KSU1087	Kansas, USA	Stalk	*MAT-2*	Klittich *et al.* 1997
KSU1106	Kansas, USA	Stalk	*MAT-2*	Klittich *et al.* 1997
KSU1137	Kansas, USA	Stalk	*MAT-1*	Klittich *et al.* 1997
KSU1183	Kansas, USA	Stalk	*MAT-1*	Klittich *et al.* 1997
KSU1321	Kansas, USA	Stalk	*MAT-1*	Klittich *et al.* 1997
KSU1377	Kansas, USA	Stalk	*MAT-1*	Klittich *et al.* 1997
KSU4093	Tester strain	-	*MAT-2*	Klittich *et al.* 1997
KSU4094	Tester strain	-	*MAT-1*	Klittich *et al.* 1997
Q4732	Texas, USA	Kernel	*MAT-2*	Prom *et al.* 2003
Q4733	Texas, USA	Kernel	*MAT-1*	Prom *et al.* 2003
Q4734	Texas, USA	Kernel	*MAT-1*	Prom *et al.* 2003
F15902	Morelos, Mexico	Mouldy grain	*MAT-2*	Montes-Belmont, *pers. com.*
F15903	Morelos, Mexico	Mouldy grain	*MAT-2*	Montes-Belmont, *pers. com.*
F15904	Morelos, Mexico	Mouldy grain	*MAT-1*	Montes-Belmont, *pers. com.*
F15905	Morelos, Mexico	Mouldy grain	*MAT-2*	Montes-Belmont, *pers. com.*
F15906	Morelos, Mexico	Mouldy grain	*MAT-1*	Montes-Belmont, *pers. com.*
F15907	Morelos, Mexico	Mouldy grain	*MAT-2*	Montes-Belmont, *pers. com.*
F15908	Morelos, Mexico	Mouldy grain	*MAT-1*	Montes-Belmont, *pers. com.*
F15909	Morelos, Mexico	Mouldy grain	*MAT-2*	Montes-Belmont, *pers. com.*
F15911	Morelos, Mexico	Mouldy grain	*MAT-1*	Montes-Belmont, *pers. com.*
F15912	Morelos, Mexico	Mouldy grain	*MAT-2*	Montes-Belmont, *pers. com.*
F15586 (S2)	South Korea	Stalk	*MAT-2*	Lim *et al.* 2001
F15587 (S9)	South Korea	Stalk	*MAT-2*	Lim *et al.* 2001
F15588 (S10)	South Korea	Stalk	*MAT-2*	Lim *et al.* 2001
F15589 (S83)	South Korea	Stalk	*MAT-2*	Lim *et al.* 2001
F15590 (S1)	South Korea	Stalk	*MAT-1*	Lim *et al.* 2001
F15591 (S22)	South Korea	Stalk	*MAT-1*	Lim *et al.* 2001
F15592 (S23)	South Korea	Stalk	*MAT-1*	Lim *et al.* 2001
F15593 (S34)	South Korea	Stalk	*MAT-1*	Lim *et al.* 2001
F. verticillioides				
KSU00149	Tester strain[c]	-	*MAT-1*	
KSU00999	Tester strain	-	*MAT-2*	
F. proliferatum				
KSU04853	Tester strain		*MAT-2*	
KSU04854	Tester strain		*MAT-1*	

[a]Accession number in the collections: Botanic Gardens and Domain Trust, Sydney, Australia (Q and RBG strain number); University of Sydney, Fusarium Research Laboratory, Sydney, Australia (F strain number); Kansas State University, Department of Plant Pathology (KSU strain number); PROMEC,

Medical Research Council, Tygerberg, South Africa (MRC strain number); Soonchunhyang University, Division of Life Sciences, Asan, Korea (S strain number)

[b]Accession numbers highlighted in bold indicate isolates used in the AFLP analysis

[c]Internationally accepted strains of mating populations for sexual compatibility studies

