The evaluation of multiplex PCR and DNA profiling methods (DGGE and SSCP) for the detection of mycotoxigenic *Fusarium* species.

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ABSTRACT

The genus *Fusarium* is an important plant pathogen that is responsible for severe yield losses of economically important plant species. In addition, some *Fusarium* species also produces mycotoxins. There is thus a need for the early detection and identification of *Fusarium* to prevent yield losses and mycotoxin contamination and consumption.

The present study investigated the potential of a multiplex PCR-DGGE and SSCP method for the detection, identification and differentiation of *Fusarium* species. Two extraction methods, i.e. the CTAB-PVP and E.Z.N.A. Fungal DNA Mini Kit were tested for the isolation of fungal DNA. The DNA Mini Kit showed the best results to successfully isolate DNA from the various *Fusarium* species that was amplifiable with the beta-tubulin, EF-1α, 18S and FUM primer sets. Optimized amplification conditions were tested and applied for each primer set. EF-1α, FUM and 18S primer sets were combined in multiplex PCR because they yielded amplification products of distinct sizes. However, preferential amplification of the 18S rDNA region occurred with this combination. Therefore, multiplex PCR was performed with the EF-1α and FUM primer sets which permitted the detection of fumonisin positive *Fusarium* species.

Subsequent DGGE and SSCP analysis of the EF-1α fragments from the multiplex PCR showed that DGGE was not sufficient in discriminating between the *Fusarium* isolates while SSCP permitted clear differentiation. However, multiple banding patterns for a single species were observed with both profiling methods. This can impede interpretation of results and may also lead to wrong conclusions.

Limits of detection were also determined for fumonisin producing *Fusarium* species individually and in combination with non-*Fusarium* species through conventional and real-time PCR. The real-time PCR method proved to be more sensitive in detecting small amounts of fungal DNA than conventional PCR. The sensitivity and accuracy of this method would allow the quantification of toxigenic *Fusarium* species in contaminated soil and plant tissues. As a result, proper control and management strategies can be executed in time to prevent the occurrence of devastating diseases and yield losses.

Sequences of the beta-tubulin and EF-1α genes were analyzed to determine phylogenetic relationships between various the *Fusarium* isolates. Sequencing of the amplified fragments indicated conflict between GenBank and MRC/PPRI identities for several *Fusarium* isolates. This conflict was observed for both protein-coding genes. Phylogenetic relationships between the various *Fusarium* isolates were more accurate with the EF-1α gene sequences than the beta-tubulin sequences.

This study demonstrated the potential of a multiplex PCR-SSCP method to detect and identify *Fusarium* species. With further careful optimization, this technique can be applied to contaminated food and feed samples to assess *Fusarium* diversity.

OPSOMMING

Die genus *Fusarium* is 'n belangrike plant patogeen omdat dit verantwoordelik is vir ernstige oes verliese en boonop vervaardig sommige *Fusarium* spesies ook mikotoksiene. Dus is daar 'n behoefte om *Fusarium* vroegtydig op te spoor en te identifiseer om sodoende oes verliese en mikotoksien kontaminasie en inname te voorkom.

Die huidige studie het ondersoek ingestel na die potensiaal van 'n veelvoudige PCR-DGGE en SSCP metode vir die deteksie, identifikasie en differensiasie van *Fusarium* spesies. Twee ekstraksie metodes, CTAB-PVP en E.Z.N.A. Fungal DNA Mini Kit, was getoets vir die isolasie van fungal DNS. Goeie kwaliteit DNS is met die DNA Mini Kit geïsoleer en was amplifiseerbaar met die beta-tubulin, EF-1α, 18S en FUM primer stelle. Optimum amplifisering kondisies was getoets en toegepas vir elke primer stel. Die EF-1α, FUM en 18S primer stelle is saamgevoeg vir 'n veelvoudige PCR omdat elke primer stel verskillende grootte amplifiserings produkte vervaardig het wat ideal is vir 'n veelvoudige PCR. Hierdie kombinasie het egter die amplifisering van die 18S rDNA geen begunstig. Dus is die veelvoudige PCR uitgevoer met net die EF-1α en FUM primer stelle wat die deteksie van fumonisin produserende *Fusarium* spesies moontlik gemaak het.

DGGE en SSCP ontleding van die EF-1α fragmente het bewys dat die DGGE metode nie in staat was om die *Fusarium* isolate van mekaar te onderskei nie, terwyl SSCP analise duidelike differensiasie toegelaat het. Veelvoudige bande vir 'n enkel spesie en strain was met albei tegnieke waargeneem. Hierdie bande kan interpretasie bemoeilik en selfs lei to foutiewe gevolgtrekkings.

Deteksie limiete vir fumonisin produserende *Fusarium* spesies, individueel en in kombinasie met nie-*Fusarium* spesies, is vasgestel met behulp van konvensionele en real-time PCR. Laasgenoemde metode was meer sensitief deurdat dit in staat was om klein hoeveelhede fungal DNA op te spoor. Die sensitiwiteit en noukeurigheid van hierdie metode sal kwantifisering van toksigeniese *Fusarium* spesies in gekontamineerde grond en plant weefsel moontlik maak. Sodoende kan behoorlike bestuur strategieë betyds toegepas word om verwoesting van oeste te voorkom.

Die nukleotied volgorde van die beta-tubulin en EF-1α gene was geanaliseer om filogenetiese verhoudings tussen die verskeie *Fusarium* isolate te bepaal. Nukleotied analise van die geamplifiseerde fragmente het konflik tussen die GenBank en MRC/PPRI identiteite vir verskeie *Fusarium* isolate aangewys. Hierdie konflik was met albei proteien-koderende gene waargeneem. Die filogenetiese verhouding tussen verskeie *Fusarium* isolate was meer akkuraat met die EF-1α geen as met die beta-tubulin geen.

Hierdie studie demonstreer die potensiaal van 'n veelvoudige PCR-SSCP metode vir die deteksie en identifikasie van *Fusarium* spesies. Indien hierdie tegniek verder geoptimiseer word, kan dit op gekontamineerde voedsel monsters toegepas word om die *Fusarium* diversiteit te bepaal.

Hierdie werk word opgedra aan my Skepper, geliefde ouers, susters, ouma en oupa en my dierbare vriend Gustav. Julle gebede, ondersteuning, positiwiteit en liefde het my gedra en gemotiveer die afgelope twee jaar.
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DECLARATION

I declare that the dissertation for the degree of Master of Environmental Science (M.Env.Sc) at the North-West University: Potchefstroom Campus hereby submitted, has not been submitted by me for a degree at this or another University, that it is my own work in design and execution, and that all material contained herein has been duly acknowledged.

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Karen Jordaan **Date**

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CHAPTER 1

INTRODUCTION

1.1 GENERAL INTRODUCTION AND PROBLEM STATEMENT

Fusarium is a phytopathogenic fungus and is globally distributed on a wide range of crop plants, including maize, wheat, barley and asparagus (Gherbawy *et al.,* 2001; Fandohan *et al.,* 2003; Krstanović *et al.,* 2005; Yergeau *et al.,* 2005; Jurado *et al.,* 2006). This is a diverse and important genus since several *Fusarium* species are responsible for damping-off, root rots, cankers and vascular wilts on a large number of economically important crops (Nelson *et al.,* 1981). A particular devastating disease caused by *Fusarium* species is *Fusarium* head blight (scab) of wheat and barley. Scab causes considerable reduction in seed yields and quality and infested seeds are often contaminated with mycotoxins creating a serious danger to animal health and food safety (McMullen *et al.,* 1997; Salas *et al.,* 1999). *Fusarium* head blight is a growing threat to the world's food supply due to outbreaks throughout much of the world (Dubin *et al.,* 1997; McMullen *et al.,* 1997; Osborne & Stein, 2007). Therefore, *Fusarium* is a major agricultural problem since quality and yield of grains can be decreased. In addition, many species are mycotoxin producers (Marasas *et al.,* 1984; Marasas, 1987; Leslie & Summerell, 2006).

Mycotoxins are secondary metabolites produced by certain fungal species that are common contaminants of agricultural products and harmful to both animals and humans (Nelson *et al.,* 1994; Sweeney & Dobson, 1998; Bennett & Klich, 2003). Five mycotoxins are considered to be economically and toxicologically important in grain and several areas throughout the world: aflatoxin and ochratoxin, produced by *Aspergillus* and *Penicillium* (Bottalico, 1998; Sweeney & Dobson, 1998), deoxynivalenol and zearalenone, produced by *Fusarium graminearum*, and

fumonisins produced by *Fusarium verticillioides* (Bottalico, 1998; Sweeney & Dobson, 1998; Leslie & Summerell, 2006) and *Fusarium proliferatum* (Castelo *et al.,* 1998; Sweeney & Dobson, 1998; Leslie & Summerell, 2006). These compounds cause diseases of animals and humans, especially in immunocompromised individuals (Rebell, 1981; Nelson *et al.,* 1994; Dignani & Anaissie, 2004; Jensen *et al.,* 2004). The most common *Fusarium* mycotoxins in cereals are considered to be fumonisins and trichothecenes (Jurado *et al.,* 2006).

Fumonisins are mainly produced in maize kernels infected with *F. verticillioides* prior to harvest and are stable through grain processing (Bullerman *et al.,* 2002). Twenty eight fumonisin analogs have been characterized and can be separated into four main groups, identified as the fumonisin A, B, C, and P series (Rheeder *et al.,* 2002). The fumonisin B (FB) analogs are the most abundant naturally occurring fumonisins (Marasas, 1996) and most studied is fumonisin B_1 (FB_1) (Nelson *et al.,* 1993; Bennett & Klich, 2003). Consumption of fumonisins has been shown to cause leucoencephalomalacia in horses (Marasas *et al.,* 1988), pulmonary edema and hydrothorax in pigs (Harrison *et al.,* 1990) and hepatotoxic and carcinogenic effects in rats (Gelderblom *et al.,* 1996). The occurrence of FB_1 has also been epidemiologically associated with human esophageal cancer and birth defects (Chu & Li, 1994; Yoshizawa *et al.,* 1994; Marasas *et al.,* 2004).

Trichothecenes comprise a large family of compounds, of which diacetoxyscirpenol, T-2 toxin, nivalenol and deoxynivalenol are most important in cereal grains (Desjardins & Proctor, 2007). At least eight fungal genera produce trichothecenes, with *Fusarium* being the most economically important group (Ueno, 1983). Of all *Fusarium* mycotoxins discovered to date, trichothecenes have been most strongly associated with chronic and fatal toxicoses of humans and animals (Hussein & Brasel, 2001; Sudakin, 2003; Pestka & Smolinski, 2005; Pinton *et al.,* 2008). They are commonly found as food and feed contaminants and consumption can result in alimentary

hemorrhage, diarrhea, vomiting and cardiovascular dysfunction resembling endotoxic shock (Hussein & Brasel, 2001; Larsen *et al.,* 2004; Parent-Massin, 2004; Pestka & Smolinski, 2005).

Taking into account the adverse effects of trichothecenes and fumonisins, early detection of *Fusarium* species on subsistence and commercial crops is vital to prevent these mycotoxins entering the food chain (Jurado *et al.,* 2006). It is also a useful tool in disease management since it provides information required for determining the need and the extent of proper control strategies (Lievens *et al.,* 2006).

The detection and identification of *Fusarium* species in pure culture or in diseased plant material are based on physiological and morphological characteristics (Nelson *et al.,* 1983; Nelson, 1992; Leslie & Summerell, 2006). These methods rely on the ability of the organism to be cultured, it is time consuming and labor intensive and require skilled taxonomical expertise (McCartney *et al.,* 2003; Lievens *et al.,* 2005). With only the use of conventional methods, the detection of the pathogen on diseased plant material is only possible in late stages of infection and the spread of the disease cannot be controlled anymore (McCartney *et al.,* 2003).

DNA-based molecular methods have been developed to study the phylogeny of *Fusarium* and to distinguish *Fusarium* species, formae speciales and strains (Donaldson *et al.,* 1995; Abdel-Satar *et al.,* 2003; Kosiak *et al.,* 2005; Yergeau *et al.,* 2005; Jurado *et al.,* 2006). Molecular methods most frequently used for the characterization of *Fusarium* species include PCR, real-time PCR, denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (t-RFLP), Amplified Fragment Length Polymorphisms (AFLP) and sequence analysis (Abdel-Satar *et al.,* 2003; Geiser *et al.,* 2004; Konstantinova & Yli-Mattila, 2004; Yergeau *et al.,* 2005; Jurado *et al.,* 2006; Fredlund *et al.,* 2008). In general, molecular techniques

are more sensitive, rapid, accurate and specific for pure *Fusarium* cultures and contaminated samples than the conventional techniques. In addition, there is no need to culture organisms prior to their identification (McCartney *et al.,* 2003; Lievens *et al.,* 2005; Jurado et al., 2006).

1.2 RESEARCH AIM AND OBJECTIVES

The aim of this study was to evaluate multiplex PCR and DNA profiling methods (DGGE and SSCP) for the detection of mycotoxigenic *Fusarium* spp.

Objectives were to:

i. Perform and optimize multiplex PCR with EF-1α, FUM and 18S primers;
ii. Optimize and evaluate PCR-DGGE methods for *Fusarium* diversity using 18S rDNA-and EF-1α fragments;
iii. Optimize and evaluate PCR-SSCP methods for *Fusarium* diversity using beta-tubulin and EF-1α fragments;
iv. Compare PCR-DGGE to PCR-SSCP for differentiation of *Fusarium* species;
v. Determine the detection limits of EF-1α and FUM DNA with RT-PCR; and
vi. Analyze the sequences of the EF-1α and beta-tubulin genes for phylogenetic relationships.

CHAPTER 2

LITERATURE OVERVIEW

2.1 Commercial and subsistence agriculture in South Africa

South Africa has a dual agricultural economy including commercial and subsistence production, with well-developed commercial farming and more subsistence-based production in the rural areas (Crawford, 2007). Commercial agriculture plays an important role in the economy and development of South Africa and is a large provider of employment, especially in the rural areas, and a major earner of foreign exchange (Directorate Agricultural Statistics, Department of Agriculture, South Africa, 2007). The value of commercial agricultural production in South Africa was R96 billion in 2007, while its contribution to the gross domestic product (GDP) was approximately R49 billion. The primary agricultural sector has grown by an average of approximately 11.8 % per annum since 1970, while the total economic growth was 14.9 % per annum over the same period, resulting in a decline in agriculture's share of the GDP from 7.1 % in 1970 to 2.3 % in 2006 (Directorate Agricultural Statistics, Department of Agriculture, South Africa, 2007).

Subsistence agriculture, especially in rural areas of South Africa, is vital in food production and food security due to mass poverty that currently exists (Khumbane, 1997; Jansen van Rensburg *et al.,* 2004). The indigenous people of South Africa depend largely on traditional food plants that vary from maize, sorghum, millet, dry beans, peanuts, melons, sweet cane, wild dark-green leafy vegetables (*morogo*) and the traditional pumpkin (Khumbane, 1997; Van Wyk & Gericke, 2000; Jansen van Rensburg *et al.,* 2007). Of these, *morogo* are probably the most widely occurring vegetables in South Africa and Africa in general (Guarino, 1997; Jansen van Rensburg *et al.,*

2004). During the production season they reportedly provide some societies with as much as a quarter of the daily protein intake (National Research Council, 2006). The leaves contain high amounts of protein, (25% for *Amaranthus cruentus*), more lysine than quality-protein maize (high-lysine corn) and more methionine than soybean meal. In addition, vitamins A and C, and minerals such as calcium and iron, occur in fair quantities (Jansen van Rensburg *et al.*, 2004; National Research Council, 2006). Thus, *morogo* are nutritionally important vegetables for subsistence households. Without these plants, the hidden hunger of malnutrition in rural areas would be much worse. With them in greater use, it can be greatly reduced (National Research Council, 2006).

Commercial and subsistence crops experience biotic and abiotic stresses, e.g. insect and pathogen attack, variation in precipitation and temperature, salinity and anaerobic conditions (Strange & Scott, 2005; Li *et al.,* 2008; Zhou & Shao, 2008). The greatest challenge to crop production is the fight against different pests, weeds and diseases (Vidhyasekaran, 2007). Plant diseases cause major yield losses every year and have impacted the well-being of humans worldwide (Agrios, 1997; Anderson *et al.,* 2004). Plant pathogens may reduce yield by causing tissue lesions; reducing leaf, root, or seed growth; or by clogging vascular tissues and causing wilt (Lucas & Dickinson, 1998; Bent, 2003; Rangaswami & Bagyaraj, 2005). Even in the absence of symptoms, pathogens can cause a general metabolic drain that reduces plant productivity. Pathogens may also cause pre- or postharvest damage to the harvested product (Bent, 2003; Strange & Scott, 2005). Thus, the effects of plant pathogens can range from mild symptoms to catastrophes in which large areas of food crops are destroyed (Strange & Scott, 2005) which worsen food supply and food insecurity, particularly in rural households.

Among the organisms responsible for plant diseases, pathogenic fungi, in particular *Fusarium* species has been studied extensively (Nelson *et al.,* 1981; Nelson *et al.,* 1983; Summerell *et al.,* 2001; Leslie & Summerell, 2006; Wakelin *et al.,* 2008). The genus *Fusarium* consists of several important pathogens that infest commercial and subsistence crops such as maize, wheat, barley, asparagus and wild *morogo* vegetables (Stack, 2003; Yergeau *et al.,* 2005; Van der Walt *et al.,* 2008; Wakelin *et al.,* 2008). Infections of grains with *Fusarium* species is of economic importance since they are responsible for severe losses yearly due to lower yield and quality of the grains (Jurado *et al.,* 2005). Apart from affecting crop yields, these species produce mycotoxins (Marasas *et al.,* 1984; Marasas, 1987; Leslie & Summerell, 2006).

Mycotoxins are secondary metabolites produced by specific fungal species that are common contaminants of agricultural products (Bennett & Klich, 2003). Poor harvesting practices, improper storage and less than optimal conditions during transportation, marketing and processing can also contribute to fungal growth and increase the risk of mycotoxin production (Magan & Aldred, 2007; Wagacha & Muthomi, 2008). These climatic conditions as well as the food production chains are characteristic in most parts of Africa (Wagacha & Muthomi, 2008). The largest mycotoxin-poisoning epidemic in a decade was reported in Africa (CDC, 2004; Lewis *et al.,* 2005). Mycotoxin management methods cannot practically be used in developing countries because of inadequate food systems and technological infrastructure resulting in uncontrolled mycotoxin levels in food (Wagacha & Muthomi, 2008). This situation is made worse by the fact that staple diets in many rural households are based on cereal crops such as maize, which are highly susceptible to mycotoxin contamination (Wagacha & Muthomi, 2008).

Mycotoxins have been known to be a hazard to human and animal health for many years, and ingestion of foods or feeds made toxic by these metabolites can cause mycotoxicoses (Nelson *et*

al., 1994; Bennett & Klich, 2003). Some of the diseases associated with toxigenic *Fusarium* species in humans and animals include alimentary toxic aleukia, Urov or Kashin-Beck disease, hemorrhagic syndrome and estrogenic syndrome (Nelson *et al.,* 1994). *Fusarium* can also cause infections which can be locally invasive or disseminated (Dignani & Anaissie, 2004). More recently, *Fusarium* species have become important pathogens of individuals with compromised immune systems (Anaissie *et al.,* 1986; Anaissie *et al.,* 1992; Rabodonirina *et al.,* 1994; Jensen *et al.,* 2004) and are associated with high morbidity and mortality (Walsh *et al.,* 1996; de Pauw & Meunier, 1999). In severely immunosuppressed patients, *Fusarium* can cause disseminated disease and has recently emerged as the second most common pathogenic mould (after *Aspergillus*) in high-risk patients with haematological cancer, and in recipients of solid organ (Musa *et al.,* 2000; Sampathkumar & Paya, 2001; Husain *et al.,* 2003) and allogeneic bone marrow or stem cell transplants (Boutati & Anaissie, 1997; Krcmery *et al.,* 1997; Ascioglu *et al.,* 2002; Marr *et al.,* 2002).

Mycotoxigenic *Fusarium* species mainly produce fumonisins, trichothecenes and zearalenones (Desjardins & Proctor, 2007). Fumonisins were discovered and characterized in 1988 by Bezuidenhout *et al.* Up to 28 fumonisin analogs have been identified of which fumonisin B (FB) group is predominant. The most abundantly produced member of the FB group is fumonisin B1 (FB1) (Marasas, 1996). Fumonisins have been described as sphingosine analogue mycotoxins because of the structural similarities they share with the sphingolipid intermediate sphingosine (Butchko *et al.,* 2003). They are responsible for the inhibition of sphingolipid metabolism, which can have diverse and complex effects in animal systems (Desjardins & Proctor, 2007). Consumption of fumonisin-contaminated feed has been associated with various mycotoxicoses including leukoencephalomalacia in horses (Desjardins & Proctor, 2007), porcine pulmonary edema (Harrison *et al.,* 1990), hepatocarcinoma in rats (Gelderblom *et al.,* 2001), and atherogenic

effects in vervet monkey (Fincham *et al.,* 1992). Although there is no direct evidence of adverse effects of fumonisins on human health (Shephard *et al.,* 1996), studies have shown that these toxins are associated with high incidences of esophageal cancer in South Africa (Marasas, 2001), China (Chu & Li, 1994), Italy (Franceschi *et al.,* 1990) and Iran (Shephard *et al.,* 2000). The Joint FAO/WHO Expert Committee on Food Additives (JEFCA) has set a tolerable daily intake of 2 μg/kg of body weight/day for the total fumonisins (B_1, B_2 and B_3), alone or in combination.

Trichothecenes consist of of a large family of compounds, of which diacetoxyscirpenol, T-2 toxin, nivalenol, and deoxynivalenol are most important in cereal grains (Desjardins & Proctor, 2007). They are commonly found as food and feed contaminants and consumption of these toxins can result in alimentary haemorrhage and vomiting (Joffe, 1986). Thrichothecenes have been associated with chronic and fatal toxicoses of humans and animals, including alimentary toxic aleukia in Russia and Central Asia, Akakabi-byo (red mold disease) in Japan, and swine feed refusal in the USA (Desjardins & Proctor, 2007). Ingestion of deoxynivalenol in high quantities by agricultural animals causes nausea, vomiting and diarrhoea. At lower quantities, pigs and other farm animals display weight loss and food refusal (Rotter *et al.,* 1996). For this reason, deoxynivalenol is sometimes called vomitoxin or food refusal factor (Miller *et al.,* 2001). The symptoms produced by the various trichothecenes include effects on almost every major system of the vertebrate body. Many of these effects are due to secondary processes that are initiated by metabolic mechanisms related to the inhibition of protein synthesis (Bennett & Klich, 2003).

Zearalenone is a non-steroidal oestrogenic mycotoxin produced by several *Fusarium* species including *F. gaminearum, F. culmorum, F. equiseti* and *F. crookwellense* (Kumar *et al.,* 2008). All of these *Fusarium* species are regular contaminants of cereal crops worldwide (Hagler *et al.,* 2001). It has been associated in numerous mycotoxicoses in farm animals causing infertility,

abortion or other breeding problems especially in pigs (Kumar *et al.,* 2008). As little as 0.1-5 mg/kg zearalenone in feed may produce estrogenic syndrome in pigs. Also, uterine prolepses can occur in young pigs at concentrations as low as 1mg/kg (Kumar *et al.,* 2008). The recommended safe human intake of zearalenone is estimated to be below 0.05 μg/kg of body weight per day (Kuiper-Goodman *et al.,* 1987). Because of its biological influence and regular dietary co-occurrence with mycotoxins such as fumonisins and trichothecenes, the potential of zearalenone to cause adverse health effects should not be ignored (Bennett & Klich, 2003).

Reflecting on adverse effects of toxigenic *Fusarium* species on agricultural crops and the implications of mycotoxins on human and animal health, it is of utmost importance for the early detection and identification of *Fusarium* species. Using culture-dependent methods for the identification of *Fusarium* species on crops and in food is problematic; the genus is diverse and conflicting taxonomic organizations have been suggested (Nelson, 1991). At present, identification and differentiation of *Fusarium* species are based on morphological characteristics such as the shape and size of the macroconidia, the presence or absence of microconidia, chlamydospores and colony morphology (Nelson *et al.,* 1983; Nelson *et al.,* 1994; Summerell *et al.,* 2003; Leslie & Summerell, 2006). This process is time consuming and requires extensive training and expertise (Bluhm *et al.,* 2002; Bluhm *et al.,* 2004). In many cases, minor morphological differences delineate species, making identification even more difficult (Nelson, 1991). Since the different *Fusarium* species also have different mycotoxin profiles, the accurate determination of the *Fusarium* species present is vital to predict the potential risk of the *Fusarium* isolate (Jurado *et al.,* 2006). Thus, a rapid and reliable method for the routine identification of toxigenic *Fusarium* species would benefit the food-processing industry (Bluhm *et al.,* 2002), subsistence agriculture, and human and animal health.