5.3. Elongation factor 1-α gene sequences obtained from GenBank

Species	GenBank Accession Number
F. acutatum	AF160276
F. anthophilum	AF160292
F. brevicatenulatum	AF160265
F. concentricum	AF160282
F. denticulatum	AF160269
F. fractiflexum	AF333931
F. globosum	AF160285
F. inflexum	AF008479
F. lactis	AF160272
F. napiforme	AF160266
F. nygamai	AF160273
F. oxysporum	AF160312
F. phyllophilum	AF160274
F. proliferatum	AF291058
F. proliferatum	AF160289
F. proliferatum	AF336913
F. proliferatum	AY660014
F. proliferatum	DQ016287
F. proliferatum	DQ016288
F. proliferatum	DQ016289
F. pseudoanthophilum	AF160264
F. pseudocircinatum	AF160271
F. pseudonygamai	AF160263
F. ramigenum	AF160267
F. sacchari	AF160278
F. subglutinans	AF160289
F. udum	AF160275
G. circinata	AF160295
G. fujikuroi	AF160279
G. thapsina	AF160270
G. moniliformis	AF160262

Appendix 6 . List of *F. thapsinum* isolates (Chapter 7)

6.1. Biological traits of *F. thapsinum* isolates of plant part and plot populations in Livingston Farm, Moree

Isolate	Site	Mating type	Fertility	Isolate	Site	Mating type	Fertility
Mesocotyl/Root							
1025	Plot F	*MAT-2*	Male	1146	Plot M	*MAT-2*	Male
1026	Plot F	*MAT-2*	Female	1155	Plot M	*MAT-1*	Male
1028	Plot F	*MAT-2*	Male	1173	Plot M	*MAT-2*	Male
1032	Plot F	*MAT-1*	Male	1191	Plot M	*MAT-2*	Male
1033[a]	**Plot F**	***MAT-2***	**Male**	1194	Plot M	*MAT-2*	Male
1039	Plot F	*MAT-2*	Male	1198	Plot M	*MAT-2*	Male
1045	Plot F	*MAT-2*	Male	1200	Plot M	*MAT-1*	Male
1051	Plot F	*MAT-2*	Male	1202	Plot M	*MAT-1*	Male
1069	Plot F	*MAT-1*	Male	1205	Plot M	*MAT-2*	Male

(*Continued*)