2.2 Molecular methods employed for fungal identification studies

Various PCR assays have been developed for rapid detection and identification of mycotoxigenic *Fusarium* species, some of them based on single copy genes directly involved in mycotoxin biosynthesis (Niessen & Vogel, 1998; Bluhm *et al.,* 2002; González-Jaén *et al.,* 2004; Kulik *et al.,* 2004). These methods are specific, since identification is made on the basis of genotypic differences, and are highly sensitive, detecting target DNA molecules in complex mixtures (Jurado *et al.,* 2006). Several researchers have used PCR-based assays to target the genes directly involved in mycotoxin biosynthesis, including *TR15*, *TR16*, *TR17* (trichothecene biosynthesis), and *FUM1* (fumonisin biosynthesis), to identify groups of toxigenic *Fusarium* spp. (Niessen & Vogel, 1998; Lee *et al.,* 2001; Bakan *et al.,* 2002; Bluhm *et al.,* 2002).

2.2.1 Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) is a sensitive, rapid and highly specific method that can be applied for the detection and screening of fungal DNA from environmental samples (Atkins & Clark, 2004; Demeke *et al.,* 2005), especially mycotoxigenic fungi (Marek *et al.,* 2003; Haughland *et al.,* 2004). Several researchers, including Bluhm *et al.* (2002, 2004); Demeke *et al.* (2005); and Jurado *et al.* (2006) have focused on the use of PCR-based techniques for the detection and identification of *Fusarium* species.

The development of primers to target specific regions of DNA has lead to greater understanding of fungal ecology, fungal-plant interactions, fungal-pest interactions and fungal-fungal interactions. As more information becomes available regarding fungal genomics and gene function, the greater the possibility of PCR technologies becomes (Atkins & Clark, 2004). Species-specific primers have been used for PCR detection and screening of various *Fusarium* species, including *F. acuminatum* (Williams *et al.,* 2002), *F. avenaceum* (Schilling *et al.,* 1996;

Doohan *et al.*, 1998; Waalwijk *et al.*, 2003; Waalwijk *et al.*, 2004), *F. crookwellense* (Yoder & Christianson 1998), *F. culmorum* (Nicholson *et al.*, 1998), *F. equiseti* (Mishra *et al.*, 2003), *F. graminearum* (Nicholson *et al.*, 1998; Waalwijk *et al.*, 2003; Waalwijk *et al.*, 2004), *F. poae* (Parry & Nicholson, 1996), and *F. pseudograminearum* (Aoki & O'Donnell, 1999).

The benefits of using PCR assays instead of culture-dependent methods for fungal diagnostics are that PCR's can be performed routinely and do not require a high level of expertise for interpretation of the results (Atkins & Clark, 2004). PCR assays also enable the assessment of the potential contamination of plant products with certain mycotoxins and to determine the potential risk to human and animal health (Kulik *et al.*, 2007).

2.2.2 Multiplex PCR

Multiplex PCR is a key technology for a wide variety of applications, including the diagnosis of infectious diseases and the identification of pathogens (Edwards & Gibbs, 1994). This PCR-based method is a modification of PCR in which two or more loci are simultaneously amplified in the same reaction (Henegariu *et al.*, 1997). This is achieved by adding more than one primer pair to the PCR reaction mixture (Schoske *et al.*, 2003). However, there are limits to how many primers can be included into a single test tube. Tettelin *et al.* (1999) had success using up to 30 primers under specific experimental conditions. The general sensitivity and specificity of multiplex PCR are significantly affected by the first few rounds of thermal cycling (Ruano *et al.*, 1991). Overall success of amplification of the target sequence depends on (i) the rate at which the primers anneal to the target sequence; and (ii) the rate at which the annealed primers are extended alongside the desired sequence during the amplification cycles (Elnifro *et al.*, 2000).

The optimization of multiplex PCR can cause several difficulties, including reduced sensitivity or specificity and/or preferential amplification of certain specific targets (Polz, & Cavanaugh, 1998). The presence of more than one primer pair in the multiplex PCR increases the chance of obtaining false amplification products, primarily because of the formation of primer dimers (Brownie *et al.,* 1997). These nonspecific products may be amplified more efficiently than the desired target, consuming reaction components and producing impaired rates of annealing and extension. Thus, the optimization of multiplex PCR should aim to minimize or reduce such nonspecific interactions (Elnifro *et al.,* 2000).

The main advantage of this method is that it saves time and effort in the laboratory without compromising the effectiveness of the test (Elnifro *et al.,* 2000). It has also been shown to be a valuable technique for the detection and identification in environmental samples and clinical specimens (Amicucci *et al.,* 2000; Fraaije *et al.,* 2001; Fujita *et al.,* 2001; Gariepy *et al.,* 2003; Nagao *et al.,* 2005). Luo & Mitchell, (2002) and Nagao *et al.* (2005) have used this method for the identification of fungi, especially pathogenic fungi. Luo & Mitchell, (2002) were able to identify up to three suspected pathogens in a single PCR reaction, and Nagao *et al.* (2005) could specifically detect pathogenic *Rhizopus* DNA from three mucormycosis patients. Multiplex PCR-based detection of trichothecene- and fumonisin-producing *Fusarium* species has been used only by a few researchers including Bluhm *et al.* (2002), Bluhm *et al.* (2004), Demeke *et al.* (2005), and Bezuidenhout *et al.* (2006a). In these studies, multiplex PCR was able to detect the genus *Fusarium* as well as group-specific trichothecene- and/or fumonisin-producing *Fusarium* species (Bluhm *et al.,* 2002).

2.2.3 Real-time PCR

Although the conventional PCR is an effective method for the detection and identification of microorganisms, it does not allow accurate quantification of DNA due to variability in the efficiency of amplification between PCR tubes (Raeymaekers, 1998). In real-time PCR, DNA amplification is monitored by measuring the fluorescence signal accumulated by amplification products (García de Viedma, 2003). One can directly observe the amplification reaction while it is occurring due to the use of fluorescence labels and the inclusion of optical devices (García de Viedma, 2003). The initial target DNA concentration/copy number can be calculated by means of a threshold cycle (Ct) defined as the cycle number at which there is a statistically significant increase in fluorescence (Atkins & Clark, 2004). By comparing the Ct value of the target DNA to the Ct value obtained from a standard curve, the amount of DNA or the copy number of target DNA initially present in the sample can be determined (Heid *et al.*, 1996).

Quantitative real-time PCR techniques, especially SYBR Green I and TaqMan probe based assays, has been successfully applied in the detection and quantification of fungi in environmental samples and clinical specimens (Kaiser *et al.*, 2001; Hsu *et al.*, 2003; Atoui *et al.*, 2007; Fredlund *et al.*, 2008; Gurjar *et al.*, 2008). Important fungal pathogens such as *Fusarium* spp., *Verticillium* spp., *Aspergillus* spp. and *Candida* spp. have been detected and quantified with this method (Haugland *et al.*, 2002; Hsu *et al.*, 2003; Bluhm *et al.*, 2004; Lievens *et al.*, 2006; Jungebloud *et al.*, 2007). Real-time PCR detection assays of toxigenic *Fusarium* species in grains and food have been developed by various researchers, including Schnerr *et al.* (2001), Bluhm *et al.* (2004), and Lopez-Errasquin *et al.* (2007). These assays can be used to quickly evaluate the toxigenic potential of grains destined for food processing procedures (Bluhm *et al.*, 2004) in which possible toxigenic *Fusarium* species can adversely affect food quality by producing mycotoxins (Marasas *et al.*, 1984; Marasas, 1987; Leslie & Summerell, 2006).

Real-time PCR has distinct advantages which makes it the choice for several studies. Compared to other PCR-based techniques available, real-time PCR allows quantification of nucleic acids over a wide range (at least 5 log units) (Atkins & Clark, 2004; Valasek & Repa, 2005; Rebrikov & Trofimov, 2006). This method is also extremely sensitive and allows the detection of less than five copies of a target sequence, making it possible to analyze small samples (Valasek & Repa, 2005; Espy *et al.,* 2006). In addition, real-time PCR is relatively quick and reactions are performed in a closed vessel that requires no post-PCR manipulations, thereby minimizing the chances for cross contamination (Atkins & Clark, 2004; Valasek & Repa, 2005; Espy *et al.,* 2006; Rebrikov & Trofimov, 2006). However, there are several limitations to real-time PCR methods. Real-time PCR is susceptible to PCR inhibition by compounds present in certain biological samples (Valasek & Repa, 2005; Carey *et al.,* 2007). Common inhibitors include food components (e.g., organic and phenolic compounds, glycogen, fats, and Ca^{2+}) and environmental compounds (e.g., phenolic compounds, humic acids, and heavy metals) (Wilson, 1997). Possibly the main limitation of real-time PCR is human error: improper assay development, incorrect data analysis, or unjustifiable conclusions (Valasek & Repa, 2005).

2.2.4 Denaturing gradient gel electrophoresis (DGGE)

DGGE is an electrophoretic method capable of detecting differences between DNA fragments of the same size but with different sequences (Muyzer & Smalla, 1998; Ercolini, 2004). The DNA fragments can be separated in a denaturing gradient gel based on their differential denaturation (melting) profile (Ercolini, 2004). The theoretical aspects of DGGE were first described by Fisher & Lerman (1983). In a polyacrylamide gel, the denaturing conditions are provided by urea and formamide. Low and high denaturing solutions are prepared and poured in a gel casting by means of a gradient former. Electrophoresis is then carried out at a constant temperature between 55 and 65°C (Ercolini, 2004).

In a DGGE gel, double-strand DNA fragments are subjected to an increasing denaturing environment (Ercolini, 2004). The double-strand fragments will start to melt according to their melting domains: stretches of base-pairs with an identical melting temperature (Muyzer & Smalla, 1998). The melting temperature (T*m*) of these domains is sequence-specific. Once the T*m* of the lowest melting domain is reached, that part of the fragment becomes partially melted, creating a branched molecule (Ercolini, 2004). This will stop the migration of the fragment in the gel (Muyzer & Smalla, 1998; Ercolini, 2004). Sequence variations within the melting domains of different DNA fragments causes the melting temperatures to differ. Thus, DNA fragments with different sequences will stop migrating at different positions in the gel (Muyzer & Smalla, 1998).

By using DGGE, 50% of the sequence variants can be detected in DNA fragments up to 500bp (Myers *et al.,* 1985). This percentage can be increased to nearly 100% by the attachment of a GC-clamp to one side of the DNA fragment (Myers *et al.,* 1985; Sheffield *et al.,* 1989). The addition of a 30- to 40-bp GC-clamp to one of the PCR primers insures that the DNA fragment will remain partially double-stranded and that the region screened is in the lowest melting domain (Myers *et al.,* 1985; Sheffield *et al.,* 1989).

Prior to DGGE analysis of DNA fragments, it is necessary to determine the melting behavior of the DNA fragments (Muyzer & Smalla, 1998). It is also necessary to optimize the gradient and the duration of electrophoresis in order to obtain the best separation of the different DNA fragments (Muyzer & Smalla, 1998). The melting behavior and optimum gradient can be determined with perpendicular gradient gels. Perpendicular gels have an increasing gradient of denaturants from left to right (Muyzer & Smalla, 1998). The sample is applied across the entire width of the gel and electrophoresed for about 3 hours at 200V. The optimal time of electrophoresis is determined by parallel gradient electrophoresis where different samples are

loaded at constant time intervals (Muyzer & Smalla, 1998; Ercolini, 2004). Parallel gradient gels have an increasing gradient of denaturants from top-to-bottom (Muyzer & Smalla, 1998). Parallel gels are the most commonly employed because they allow multiple samples to be loaded on the same gel (Ercolini, 2004).

DNA bands in DGGE profiles can be visualized by ethidium bromide staining (Muyzer & Smalla, 1998), SYBR Green I (Muyzer *et al.,* 1997), and silver staining (Felske *et al.,* 1996). SYBR Green staining does not give background staining, thus allowing the detection of DNA fragments even at very low concentrations (Ercolini, 2004). Silver staining is a more sensitive detection method (Felske *et al.,* 1996), however, it also stains single stranded DNA and gels cannot be used for subsequent hybridization analysis (Heuer & Smalla, 1997).

DGGE has been widely used by several researchers for the identification of fungi and fungal communities, including Kowalchuk *et al.* (1997), Schabereiter-Gurtner *et al.* (2001), and Oros-Sichler *et al.* (2006). Studies that used PCR-DGGE specifically for the identification of *Fusarium* species include Mach *et al.* (2004), Yergeau *et al.* (2005), and Wakelin *et al.* (2008). Yergeau *et al.* (2005) used PCR-DGGE for the detection and identification of *Fusarium* species directly for environmental asparagus samples without an isolation step. This method allowed the easy and rapid differentiation between the majority *Fusarium* species and/or isolates tested in pure culture. A further sequencing step permitted the differentiation between the few species that showed similar migration patterns. In another study of Yergeau *et al.* (2006), PCR-DGGE was used to determine changes in communities of *Fusarium* and arbuscular mycorrhizal fungi on asparagus samples. Mach *et al.* (2004) developed a PCR-DGGE method for the early and specific detection of *F. langsethiae* and distinguishing it from related species of section *Sporotrichiella* and Discolor (*F. poae*, *F. sporotrichioides*, *F. kyushuense*, *F. robustum*, *F. sambucinum* and *F.*

tumidum). They demonstrated that DGGE reliably separated all these strains, even from mixtures and in the presence of DNA from their natural hosts *Zea mais*, *Triticum aestivum* and *Avena sativa*. Wakelin *et al.* (2008) used DGGE to determine the *Fusarium* communities in soil. Their study also showed that abundance and species of *Fusarium* present in soil are highly responsive to agricultural management practices.

2.2.5 Single-Stranded Conformational Polymorphisms/Heteroduplex Analysis

SSCP is a simple technique that relies on the susceptibility for single-stranded DNA in non-denaturing conditions to take on a three-dimensional structure that is highly sequence dependent (Wallace, 2002). Differences in a single base can cause alterations to the DNA's secondary structure and thus result in different migration rates of the DNA strands (Wallace, 2002; Han & Robinson, 2003). Thus, SSCP is a simple and reliable method for the detection of uncharacterized mutations. A few hundred bases can be screened at once, and may thus reduce the requirement for nucleotide sequencing (Kerr & Curran, 1996; Taylor, 1997).

The basic SSCP procedure involves four steps. First, the region of interest is amplified through PCR. The success of SSCP is more likely when shorter DNA fragments (100-300bp) are used because migration differences are better resolved with shorter fragments. Therefore, primers should be designed to amplify 100-300bp DNA fragments (Han & Robinson, 2003). Larger fragments can be analyzed by cutting them with an appropriate restriction enzyme to yield shorter fragments (Wallace, 2002). Second, the amplified PCR products are heated to denature the double-stranded DNA into single-stranded DNA. The amplified products are mixed with gel loading buffer and dye containing formamide to prevent reannealing of the DNA and to visualize bands in the non-denaturing gel. The third step is to separate the single-stranded DNA strands on

a non-denaturing polyacrylamide gel through electrophoresis. The final step is visualizing the bands in the gel (Han & Robinson, 2003).

The most common factors influencing the success of the SSCP procedure include (i) length of the PCR fragments. Up to 90% of mutations are detected when shorter fragments are used (Sheffield *et al.,* 1993; Humphries *et al.,* 1997). (ii) Temperature: typical running conditions are either at room temperature or 4°C. Higher temperatures change the migration of the bands to such a degree that certain SSCPs are no longer detected (Humphries *et al.,* 1997). A temperature rise can also affect reproducibility (Kerr & Curran, 1996). (iii) The level of cross linking within the gel: separation of single-stranded DNA is better in polyacrylamide gels with low cross linking. The low level of cross linking gives a bigger pore size, thus allowing efficient separation of the single-stranded DNA (Kerr & Curran, 1996; Humphries *et al.,* 1997). (iv) Method of PCR denaturation (Humphries *et al.,* 1997). Usually, PCR products are used without purification prior to a SSCP run. This sometimes results in false bands due to excessive PCR cycles or excessive residual PCR primer that may anneal to single strands (Cai & Touitou, 1993). These problems can be reduced by reducing the number of PCR reaction cycles and diluting the sample 10 to 30 fold. Stronger denaturants can also be added to the sample if less sensitive detection methods are used (Humphries *et al.,* 1997). Formamide, sodium hydroxide, urea, and methylmercuric hydroxide (Hongyo *et al.,* 1993) have been used. Most SSCP protocols involve heating the sample, cool rapidly on ice, then loading the sample at between 4°C and 25 °C (Humphries *et al.,* 1997).

Heteroduplexes are hybrid DNA molecules that have one or more mismatched base pairs (Wallace, 2002). The formation of heteroduplex DNA is dependent on spontaneous reannealing in the formamide loading buffer or during the earliest phase of electrophoresis (Wallace, 2002).

These DNA molecules have been used to search for point mutations since 1992 (White *et al.*, 1992). They appear on polyacrylamide gels as one or two bands of reduced mobility relative to homoduplex DNA (Wallace, 2002). The mismatched base pairs in heteroduplexes are thought to affect electrophoretic mobility by inducing bends in the DNA (Wallace, 2002). The optimum fragment size for heteroduplex analysis can range between 250-500bp (Wallace, 2002). The detection efficiency of heteroduplexes has been reported to be 90% under ideal conditions (Ganguly *et al.*, 1993).

PCR-SSCP has been applied for fungal identification in environmental samples and clinical specimens. Walsh *et al.* (1995) demonstrated that the PCR-SSCP technique was a useful tool to recognize and distinguish medically important opportunistic fungi, including *Candida* spp., *Aspergillus* spp., *Cryptococcus neoformans*, *Pseudallescheria boydii*, and *Rhizopus arrhizus*. Hauser *et al.* (1997) used PCR-SSCP for molecular typing of *Pneumocystis carinii* f. sp. *hominis* epidemiology. They proposed that multitarget target typing of *P. carinii hominis* by PCR-SSCP should allow the investigation of strain diversity and thus be useful for future epidemiological studies.

Studies that made use of PCR-SSCP to investigate mutations between medically important fungal pathogens include Hui *et al.* (2000), Nahimana *et al.* (2000) and Kumar & Shukla, (2005). However, only a few studies applied this method for *Fusarium* identification and differentiation (Dong *et al.*, 2005; Wong & Jeffries 2006). Sequence data obtained from Dong *et al.* (2005) confirmed that the SSCP method was capable in detecting one single base change within the 550bp PCR fragment from the ITS region of *Fusarium oxysporum*. Wong & Jeffries (2006) was able to identify over 360 fusarial isolates from symptomatic asparagus plants in Spain and the

UK. The isolates were easily differentiated by SSCP into four principal species, *Fusarium oxysporum* f. sp. asparagi, *Fusarium proliferatum*, *Fusarium redo* and *Fusarium solani*.

2.3 Genes employed for fungal phylogenetic studies

2.3.1 Ribosomal genes vs. protein coding genes

2.3.1.1 Ribosomal genes

The ribosomal DNA (rDNA) of a eukaryote nuclear genome consists of several hundred tandemly repeated copies of transcribed and non-transcribed regions (Long & Dawid, 1980). The transcribed regions consist of conserved sequences which include the 18S, 5.8S, and 28S genes (Appel & Gordon, 1996). The 18S, 5.8S and 28S genes are separated by two internal transcribed spacers (ITS-1 and ITS-2) (Hillis & Dixon, 1991). The transcribed spacers contain signals for processing the rRNA transcript. Adjacent copies of the rDNA repeat unit are separated by non-transcribed regions, called the intergenic spacer regions (IGS) (Hillis & Dixon, 1991). This region contains subrepeating elements that serve as enhancers of transcription (Kohorn & Rae, 1982).

Ribosomal genes are useful for phylogenetic analysis because it is a very conserved region of the genome (Ercolini, 2004) and the different regions of the rDNA repeat unit evolve at very different rates (Hillis & Dixon, 1991). Thus, rDNA regions that are likely to yield information for almost any systematic question can be used for analysis (Hillis & Dixon, 1991). Also, the highly conserved sequences within most rDNA genes are very useful for constructing universal primers that can be used to (i) sequence the rRNA or rDNA regions for many species; (ii) amplify regions of interest; and (iii) use them as probes in restriction enzyme analysis (Vilagalys & Hester, 1990; Kowalchuk *et al.*, 1997).

2.3.1.1.1 18S rDNA

The most commonly used gene in fungal phylogenetic analysis is the 18S rDNA gene since its database is the most complete for all living organisms and it is the most slowly evolving of the rDNA genes (Hillis & Dixon, 1991). This makes the 18S rDNA a good candidate for finding consensus conserved regions suitable for genus or higher taxonomic level detections (Wu *et al.*, 2003). It is fairly easy to amplify this gene because of its large number of copies per genome. Also, the alternation of the variable regions in a single molecule makes 18S rDNA a powerful tool for molecular phylogeny at different taxonomic levels (Berney *et al.*, 2000). However, identification of fungi by means of the 18S rDNA gene is limited to genus or family level (Anderson & Cairney, 2004). This is mainly due to the relative lack of variation within the 18S rDNA gene between closely related fungal species as a result of the relatively short period of evolution of the kingdom fungi (Hugenholtz & Pace, 1996).

Studies that focused on the use of 18S rDNA for the identification and characterization of *Fusarium* species include Mule *et al.* (1997), Jaeger *et al.* (2000), Leeflang *et al.* (2002), and Bezuidenhout *et al.* (2006a). Mule *et al.* (1997) used the 28S rDNA gene to determine the genetic relatedness of trichothecene-producing *Fusarium* species. Bezuidenhout *et al.* (2006a) included 18S rDNA target sequence in the multiplex PCR to eliminate the limitation of potential false negatives that could be associated with general PCR failure. Leeflang *et al.* (2002) used 18S rDNA analysis to study the *Fusarium* population in the soil of a wheat field in the Netherlands through the construction of clone libraries. They also measured effects of *Pseudomonas putida* WCS358r and its genetically modified phenazine producing derivative on the *Fusarium* population.

2.3.1.1.2 Internal Transcribed Spacer (ITS) region

Until recently, the molecular identification of fungi to species level has been based mostly on the use of variable internal transcribed spacer (ITS) regions (Michealsen *et al.,* 2006). The non-coding ITS regions have a high copy number in the fungal genome as part of tandemly repeated nuclear rDNA (Michealsen *et al.,* 2006). These regions benefit from a fast rate of evolution, which results in higher variation in sequence between closely related species, in comparison with the more conserved coding regions of the rRNA genes (Michealsen *et al.,* 2006). As a consequence, the DNA sequences in the ITS region generally provide greater taxonomic resolution than those from coding regions (Lord *et al.,* 2002; Anderson *et al.,* 2003). In addition, the DNA sequences in the ITS region are highly variable, divergent, and distinctive, and might serve as markers for taxonomically more distant groups (Michealsen *et al.,* 2006).

Taxon-selective ITS amplification has been used for detection of the fungal pathogens such as *Fusarium* spp., *Verticillium* spp. (Abd-Elsalam *et al.,* 2003), *Phytophthora* spp., *Mycosphaerella* spp. (Larena *et al.,* 1999), *Pythium* spp., and *Rhizoctonia solani* (Lievens *et al.,* 2006). Lee *et al.* (2000) demonstrated that analysis of the ITS region was useful for investigating the genetic relationship among 12 species belonging to the *Fusarium* section *Martiella, Dlaminia, Gibbosum, Arthrosporiella, Liseola* and *Elegans*. A study conducted by Tan & Niessen (2003), showed that the phylogenetic analysis with the ITS sequences were able to resolve most of the *Fusarium* species examined. But the following recommendations were made: (i) the ITS information can be combined with morphological classification and species-specific assays for accurate diagnosis and identification of most of the *Fusarium* species, and (ii) genus-specific primers can be designed to allow the amplification of *Fusarium* species in infected plant material.