Isolate	Site	Mating type	Fertility	Isolate	Site	Mating type	Fertility
1088	Plot F	*MAT-2*	Male	1212	Plot M	*MAT-1*	Male
1091	Plot F	*MAT-1*	Male	1217	Plot M	*MAT-1*	Male
1093	Plot F	*MAT-2*	Male	1236	Plot M	*MAT-1*	Male
1367	Plot F	*MAT-2*	Male	1240•	Plot M	*MAT-2*	Female
1377	Plot F	*MAT-1*	Male	1241•	Plot M	*MAT-2*	Female
1381	Plot F	*MAT-1*	Male	1243	Plot M	*MAT-2*	Male
1388	Plot F	*MAT-2*	Male	1247	Plot M	*MAT-1*	Male
1390	Plot F	*MAT-1*	Male	1433	Plot M	*MAT-1*	Male
1399	Plot F	*MAT-1*	Male	1270	Plot S	*MAT-2*	Male
1401	Plot F	*MAT-1*	Male	1273	Plot S	*MAT-1*	Male
1416	Plot F	*MAT-2*	Male	1279	Plot S	*MAT-2*	Female
1421■	Plot F	*MAT-1*	Male	1280■	Plot S	*MAT-1*	Male
1422■	Plot F	*MAT-2*	Male	1281■	Plot S	*MAT-1*	Male
1428	Plot F	*MAT-2*	Male	1306	Plot S	*MAT-1*	Male
1430	Plot F	*MAT-2*	Male	1310	Plot S	*MAT-1*	Male
1099	Plot M	*MAT-1*	Male	1312	Plot S	*MAT-1*	Male
1114	Plot M	*MAT-1*	Male	1321	Plot S	*MAT-1*	Male
1118	Plot M	*MAT-1*	Male	1325	Plot S	*MAT-2*	Female
1121	Plot M	*MAT-2*	Female	1329	Plot S	*MAT-2*	Male
1127	Plot M	*MAT-1*	Male	1340♦	Plot S	*MAT-1*	Male
1135	Plot M	*MAT-2*	Male	1341♦	Plot S	*MAT-1*	Male
1143	Plot M	*MAT-1*	Male				
Crown							
1024	Plot F	*MAT-1*	Male	1223	Plot M	*MAT-2*	Female
1037□	Plot F	*MAT-2*	Male	1245	Plot M	*MAT-2*	Male
1041	Plot F	*MAT-1*	Male	1252	Plot M	*MAT-2*	Male
1042□	Plot F	*MAT-2*	Male	1267	Plot S	*MAT-1*	Male
1044	Plot F	*MAT-2*	Male	1268	Plot S	*MAT-2*	Male
1050	Plot F	*MAT-2*	Female	1271	Plot S	*MAT-1*	Male
1061	Plot F	*MAT-1*	Male	**1275**	**Plot S**	***MAT-1***	**Male**
1077■	Plot F	*MAT-2*	Male	1276	Plot S	*MAT-2*	Male
1078■	Plot F	*MAT-2*	Male	1277	Plot S	*MAT-1*	Female
1094	Plot F	*MAT-1*	Male	1283	Plot S	*MAT-1*	Male
1369	Plot F	*MAT-2*	Male	1285	Plot S	*MAT-1*	Male
1383	Plot F	*MAT-2*	Male	1296	Plot S	*MAT-1*	Male
1396	Plot F	*MAT-1*	Male	1297	Plot S	*MAT-2*	Female
1398	Plot F	*MAT-1*	Male	1302	Plot S	*MAT-2*	Male
1408	Plot F	*MAT-1*	Male	1307	Plot S	*MAT-1*	Male
1417	Plot F	*MAT-1*	Male	1311	Plot S	*MAT-1*	Male
1423	Plot F	*MAT-2*	Male	1313	Plot S	*MAT-1*	Male
1431	Plot F	*MAT-1*	Male	1319	Plot S	*MAT-2*	Male
1103	Plot M	*MAT-2*	Male	1322	Plot S	*MAT-1*	Male
1112	Plot M	*MAT-2*	Male	1342	Plot S	*MAT-2*	Male
1156	Plot M	*MAT-2*	Male	1344	Plot S	*MAT-2*	Male
1158	Plot M	*MAT-1*	Male	1350	Plot S	*MAT-1*	Male
1182	Plot M	*MAT-1*	Male	1354	Plot S	*MAT-2*	Male
1220	Plot M	*MAT-1*	Male				
Stalk							
1038	Plot F – 6th n.	*MAT-1*	Male	1176☼☼	Plot M – 4th n.	*MAT-1*	Male
1046	Plot F – 4th n.	*MAT-1*	Male	1177☼☼	Plot M – 6th n.	*MAT-2*	Male
1047*	Plot F – 4th n.	*MAT-1*	Male	1183	Plot M – 6th n.	*MAT-1*	Male
1048*	Plot F – 6th n.	*MAT-1*	Male	1190	Plot M – 4th n.	*MAT-1*	Male
1052	Plot F – 4th n.	*MAT-2*	Male	1193	Plot M – 6th n.	*MAT-2*	Female
1060	Plot F – 6th n.	*MAT-1*	Male	1209	Plot M – 4th n.	*MAT-1*	Male
1062	Plot F – 6th n.	*MAT-1*	Male	1222	Plot M – 4th n.	*MAT-1*	Male
1066	Plot F – 4th n.	*MAT-1*	Male	1227	Plot M – 6th n.	*MAT-1*	Male

(*Continued*)