2.3.1.2 Protein-coding genes

Protein-coding genes (e.g. tubulin genes, EF-1 gene, histones) are the markers of choice for species-level phylogenetics in fungi (Geiser, 2003). These gene regions tend to evolve at a higher rate than more commonly applied markers such as the internal transcribed spacer (ITS) regions of the nuclear ribosomal RNA gene repeat (O'Donnell *et al.,* 1998a; O'Donnell, 2000).

Protein-coding genes offer several advantages above ribosomal genes for phylogenetic analysis. Homology and convergence are easier to recognize in protein-coding regions, which are made up of the 20 amino acids, than in DNA sequences. Length changes are uncommon in protein-coding genes since insertions and deletions often lead to fatal frame shifts and elimination/exclusion through natural selection. Although a wide range of eukaryotic protein-coding genes is available for phylogenetic studies, they must overcome some minor obstacles like primer design. A primer that works for one fungus may be unsuccessful with its close relatives as a result of substitutions that destroy the priming site in the DNA without changing the amino acid sequence. (Berbee & Taylor, 1994).

2.3.1.2.1 Translation Elongation Factor-1α (EF-1) gene

The elongation factor-1α (EF-1α) gene, which encodes an essential part of the protein translation system (Geiser *et al.,* 2004), has frequently been used in phylogenetic analyses by several researchers (Geiser *et al.,* 2004; Glynn *et al.,* 2005; Yergeau *et al.,* 2005; Bezuidenhout *et al.,* 2006a). EF-1α is an ideal phylogenetic tool due to its universal occurrence and good amino acid sequence preservation (Berney *et al.,* 2000). It can be used to determine very ancient relationships, such as the relative branching order of the most primitive eukaryotes (Hashimoto *et al.,* 1994; Nordnes *et al.,* 1994; Baldauf, *et al.,* 1996). EF-1α gene has also been employed for determining the phylogenetic relationships between animal phyla and classes as a method to

confirm or shed uncertainty on results based on other genes (Kojima *et al.,* 1993; Regier & Shultz 1997; Kojima, 1998).

Studies that used this gene for detection and/or identification of *Fusarium* species include O'Donnell *et al.* (1998b), Geiser *et al.* (2004), Knutsen *et al.* (2004), Yergeau *et al.* (2005), Bezuidenhout *et al.* (2006a), and Bogale *et al.* (2006). In *Fusarium*, the gene appears to be consistently single-copy, and it shows a high level of sequence polymorphism among closely related species (Geiser *et al.,* 2004). The high level of sequence variation can be used to discriminate between most species. This genetic variability can be resolved electrophoretically (Wakelin *et al.,* 2008). O'Donnell *et al.* (1998b) demonstrated that the EF-1α gene proved to be a good phylogenetic marker for resolving relationships within the *F. oxysporum* complex. They also showed that the EF-1α gene possessed 50% more phylogenetic information than the mtSSU rDNA, and that the gene recovered substantiated phylogenetic relationships within the *Gibberella fujikuroi* complex of *Fusarium*. Yergeau *et al.* (2005) have described a PCR-DGGE method with the EF-1α gene to detect the presence of multiple *Fusarium* species in environmental samples. Over 19 different *Fusarium* species were correctly categorized.

2.3.1.2.2 Beta-tubulin (BT) gene

The tubulin gene family consists of three major highly conserved subfamilies, alpha-, beta-, and gamma-tubulin, which arose from a series of gene duplications in early eukaryotic evolution (Edlind *et al.,* 1996; Keeling & Doolittle, 1996). The tubulin genes are made up of highly conserved proteins which are the principle structural and functional components of eukaryotic microtubules that are major components of the cytoskeleton, mitotic spindles, and flagella of eukaryotic cells (Keeling & Doolittle, 1996; Thon & Royse, 1999). Tubulin genes are frequently

found in multiple copies in a genome, and any two genes from a single organism can range from being identical to being highly divergent (Keeling *et al.,* 2000).

Several studies have made use of beta-tubulin genes to examine relationships among fungi at all levels, and has been found to be a useful tool in both deep level phylogenetic studies and studies of complex species groups (Baldauf & Palmer, 1993; Edlind *et al.,* 1996; Baldauf & Doolittle, 1997; O'Donnell *et al.,* 1998a). Studies that have used the beta-tubulin gene for characterization of *Fusarium* species include Geiser *et al.* (2001), Mach *et al.* (2004), Reischer *et al.* (2004), and Yli-Mattila *et al.* (2004). Geiser *et al.* (2001) used the beta-tubulin gene, as well as the EF-1α gene, to identify and characterize *F. hostae*. Mach *et al.* (2004) employed the beta-tubulin gene for the early and specific detection of *Fusarium langsethiae*, and distinguishing it from related species of section *Sporotrichiella* and *Discolor* (*Fusarium poae*, *Fusarium sporotrichioides*, *Fusarium kyushuense*, *Fusarium robustum*, *Fusarium sambucinum* and *Fusarium tumidum*). Yli-Mattila *et al.* (2004) demonstrated that the beta-tubulin gene was able to distinguish between *Fusarium poae*, *Fusarium sporotrichioides*, *Fusarium langsethiae* and *Fusarium kyushuense*, but it did not resolve the phylogenetic relationship between *Fusarium sporotrichioides* and *Fusarium langsethiae*.

2.4 Summary

Fusarium spp., in particular toxigenic species, has become a major concern because of the significant economic losses associated with their impact on agriculture production and human and animal health. *Fusarium* mycotoxins are responsible for several diseases in humans and animals, including alimentary toxic aleukia, esophageal cancer, and hemorrhagic syndrome. Recently, *Fusarium* infections in immunocompromised patients have emerged resulting in high morbidity and mortality. Early detection of *Fusarium* spp. on commercial and subsistence crops

is vital in preventing yield losses and mycotoxins contaminating foodstuffs and feed. The use of culture dependent methods for the detection and identification of *Fusarium* spp. are time consuming and skilled expertise is required. In many cases, minor morphological differences can define species, making identification even more problematic (Nelson, 1991). Culture independent methods are more rapid and specific, and can be used to detect small quantities of fungal DNA from environmental and clinical samples. Thus, culture independent methods can quicken the detection and identification of *Fusarium* and their mycotoxins in foods and feed which allow proper control measures to be carried out in time.

CHAPTER 3

METHODS AND MATERIALS

3.1 Fungal isolates and culture conditions

The reference *Fusarium* species employed in this study (Table 3.1) were obtained from the Medical Research Council (PROMEC), identified by the Agricultural Research Council's Plant Protection Research Institute (ARC-PPRI) of South Africa, or isolated from *morogo* vegetables (Alli, 2007). Isolates were grown on Potato Dextrose Agar (PDA) for 4-7 days at 25°C prior to DNA isolation.

Table 3.1: The fungal species and strains employed in this study. *Acremonium strictum* (VBSIL 12b) and *Alternaria* spp. (VASIL II A) were from the North-West University culture collection.

Taxon	**Isolate**
F. verticillioides	MRC 8559, 8560, 826, 4317, 4319
	PPRI 7877, 7899, 7897
	17-13, 17-45, 17-65, 17-69
F. nygamai	MRC 3997, 8546, 8547
F. proliferatum	MRC 8549, 8550
F. oxysporum	PPRI 7376
F. subglutinans	PPRI 7365, 7383
Aspergillus niger	ATTC 16404
Acremonium strictum	VBSIL 12b
Alternaria spp.	VASIL II A

3.2 DNA isolation

3.2.1 CTAB-PVP DNA Extraction Method

The mycelium mats were scraped from the surface and transferred to 2ml microfuge tubes. DNA was obtained by using the standard CTAB-PVP, phenol-chloroform sodium chloride, alcohol precipitation extraction procedure as previously described (O'Donnell *et al.,* 1997; Appendix A).

The CTAB-PVP method and E.Z.N.A Fungal DNA Mini Kit (PeQLab, Germany) were compared for quantity and quality of the isolated DNA by comparing the 260:280 ratios and DNA concentrations. The 260:280 ratios and DNA concentrations were determined using the NanoDropTM 1000 Spectrophotometer (Thermo Fischer Scientific, US).

3.2.2 E.Z.N.A Fungal DNA Mini Kit (PeQLab, Germany)

The mycelium mats were scraped from the surface and transferred to 2ml microfuge tubes and beaded with glass beads for 1-2 hours prior to DNA isolation. The E.Z.N.A Fungal DNA Mini Kit (PeQLab, Germany) was used for DNA isolation according to the manufacturers' instructions (Appendix A).

3.3 DNA amplification

3.3.1 Optimization of PCR conditions

DNA was amplified using an ICycler (Bio-Rad, UK) thermal cycler. Optimized conditions for DNA amplification with the individual primer sets were as follows: single strength PCR master mix (4U/µl *Taq* DNA Polymerase (recombinant) in reaction buffer, 2mM $MgCl_2$, 0.2mM of each dNTP); 0.25µl of forward and reverse primers (50pmol each) (Table 3.2); Bovine serum albumin (100ng); additional 1µl $MgCl_2$ (1mM); additional 0.2µl Supertherm *Taq* polymerase (1U; JM Holdings, UK); 100ng DNA and PCR-grade water (Fermentas Life Sciences, US) to give a final

reaction volume of 25µl. Cycling conditions were set at 95°C for 10 min; 35 cycles of 30 seconds of melting at 95°C; 30 seconds of annealing; 60 seconds of extension at 72°C; followed by a 10 min final extension at 72°C.

3.3.2 Multiplex PCR

Conditions for the multiplex PCR were optimized as describe in Section 3.3.1. The annealing temperature of the reaction was 55°C. The primers employed, their target sequences, and expected product sizes are listed in Table 3.2 (Bluhm *et al.,* 2002). Ratios of 1:1:1, 1:1:2 and 1:1:3 (EF-1α:FUM:18S) were tested on all of the *Fusarium* species and strains. The EF-1α bands were excised from gels using a sterile scalpel blade. The DNA was purified using the NucleoSpin® Extract II purifying kit according to the manufacturers' instructions. Purified DNA fragments were subjected to a second round of amplification.

Table 3.2: Primer sets employed in this study.

Primer	Sequence	Gene Target	Product Size (bp)	Reference
EF-1α*	5'-ATG GGTAAGGAGGACAAGAC-3'	Translation Elongation	700	Geiser *et al.*, 2004
EF-2*	5'-GGA AGT ACC AGT GAT CAT GTT-3'	factor 1-α		
FUM-1 F*	5'-GTC GAG TTG TTG ACC ACT GCG-3'	Polyketide Synthase	800	Bluhm *et al.*, 2002
FUM-1 R*	5'-CGT ATC GTC AGC ATG ATA GC-3'	(PKS)		
SSU 0017F GC**	5'-AGT AGT CAT ATG CTT GTC -3'	18S Small Sub-unit	570	Kowalchuk *et al.*,
SSU 0583R	5'-TCT GGA CCT GGT GAG TTT CC-3'			1997
EF-1α GC**	5'-ATG GGT AAG GAG GAC AAG AC-3'	Translation Elongation	500	Geiser *et al.*, 2004
EF-3	5'-AGG AAC CCT TAC CGA GCT C-3'	factor 1-α		
BT-1	5'-AAC ATG CGT GAG ATT GTA AGT-3'	β-tubulin	564-700	O'Donnell &
BT-2	5'-TAG TGA CCC TTG GCC CAG TTG-3'			Cigelnik, 1997
Alfie-1 GC**	5'-TCG TCA TCG GCC ACG TCG ACT C-3'	Translation Elongation	500	Yergeau *et al.*,2005
Alfie-2	5'-CCT TAC CGA GCT CRG CGG CTT C-3'	factor 1-α		

*Primers used for multiplex PCR

**SSU 0017F, EF-1α and Alfie-1 contained the GC-clamp CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC for DGGE purposes (Muyzer *et al.*, 1996).

3.4 Agarose electrophoresis

PCR amplifications were confirmed by 1.5% (w/v) agarose gel in 1 x TAE buffer (40mM Tris, 20mM Acetic acid, 1mM EDTA, pH 8.0). EtBr (1μg/ml) was added for visualization under UV light (Bluhm *et al.,* 2002). Five microliter (μl) PCR product was mixed with 5μl loading buffer (6x Orange Loading Dye, Fermentas Life Sciences, US) and loaded into a gel slot. Three microliter (μl) of a 100bp molecular marker (O'GeneRuler, Fermentas Life Sciences, US) was also loaded into each gel to confirm the sizes of the fragments. Electrophoresis was performed for 45-60 min at 80V using 1 x TAE buffer. Gel images were captured using a Gene Bio Imaging System (Syngene, Synoptics, UK) and GeneSnap (version 6.00.22) software (Bezuidenhout *et al.,* 2006a).

3.5 Denaturing Gradient Gel Electrophoresis of the *Fusarium* species and strains

Denaturing gradient gel electrophoresis (DGGE) of amplified EF-1α DNA and 18S rDNA were used to investigate the potential of this technique to discriminate between various *Fusarium* species and strains. The EF-1α fragments obtained in Section 3.3.2 were excised from agarose gels and purified by means of a kit. The EF-1α DNA and 18S rDNA were amplified with primers listed in Table 3.2. Primer sets for DGGE all contained a GC clamp. PCR conditions described in Section 3.3.1 were used except that no additional *Taq* DNA polymerase was added. Annealing temperatures for EF-1α and 18S rDNA were 67°C and 58°C, respectively.

A DCode Universal Mutation Detection System (Bio-Rad, UK) was used for DGGE analysis. Electrophoresis was conducted on a 6% (w/v) acrylamide gel for 16 hours at 80V for the EF-1α fragments and 100V for the 18S fragments. A 40-60% and 30-50% gradient was used for EF-1α and 18S rDNA, respectively. EtBr (1μg/ml) stained gels were visualized using a Gene Genius Bio Imaging System (Syngene, Synoptics, UK) and GeneSnap software (version 6.00.22).

3.6 Single-stranded conformation polymorphism of the *Fusarium* species and strains

Single-strand conformation polymorphism (SSCP) of amplified EF-1α and β-tubulin (BT) fragments was used to investigate the potential of this technique to discriminate between various *Fusarium* species and strains. The beta-tubulin and EF-1α genes from *F. proliferatum*, *F. oxysporium*, *F. subglutinans*, five *F. verticillioides* (MRC 8559, 8560, 826, 4317 & 4319) and three *F. nygamai* strains (MRC 3997, 8546 & 8547) were amplified with the primers BT-1 and BT-2, and Alfie-1 (without the GC clamp) and Alfie-2 (Table 3.2). The re-extracted EF-1α fragments (Section 3.3.2), as well as pure DNA, were used for amplifications with the Alfie-1 primer set. PCR conditions described in Section 3.3.1 were used for amplification with the Alfie-1 and BT primer sets, except that no additional *Taq* DNA polymerase was added to the Alfie-1 primer set. Annealing temperatures for Alfie-1 and BT were 62°C and 55°C, respectively. Amplification was confirmed on a 1.5% (w/v) agarose gel as described in Section 3.4. The BT fragments were then restricted with an enzyme, KpnI, to yield 350bp fragments. The presence of the restricted fragments was confirmed on a 1.5% (w/v) agarose gel as described in Section 3.4.

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Ten microliters (μl) of each PCR product (i.e. the amplified EF-1α and beta-tubulin) were mixed with an equal volume of 2 × loading buffer (0.05% bromophenol blue, 0.05% xylene cyanol, 95% formamide, and 20mM EDTA), with subsequent incubation of 10 min at 95°C and quenching on ice. The BT samples were applied to a 6% (w/v) polyacrylamide gel with 10% (w/v) glycerol and electrophoresed at 500V in 1 × TBE buffer (99mM Tris, 99mM Boric acid, 2.2mM EDTA, pH 8.0) at room temperature for 3.5-4 hours. The EF-1α samples were applied to a 6% (w/v) and 8% (w/v) polyacrylamide gel with 10% (w/v) glycerol and electrophoresed at 500V in 1 × TBE buffer at room temperature for 4.5 hours. The gels were stained for 45 min with EtBr (1μg/ml) and visualized using a Gene Genius Bio Imaging System (Syngene, Synoptics, UK) and GeneSnap software (version 6.00.22).

3.7 Sequence analysis

Amplified EF-1α and beta-tubulin fragments were sequenced by Inqaba Biotech (South Africa) using Base Spectrum software. Geospiza Finch TV (version 1.4) software was used to view the chromatograms. Blastn searches (http://www.ncbi.nlm.nih.gov/BLAST) were used to confirm the identity of the amplified sequences (Bezuidenhout *et al.*, 2006a). Mega 4 Sequence Alignment (version 4.0.2) software was used to determine phylogenetic relationships for the EF-1α and beta-tubulin genes of the various *Fusarium* species and strains. A neighbor-joining phylogenetic tree was constructed for both protein-coding genes. Branch lengths were indicated on tree the branches.

The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Codon positions included were 1st, 2nd and 3rd, as well as non-coding regions. All positions containing gaps and missing data were eliminated from the datasets (Tamura *et al.*, 2004).

3.8 Detection limits of fungal genomic DNA using conventional and quantitative PCR protocols

The detection limit for a PCR primer set is defined by Bluhm *et al.* (2002) as the minimal amount of DNA template that will yield clear visible product on agarose gels containing ethidium bromide (EtBr). This parameter was also used in the present study.

3.8.1 Detection limits for individual *Fusarium* species DNA using conventional PCR

Detection limits of genomic DNA for *F. verticillioides* and *F. subglutinans* were tested by means of a dilution series (100ng, 10ng, 1ng, 0.1ng, and 0.01ng). Genomic DNA was amplified with primers EF-1α and FUM (Table 3.2). The PCR conditions described in Section 3.3.1 were used

except that no additional *Taq* DNA polymerase was added. The annealing temperature of the reaction was 58°C. Amplification was confirmed on a 1.5% (w/v) agarose gel as described in Section 3.4.

3.8.2 Detection limits of *Fusarium* species DNA in the presence of non-*Fusairum* species DNA using conventional PCR

Detection limits of genomic DNA for *F. verticillioides* and *F. subglutinans* in the presence of non-*Fusarium* species (*Aspergillus niger, Acremonium strictum, Alternaria* spp, and *morogo*, Table 3.1) were tested by means of a dilution series (100ng, 10ng, 1ng, 0.1ng, and 0.01ng). DNA concentrations of the non-*Fusarium* species were constant a 100ng per reaction volume. Primers EF-1α and FUM (Table 3.2) were used. The PCR conditions described in Section 3.3.1 were used except that no additional *Taq* DNA polymerase was added. The annealing temperature of the reaction was 58°C. Amplification was confirmed on a 1.5% (w/v) agarose gel as described in Section 3.4.

3.8.3 Quantitative detection of individual *Fusarium* species DNA

Real-time PCR was used to test the detection limits of *F. verticillioides* and *F. subglutinans* by means of a dilution series (100ng, 10ng, 1ng, 0.1ng, and 0.01ng). DNA was amplified with primers EF-1α and FUM (Table 3.2). The PCR conditions described in Section 3.3.1 were used except that SYBR Green PCR master mix (Bio-Rad, UK) was used, no additional *Taq* DNA polymerase and $MgCl_2$ were added. A total of 40 PCR cylces were used. The annealing temperature of the reaction was 58°C. To detect the specific product from non-specific products and primer dimers, melting curve analysis was done immediately after amplification. The set point temperature was increased after cycle 2 from 40°C to 94°C at 0.5°C/s for 110 cycles to obtain the melting curve.

Standard curves based on threshold cycles (Ct) for 10-fold dilution series of fungal genomic DNA (100ng, 10ng, 1ng, 0.1ng, and 0.01ng)) were constructed for each *Fusarium* species. The standard curve is a plot of the Ct value versus log DNA concentration (Atoui *et al.,* 2007). Ct values were calculated by the ICycler™ iQ Optical System (version 3.0a) software (Bio-Rad, UK) to indicate significant fluorescence signals rising above background during the early cycles of the exponentially growing phase of the PCR amplification process. Amplification was confirmed on a 1.5% (w/v) agarose gel as described in Section 3.4.

The PCR efficiency of an ideal standard curve should be between 90 and 100% (-3.6 > slope > -3.1) (Dorak, 2006). The PCR efficiency for the EF-1α and FUM primer sets in this study were calculated to be 10 × higher than the proposed value. The high PCR efficiency values could have resulted from handling and/or pipetting errors. Therefore, the Ct values could not be used to determine DNA concentrations or for any further analysis.

3.8.4 Quantitative detection of *Fusarium* species DNA in the presence of non-*Fusarium* species DNA

Real-time PCR was used to test the detection limits of *F. verticillioides* and *F. subglutinans* in the presence of non-*Fusarium* species (*Aspergillus niger, Acremonium strictum, Alternaria* spp, and *Marogo*, Table 3.2) by means of a dilution series (100ng, 10ng, 1ng, 0.1ng, and 0.01ng). DNA concentrations of the non-*Fusarium* species were 100ng per reaction volume. DNA was amplified with primers EF-1α and FUM (Table 3.2). The PCR conditions described in Section 3.3.1 were used except that SYBR Green PCR master mix (Bio-Rad, UK) was used, no additional *Taq* DNA polymerase and $MgCl_2$ were added. A total of 40 PCR cycles were used. The annealing temperature of the reaction was 58°C. Melting curve analysis was done as described in Section 3.7.3

CHAPTER 4

RESULTS

4.1 DNA isolation

Genomic DNA was isolated from the mycelium mats of the various *Fusarium* species and strains (Table 3.1) by means of the CTAB-PVP method (Section 3.2.1) and E.Z.N.A Fungal DNA Mini Kit (Section 3.2.2). Camera aperture settings were alike for all gel images.

Figure 4.1 are images of ethidium bromide stained 1.5% (w/v) agarose gels that show the quantity and quality of DNA isolated by these two methods. In Figure 4.1A it is evident that a large quantization of DNA was isolated using the CTAB-PVP method. However, fragmentation and RNA contamination is also observed in this image. PCR amplification using this DNA was inconsistent although optimized PCR conditions were used as described in Section 3.3.1.

DNA isolated by the E.Z.N.A Fungal DNA Mini Kit was consistently of lower concentration, but of higher quality (Figure 4.1B). There were no RNA or fragmentation observed. This method was also less time consuming than the CTAB-PVP method.

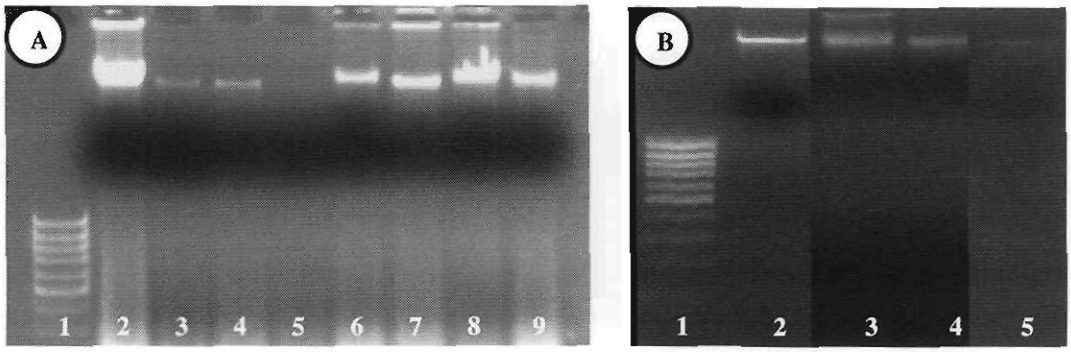

Figure 4.1: DNA isolation of various *Fusarium* species and strains by means of the (A) CTAB-PVP method and (B) E.Z.N.A Fungal DNA Mini Kit (PeQLab, Germany) on a 1.5% (w/v) agarose gel.

This observation was confirmed by the 260:280 ratios and concentrations of the DNA isolated by the respective methods. The 260:280 ratios for the CTAB-PVP method and the E.Z.N.A Fungal DNA Mini Kit were in the region of 2.00 and 1.9, respectively. High 260:280 ratios are usually an indication of RNA contamination. This was evident for the DNA isolated with the CTAB-PVP method (Figure 4.1A). DNA concentrations for the CTAB-PVP method were higher than for the Fungal DNA Mini Kit with values ranging between 80 and 300ng/µl, while DNA concentrations for the Fungal DNA Mini Kit varied between 9 and 150ng/µl. Typically, 20-50µg DNA with a A_{260}/A_{280} ratio of 1.7-1.9 can be isolated using 200mg fresh or frozen specimens. Although the DNA quantity of the CTAB-PVP method was higher than for the Fungal DNA Mini Kit, the quality of the DNA was not as good as for the DNA Mini Kit. Therefore, DNA isolated with the E.Z.N.A Fungal DNA Mini Kit was used for further analysis.