Isolate	Site	Mating type	Fertility	Isolate	Site	Mating type	Fertility
1071**	Plot F – 4th n.	*MAT-2*	Male	1228	Plot M – 6th n.	*MAT-2*	Male
1072**	Plot F – 6th n.	*MAT-1*	Male	1231	Plot M – 6th n.	*MAT-2*	Male
1073	Plot F – 6th n.	*MAT-2*	Male	1237	Plot M – 4th n.	*MAT-2*	Male
1095	Plot F – 6th n.	*MAT-1*	Male	**1242**	**Plot M – 6th n.**	***MAT-2***	**Male**
1096	Plot F – 4th n.	*MAT-2*	Male	1249	Plot M – 4th n.	*MAT-2*	Male
1372	Plot F – 4th n.	*MAT-1*	Male	1256	Plot M – 6th n.	*MAT-1*	Male
1379	Plot F – 4th n.	*MAT-1*	Male	1259	Plot M – 6th n.	*MAT-1*	Female
1386	Plot F – 4th n.	*MAT-1*	Male	1284	Plot S – 4th n.	*MAT-1*	Male
1405	Plot F – 4th n.	*MAT-2*	Female	1293Δ	Plot S – 4th n.	*MAT-1*	Male
1411	Plot F – 4th n.	*MAT-1*	Male	1294Δ	Plot S – 6th n.	*MAT-1*	Male
1414	Plot F – 4th n.	*MAT-2*	Male	1298••	Plot S – 4th n.	*MAT-1*	Male
1420	Plot F – 4th n.	*MAT-2*	Male	1299••	Plot S – 6th n.	*MAT-1*	Male
1100	Plot M – 6th n.	*MAT-2*	Male	1301	Plot S – 6th n.	*MAT-2*	Male
1105	Plot M – 6th n.	*MAT-1*	Male	1303▪▪	Plot S – 6th n.	*MAT-1*	Male
1108	Plot M – 6th n.	*MAT-1*	Male	1304▪▪	Plot S – 4th n.	*MAT-2*	Male
1110	Plot M – 6th n.	*MAT-2*	Male	1305	Plot S – 6th n.	*MAT-2*	Male
1126	Plot M – 6th n.	*MAT-1*	Male	1309	Plot S – 4th n.	*MAT-1*	Male
1131	Plot M – 6th n.	*MAT-2*	Male	1314	Plot S – 6th n.	*MAT-2*	Male
1133‡	Plot M – 4th n.	*MAT-1*	Male	1316	Plot S – 4th n.	*MAT-1*	Male
1134‡	Plot M – 6th n.	*MAT-2*	Male	1326■■	Plot S – 4th n.	*MAT-1*	Male
1141‡‡	Plot M – 4th n.	*MAT-2*	Male	1327■■	Plot S – 6th n.	*MAT-2*	Male
1142‡‡	Plot M – 6th n.	*MAT-2*	Male	1332♦♦	Plot S – 4th n.	*MAT-1*	Male
1149—	Plot M – 4th n.	*MAT-1*	Male	**1333♦♦**	**Plot S – 4th n.**	***MAT-1***	**Male**
1150—	**Plot M – 6th n.**	***MAT-1***	**Male**	1339	Plot S – 6th n.	*MAT-1*	Male
1152	Plot M – 4th n.	*MAT-1*	Male	1343	Plot S – 4th n.	*MAT-2*	Male
1160	Plot M – 6th n.	*MAT-1*	Female	1347	Plot S – 4th n.	*MAT-2*	Male
1162	Plot M – 6th n.	*MAT-2*	Male	1352	Plot S – 6th n.	*MAT-2*	Male
1169	Plot M – 4th n.	*MAT-2*	Male	1356○○	Plot S – 4th n.	*MAT-1*	Male
1170☼	Plot M – 4th n.	*MAT-1*	Female	1357○○	Plot S – 6th n.	*MAT-1*	Male
1171☼	Plot M – 6th n.	*MAT-1*	Male	1359	Plot S – 4th n.	*MAT-1*	Male

[a]Isolates marked in bold type are included into the country/continent levels of comparison

♦, ■, •, ■, □, ■ : Isolates from the same part of plant

*, **, ‡, ‡‡, —, ☼, ☼☼, Δ, ••, ▪▪, ■■, ♦♦, ○○ : Isolates from the 4th and 6th node of the same plant

6.2. Biological traits of *F. thapsinum* isolates of geographic populations Goondiwindi and Quirindi recovered from stalks in the northern grain belt of eastern Australia. Isolates of the Moree population recovered from stalks are listed in Appendix 6.1

Isolate	Site	Mating type	Fertility	Isolate	Site	Mating type	Fertility
Goondiwindi population							
1945	Site 3	*MAT-2*	Male	2036■	Site 1	*MAT-1*	Male
1953♦	Site 3	*MAT-2*	Male	2039	Site 1	*MAT-2*	Male
1954♦	Site 3	*MAT-2*	Male	2040	Site 1	*MAT-2*	Male
1955	Site 3	*MAT-1*	Male	2041	Site 2	*MAT-2*	Male
1957	Site 3	*MAT-1*	Female	2044▲	Site 2	*MAT-2*	Male
1959	Site 3	*MAT-2*	Male	2045▲	Site 2	*MAT-2*	Male
1962	Site 3	*MAT-1*	Male	2046	Site 2	*MAT-1*	Male
1966	Site 3	*MAT-1*	Male	2047◊	Site 2	*MAT-2*	Male
1967	Site 3	*MAT-1*	Male	2048◊	Site 2	*MAT-2*	Male
1969	Site 3	*MAT-2*	Male	2049◊	Site 2	*MAT-2*	Male
1971	Site 3	*MAT-1*	Male	2050	Site 2	*MAT-2*	Male
1972	Site 3	*MAT-1*	Male	2051	Site 2	*MAT-1*	Male
1974	Site 3	*MAT-2*	Male	2054▼	Site 2	*MAT-1*	Male
1976	Site 3	*MAT-1*	Male	2055▼	Site 2	*MAT-1*	Male
1977	Site 3	*MAT-2*	Male	2056	Site 2	*MAT-2*	Male

(*Continued*)