4.2 Amplification of fungal DNA

Fungal genomic DNA was amplified by PCR using the procedures described in Section 3.3.1. PCR conditions were optimized for the EF-1α, FUM and beta-tubulin primer sets to yield high-quality PCR products for further analysis.

4.2.1 Amplification with individual primer sets

Figure 4.2 illustrates the sizes of the amplification products of the four gene fragments employed in this study: EF-1α (A), beta-tubulin (B), 18S rDNA (C) and FUM (D). Amplification products were confirmed by agarose electrophoresis as described in Section 3.4. Genomic DNA was from various *Fusarium* strains and species as indicated in Table 3.1. Sizes of the fragments obtained were as expected (Table 3.2).

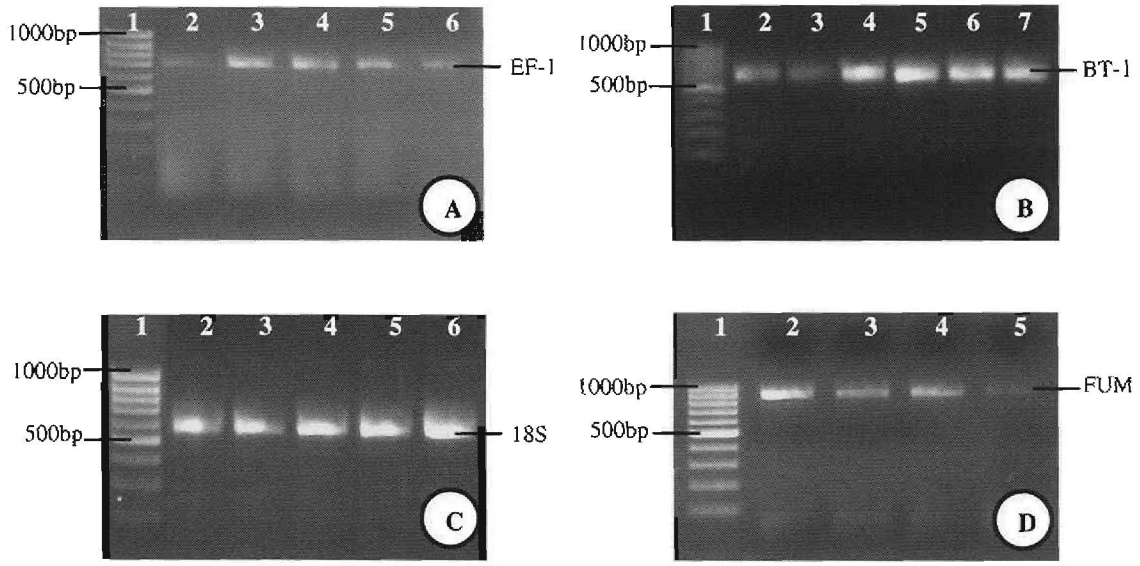

Figure 4.2: Four 1.5% (w/v) ethidium bromide stained agarose gels illustrating the (A) EF-1α, (B) beta-tubulin, (C) 18S and (D) FUM gene fragments of various *Fusarium* strains and species. Sizes of the fragments were as expected (Table 3.2). Lane 1 of all four gel images indicates a 100bp molecular size marker (O'GeneRuler™ 100bp DNA ladder, Fermentas Life Sciences, US). (A) Lane 2 – *F. proliferatum*; lane 3 – *F. oxysporum*; lane 4 – *F. subglutinans*; lane 5 – *F. verticillioides*; and lane 6 – *F. nygamai*. (B) Lane 2 to 7 – *F. verticillioides*. (C) Lane 2 – *Fusarium* mixture (*F. proliferatum*, *F. oxysporum*, *F. subglutinans*, *F. verticillioides* and *F. nygamai*); lane 3 – *F. proliferatum*; lane 4 – *F. oxysporum*; lane 5 – *F. subglutinans*; and lane 6 – *F. verticillioides*. (D) Lane 2 to 5 – *F. verticillioides*.

Yield of the EF-1α products (Figure 4.2A) for *F. oxysporum* (Lane 3), *F. subglutinans* (Lane 4) and *F. verticillioides* (Lane 5) was generally higher than for *F. proliferatum* (Lane 2) and *F.*

nygamai (Lane 6), although the same amount of genomic DNA (100ng) was used in standard PCR reactions. Amplification products obtained were 700bp in length. Diffused smears along the length of the lanes for the various *Fusarium* strains and species were frequently observed with this primer set (Figure 4.2A, Lanes 2 to 6). This could have been due to insufficient $MgCl_2$ and enzyme concentrations, cycling conditions, and/or high DNA template concentrations.

The quantity of amplification product with the beta-tubulin primer set (Figure 4.2B) for *F. verticillioides* (Lanes 2 to 7) was high. Annealing temperature was lower for this primer set (Section 3.3.1). Amplification products obtained were ~640bp in length. PCR amplification of the beta-tubulin region for *F. proliferatum* and *F. nygamai* was inconsistent and difficult even though the same amount of genomic DNA (100ng/reaction volume) was used in PCR reactions (results not shown).

Genomic DNA of the various *Fusarium* strains and species amplified easily with the 18S primer set and yielded high quantities of the amplification product without the additional *Taq* polymerase (Figure 4.2C). Amplification products obtained were 570bp in length. Yield of the 18S rDNA fragments were similar for all of the *Fusarium* strains and species employed (Figure 4.2C, Lanes 2 to 6). This primer set produced clear, sharp bands devoid of non-specific products and/or primer dimers.

PCR amplification with the FUM primer set was problematic for most of the *Fusarium* strains and species. However, DNA of *F. verticillioides* amplified more easily with this primer set (Figure 4.2D, Lanes 2 to 5). Amplification products obtained were 800bp in length. *F. nygamai* and *F. subglutinans* produced either low quantities or not any FUM products using conventional PCR (results not shown). When optimized PCR conditions were used as described in Section

3.3.1, non-specific bands were evident (results not shown). *F. proliferatum* and *F. oxysporum* did not yield PCR product with the FUM primer set although optimized PCR conditions were applied (results not shown). In a study conducted by Bluhm *et al.* (2002), FUM product was detected for *F. proliferatum* by multiplex PCR. Amplification of *F. nygamai*, *F. subglutinans*, *F. proliferatum* and *F. oxysporum* were less efficient with the FUM primer set than *F. verticillioides*.

The EF-1α, FUM and 18S primer sets were selected for multiplex PCR since PCR products yielded by these primer sets were of distinct sizes. This property makes them ideal for multiplex PCR analysis.

4.2.2 Multiplex PCR analysis

With the EF-1α and FUM primer sets the *Fusarium* species (Table 3.1) produced the expected fragments sizes (Table 3.2) after the multiplex PCR under optimized conditions to ensure amplification of the desired gene regions and to eliminate non-specific primer interactions and amplification biases. Bezuidenhout *et al.* (2006a) reported that a 1:1 primer ratio and annealing temperature of 55°C most suitable for multiplex PCR with the EF-1α and FUM primer sets. These conditions were also applied in the present study.

The 18S primer set was not included in the multiplex PCR. The main reason for this was that preferential amplification occurred with this primer set. Although the method described by Bezuidenhout *et al.* (2006a) for the multiplex PCR with the selected primer sets was applied, in the present study only the 18S rDNA fragment amplified (results not shown). A possible explanation for preferential amplification of the 18S rDNA region is because of its large number of copies per genome (Berney *et al.*, 2000).

Successful amplification with the combined EF-1α and FUM primer sets for five *F. verticillioides* strains (Lanes 2 to 6) and three *F. nygamai* strains (Lanes 7 to 9) is illustrated in Figure 4.3. Amplification products of *F. nygamai* were of a lower yield when compared to *F. verticillioides* products even though optimized PCR conditions (Section 3.3.1) were used. Faint EF-1α and FUM bands were observed for *F. nygamai* whereas *F. verticillioides* produced two clear bands.

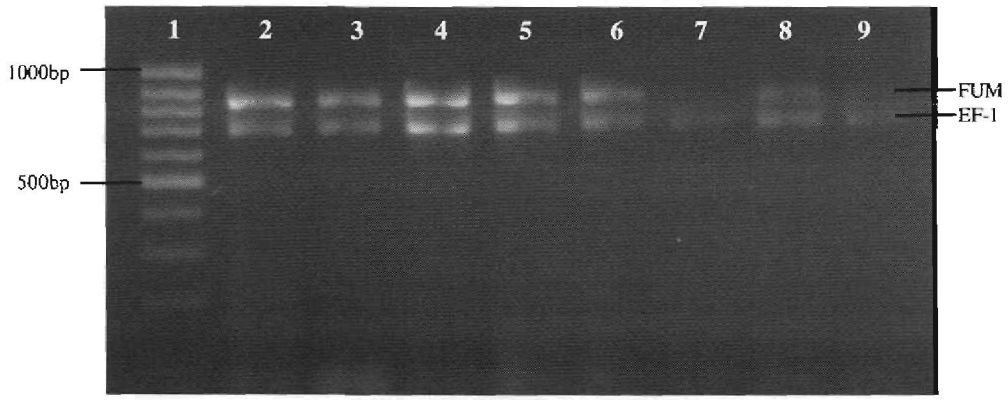

Figure 4.3: Multiplex PCR results for five *F. verticillioides* and three *F. nygamai* strains on a 1.5% (w/v) agarose gel. Lane 1 indicates a 100bp molecular size marker (O'GeneRuler^{TM} 100bp DNA ladder, Fermentas Life Sciences, US). Lane 2 to 6 represent the five *F. verticillioides* (MRC 8559, 8560, 826, 4317 & 4319) strains, and lane 7 to 9 represent the three *F. nygamai* (MRC 3997, 8546 & 8547) strains.

The multiplex PCR results presented in Figure 4.4 are for *F. proliferatum*, *F. oxysporum*, *F. subglutinans*, *F. verticillioides* and *F. nygamai* on a 1.5% (w/v) agarose gel. The *Fusarium* mixture (Lane 2), *F. verticillioides* (Lane 6) and *F. nygamai* (Lane 7) produced both the EF-1α and FUM bands. *F. proliferatum* (Lane 3), *F. oxysporum* (Lane 4) and *F. subglutinans* (Lane 5) only produced the EF-1α band. Various PCR conditions were tested to obtain both fragments for the latter three species, however only the EF-1α fragment was yielded (results not shown).

The multiplex PCR approach described here allowed the detection and identification of fumonisin producing *Fusarium* species although the amplification efficiency was lower compared to PCR described in Section 4.2. Further careful optimization of this method is necessary.

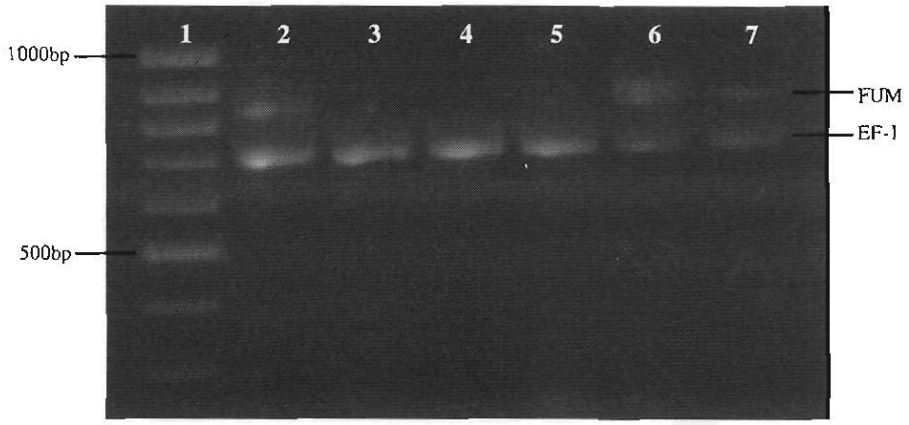

Figure 4.4: A 1.5% (w/v) ethidium bromide stained gel indicating multiplex PCR results for various *Fusarium* species. Lane 1 indicates a 100bp molecular marker (O'GeneRuler™ 100bp DNA ladder, Fermentas Life Sciences, US). Lane 2 represents a *Fusarium* mixture (*F. proliferatum*, *F. oxysporum*, *F. subglutinans*, *F. verticillioides*, and *F. nygamai*). Lane 3 - *F. proliferatum*; lane 4 - *F. oxysporum*; lane 5 - *F. subglutinans*; lane 6 - *F. verticillioides*; and lane 7 - *F. nygamai*.