Isolate	Site	Mating type	Fertility	Isolate	Site	Mating type	Fertility
1979	Site 3	*MAT-1*	Male	2057	Site 2	*MAT-2*	Male
1981	Site 3	*MAT-2*	Male	2058	Site 2	*MAT-2*	Male
1987	Site 3	*MAT-1*	Male	2059	Site 2	*MAT-2*	Male
1993	Site 3	*MAT-2*	Male	2062	Site 2	*MAT-2*	Male
1997	Site 3	*MAT-1*	Male	2063◄	Site 2	*MAT-1*	Female
1998	Site 1	*MAT-2*	Male	2064◄	Site 2	*MAT-1*	Male
1999	Site 1	*MAT-1*	Male	2065■	Site 2	*MAT-1*	Male
2003	Site 1	*MAT-1*	Male	2066■	Site 2	*MAT-1*	Male
2004[a]	**Site 1**	***MAT-2***	**Male**	2067►	Site 2	*MAT-2*	Male
2009	Site 1	*MAT-2*	Female	2068►	Site 2	*MAT-2*	Male
2011	Site 1	*MAT-2*	Male	2069◯	Site 2	*MAT-2*	Male
2012	Site 1	*MAT-1*	Male	2070◯	Site 2	*MAT-2*	Male
2013	Site 1	*MAT-1*	Male	2071	Site 2	*MAT-2*	Male
2015	Site 1	*MAT-2*	Male	2075	Site 2	*MAT-1*	Male
2017	Site 1	*MAT-1*	Male	2077	Site 2	*MAT-1*	Male
2018	Site 1	*MAT-1*	Male	2080	Site 2	*MAT-2*	Male
2019	**Site 1**	***MAT-1***	**Male**	2081	Site 2	*MAT-2*	Male
2020	Site 1	*MAT-1*	Male	2082	Site 2	*MAT-2*	Male
2021	**Site 1**	***MAT-2***	**Male**	2083	Site 2	*MAT-1*	Male
2023	Site 1	*MAT-2*	Male	2089	Site 2	*MAT-2*	Male
2025	Site 1	*MAT-1*	Male	2090	Site 2	*MAT-1*	Male
2030	Site 1	*MAT-1*	Male	2091	Site 2	*MAT-2*	Male
2034	Site 1	*MAT-1*	Male	2094	Site 2	*MAT-1*	Male
2035■	Site 1	*MAT-2*	Male	2096	Site 2	*MAT-2*	Male
Quirindi population							
2099	Site 2	*MAT-1*	Male	**2160**	**Site 2**	***MAT-2***	**Male**
2104	Site 2	*MAT-2*	Male	2168	Site 3	*MAT-2*	Male
2109	Site 2	*MAT-1*	Male	2169	Site 3	*MAT-1*	Male
2111	Site 2	*MAT-1*	Male	2173	Site 3	*MAT-2*	Male
2114	Site 2	*MAT-2*	Male	2175◎	Site 3	*MAT-2*	Male
2117	Site 2	*MAT-1*	Male	2176◎	Site 3	*MAT-1*	Male
2120◆	Site 2	*MAT-1*	Male	2182	Site 3	*MAT-1*	Male
2121◆	Site 2	*MAT-1*	Male	2198	Site 3	*MAT-2*	Male
2124	Site 2	*MAT-1*	Male	**2199**	**Site 3**	***MAT-1***	**Male**
2125●	**Site 2**	***MAT-1***	**Male**	2204○	Site 3	*MAT-2*	Male
2126●	Site 2	*MAT-1*	Male	**2205○**	**Site 3**	***MAT-2***	**Male**
2127●	Site 2	*MAT-1*	Male	2210	Site 3	*MAT-1*	Male
2128	Site 2	*MAT-1*	Male	2212□	Site 1	*MAT-2*	Male
2131	Site 2	*MAT-1*	Male	2213□	Site 1	*MAT-2*	Male
2136	Site 2	*MAT-1*	Male	2214■	Site 1	*MAT-2*	Male
2137	Site 2	*MAT-1*	Male	2215■	Site 1	*MAT-2*	Male
2139	Site 2	*MAT-1*	Male	2227	Site 1	*MAT-2*	Female
2140	Site 2	*MAT-1*	Female	2230	Site 1	*MAT-1*	Male
2144	Site 2	*MAT-1*	Male	2237	Site 1	*MAT-1*	Female
2146	Site 2	*MAT-2*	Male	2239	Site 1	*MAT-1*	Male
2147	Site 2	*MAT-1*	Male	2244†	Site 1	*MAT-2*	Male
2150	Site 2	*MAT-1*	Male	2245†	Site 1	*MAT-1*	Male
2151	Site 2	*MAT-1*	Male	2252	Site 1	*MAT-2*	Male
2153	Site 2	*MAT-2*	Male	2256	Site 1	*MAT-1*	Male
2157	Site 2	*MAT-1*	Male				

[a] Isolates marked in bold type are included into the country/continent levels of comparison

◆, ■, ▲, ◊, ▼, ◄, ■, ►, ◯, ◆, ●, ◎, ○, □, ■, † : Isolates from the same 4th node of the plant

6.3. Isolates of *F. thapsinum* from agricultural/non-agricultural ecosystem and different hosts

Isolate	Host	Location	Mating type	Fertility
Agricultural ecosystem				
1033	*Grain sorghum*	Moree, NSW	*MAT-2*	Male
1150	*Grain sorghum*	Moree, NSW	*MAT-1*	Male
1242	*Grain sorghum*	Moree, NSW	*MAT-2*	Male
1275	*Grain sorghum*	Moree, NSW	*MAT-1*	Male
1333	*Grain sorghum*	Moree, NSW	*MAT-1*	Male
2004	*Grain sorghum*	Goondiwindi, QLD	*MAT-2*	Male
2019	*Grain sorghum*	Goondiwindi, QLD	*MAT-1*	Male
2021	*Grain sorghum*	Goondiwindi, QLD	*MAT-2*	Male
2125	*Grain sorghum*	Quirindi, NSW	*MAT-1*	Male
2199	*Grain sorghum*	Quirindi, NSW	*MAT-1*	Male
2160	*Grain sorghum*	Quirindi, NSW	*MAT-2*	Male
2205	*Grain sorghum*	Quirindi, NSW	*MAT-2*	Male
Sh-10	*Sorghum halepense*	Quirindi, NSW	*MAT-2*	Male
Sh-11	*Sorghum halepense*	Breeza, NSW	*MAT-1*	Male
Sh-13	*Sorghum halepense*	Breeza, NSW	*MAT-1*	Male
Sh-14	*Sorghum halepense*	Breeza, NSW	*MAT-1*	Male
Sh-16	*Sorghum halepense*	Breeza, NSW	*MAT-2*	Male
Sh-19	*Sorghum halepense*	Breeza, NSW	*MAT-2*	Female
Sh-28	*Sorghum halepense*	Breeza, NSW	*MAT-2*	Male
Sh-36	*Sorghum halepense*	Moree, NSW	*MAT-1*	Male
Sh-41	*Sorghum halepense*	Moree, NSW	*MAT-1*	Male
Sh-46	*Sorghum halepense*	Moree, NSW	*MAT-1*	Male
Sh-69	*Sorghum halepense*	-, NSW	*MAT-1*	Male
Sh-76	*Sorghum halepense*	-, NSW	*MAT-2*	Male
Sh-77	*Sorghum halepense*	-, NSW	*MAT-1*	Male
Sh-91	*Sorghum halepense*	Livingston Farm, Moree, NSW	*MAT-1*	Male
Sh-97	*Sorghum halepense*	Livingston Farm, Moree, NSW	*MAT-1*	Male
Sh-113	*Sorghum halepense*	Tathra, NSW	*MAT-1*	Male
Sh-114	*Sorghum halepense*	Tathra, NSW	*MAT-1*	Male
Sh-115	*Sorghum halepense*	Tathra, NSW	*MAT-1*	Male
Sh-116	*Sorghum halepense*	Tathra, NSW	*MAT-2*	Male
Sh-117	*Sorghum halepense*	Tathra, NSW	*MAT-1*	Male
Ecg-127	*Echinochloa crus-galli*	Tathra, NSW	*MAT-1*	Male
Non-Agricultural ecosystem				
F14595	*Austrostipa aristiglumis*	Spring Ridge, NSW	*MAT-1*	Male
F14596	*Austrostipa aristiglumis*	Spring Ridge, NSW	*MAT-2*	Male
F14597	*Austrostipa aristiglumis*	Spring Ridge, NSW	*MAT-2*	Male
F14598	*Austrostipa aristiglumis*	Spring Ridge, NSW	*MAT-2*	Male
F14599	*Austrostipa aristiglumis*	Spring Ridge, NSW	*MAT-2*	Male
F14600	*Austrostipa aristiglumis*	Spring Ridge, NSW	*MAT-2*	Male
F14585	*Coix gasteenii*	Lakefield NP, QLD	*MAT-1*	Female
F14586	*Coix gasteenii*	Lakefield NP, QLD	*MAT-1*	Male
F14587	*Coix gasteenii*	Lakefield NP, QLD	*MAT-1*	Male
F14588	*Coix gasteenii*	Lakefield NP, QLD	*MAT-1*	Male
F14589	*Coix gasteenii*	Lakefield NP, QLD	*MAT-1*	Male
F14590	*Coix gasteenii*	Lakefield NP, QLD	*MAT-1*	Male
F14591	*Coix gasteenii*	Lakefield NP, QLD	*MAT-2*	Male
F14592	*Coix gasteenii*	Lakefield NP, QLD	*MAT-2*	Male
F14593	*Coix gasteenii*	Lakefield NP, QLD	*MAT-2*	Male
F15236	*Heteropogon triticeus*	Davies Creek, Mareeba, QLD	*MAT-2*	Male
F14338	*Sorghum interjectum*	Litchfield NP, NT	*MAT-2*	Male
F14339	*Sorghum interjectum*	Litchfield NP, NT	*MAT-2*	Male

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