Bezuidenhout *et al.* (2006a) suggested that the EF-1α and 18S fragments can be excised from multiplex PCR agarose gels and reanalyzed by PCR-DGGE. This approach was evaluated in the present study. However, only the EF-1α fragments were used because of its high phylogenetic utility as described in Section 2.3. Amplification conditions were as described in Section 3.5

Figure 4.5 illustrates the amplified 500bp EF-1α fragments with either the EF-1α GC or Alfie-1 GC primer set on a 1.5% (w/v) agarose gel. Both primer sets produced the expected fragment size (Table 3.2) for DGGE purposes. The EF-1α primer set produced good quality PCR products with

no primer dimers and/or non-specific products (Figure 4.5A). Primer dimers were observed for the Alfie-1 primer set (Figure 4.5B) although a higher annealing temperature was used.

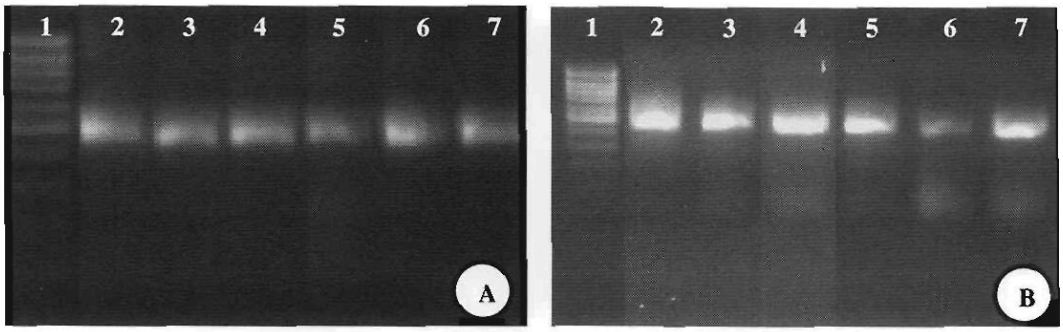

Figure 4.5: Amplified EF-1α fragments (500bp) of various *Fusarium* species and strains on a combined 1.5% (w/v) agarose gel. (A) The EF-1α GC primer set was used for amplification. Lane 2 represents a *Fusarium* mixture (*F. proliferatum, F. oxysporum, F. subglutinans, F. verticillioides* and *F. nygamai*). Lane 3 - *F. proliferatum*; lane 4 - *F. oxysporum*; lane 5 - *F. subglutinans*; lane 6 - *F. verticillioides* (MRC 826); and lane 7 - *F. nygamai* (MRC 3997). (B) The Alfie-GC primer set was used for amplification. The order of the amplification products is the same as in (A).

4.3 Denaturing gradient gel electrophoresis of the *Fusarium* species and strains

The 500bp EF-1α fragments (Section 3.3.2 & 3.5) were subjected to DGGE analysis. A 6% (w/v) polyacrylamide gel with a 40-60% denaturing gradient was used.

No discrimination was observed in the migration positions of amplicons obtained from DNA of the various *Fusarium* species and strains using the EF-1α GC (Figure 4.6A) and Alfie-1 GC (Figure 4.6B). Pure *Fusarium* species and strains should produce a single DGGE band, as demonstrated by Yergeau *et al.* (2005). However, in the present study multiple bands for a single strain and/or species were observed (Figure 4.6A & B). The DGGE profiles in Figure 4.6A & B illustrate a prominent band accompanied at close distance by another band. A faint third and fourth band was also observed. Occurrence of these multiple bands may be due to non-specific

products (heteroduplexes and chimeras) and/or cross contamination obtained through multiplex PCR. Because more than one primer set was included in the multiplex PCR, formation of artifacts (heteroduplexes and chimeras) could have taken place providing additional bands on DGGE gels (Kanagawa, 2003). Due to the presence of these bands, interpretation and analysis of DGGE gels were impeded as a method to discriminate between the various *Fusarium* species and strains.

Different TAE running buffer and polyacrylamide gel concentrations were also tested to determine if these would not improve the quality of the DGGE profiles. Despite these attempts, the quality of the DGGE profiles could not be improved (results not shown). Similar DGGE results for the EF-1α and Alfie-1 primer sets were repeatedly obtained as illustrated in Figure 4.6.

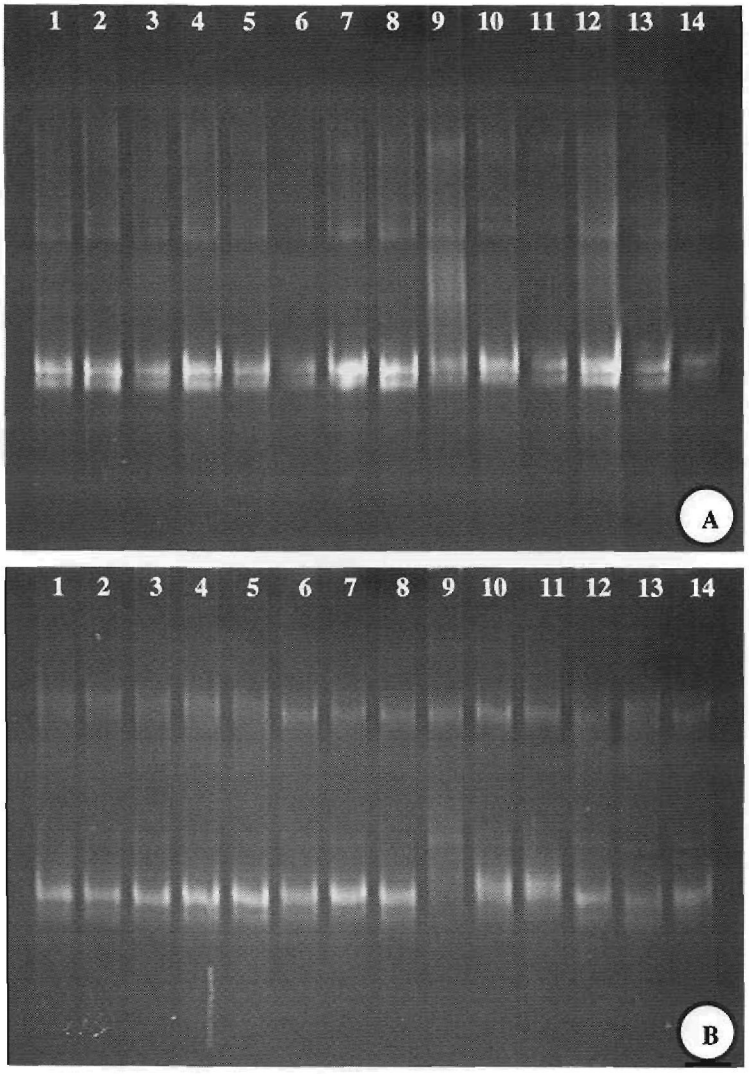

Figure 4.6: DGGE separations of EF-1α fragments for various *Fusarium* strains and species. A 6% (w/v) polyacrylamide gel with a 40-60% gradient was used. Electrophoresis was carried out at 80V for 16 hours. The re-extracted EF-1α fragments from the multiplex PCR were used for a second round of amplification with the (A) EF-1α GC primer set (Table 3.2) and (B) Alfie-1 GC primer set (Table 3.2). (A & B) Lane 1 to 5 – five *F. verticillioides* strains (MRC 8559, 8560, 826, 4317 & 4319); lane 6 to 8 – three *F. nygamai* strains (MRC 3997, 8546 & 8547); lane 9 – *Fusarium* mixture (*F. proliferatum*, *F. oxysporum*, *F. subglutinans*, *F. verticillioides* (MRC 826) and *F. nygamai* (MRC 3997)); lane 10 – *F. proliferatum*; lane 11 – *F. oxysporum*; lane 12 – *F. subglutinans*; lane 13 – *F. verticillioides* (MRC 826); and lane 14 – *F. nygamai* (MRC 3997).

The 18S rDNA genes were also used to investigate the potential of the DGGE technique to discriminate between various *Fusarium* species and strains. Optimized PCR conditions were used for amplification with the 18S primer set (Section 3.3.1). The 18S rDNA fragments were then subjected to DGGE analysis. A 6% (w/v) polyacrylamide gel with a 30-50% denaturing gradient was used.

DGGE profiles of the amplified 18S rDNA fragments (Figure 4.7A & B) were similar to DGGE profiles of the EF-1α fragments (Figure 4.6A). Most of the amplicons migrated to similar positions. Discrimination between the various *Fusarium* species and strains was impossible based on these DGGE profiles, except for *F. proliferatum* which was the only species showing a different migration pattern (Figure 4.7B, Lane 2). High intensity bands were observed for three of the *F. verticillioides* strains (Figure 4.7A, Lanes 2, 4 & 5), the *Fusarium* mix (Figure 4.7B, Lane 1), *F. proliferatum* (Figure 4.7B, Lane 2) and *F. subglutinans* (Figure 4.7B, Lane 5). The remainder of the *Fusarium* strains and species produced faint bands, but they were still visible (Figure 4.7A & B). Diffused smears along the length of each lane for both 18S DGGE profiles were observed (Figure 4.7A & B). Lanes which demonstrated higher intensity bands (Figure 4.7A, Lane 2, 4 & 5; Figure 4.7B, Lane 1, 2 & 5) showed more diffused smearing along the length of the lane.

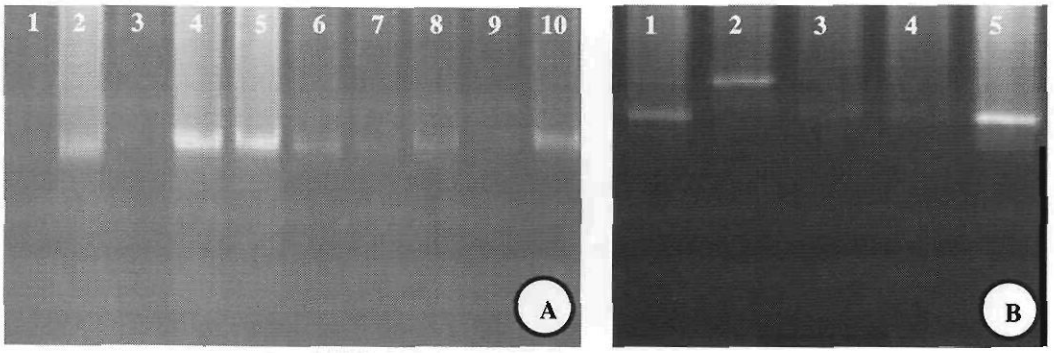

Figure 4.7: DGGE separations of 18S rDNA amplicons for various (A) *Fusarium* strains and (B) species. A 6% (w/v) polyacrylamide gel with a 30-50% gradient was used. Electrophoresis was carried out at 100V for 16 hours. (A) Analysis of five *F. verticillioides* and three *F. nygamai* strains. Lanes 1 & 7 are molecular markers composed of the five *F. verticillioides* and three *F. nygamai* strains, respectively. Lanes 2 to 6 represent the five *F. verticillioides* strains, and lanes 8 to 10 represent the three *F. nygamai* strains. (B) Analysis of various *Fusarium* species. Lane 1 represents a *Fusarium* mixture (*F. proliferatum*, *F. oxysporum*, *F. verticillioides* and *F. subglutinans*). Lane 2 - *F. proliferatum*; lane 3 - *F. oxysporum*; lane 4 - *F. verticillioides* (PPRI 7367); and lane 5 - *F. subglutinans*.

4.4 Single-strand conformation polymorphism of the *Fusarium* species and strains

SSCP analysis of the amplified EF-1α and beta-tubulin fragments (Section 3.6) was performed to investigate the potential of this technique to differentiate between various *Fusarium* species and strains.

On the 6% (w/v) polyacrylamide gel and under room temperature conditions (22-25°C), the SSCP patterns for the complete and restricted beta-tubulin fragments were absent or indistinct (Figure 4.8A & B). The SSCP process was repeated several times for the beta-tubulin gene. However, poor quality results were continuously obtained. The electrophoretic conditions described in this study may have changed the migration of the bands to such a degree that SSCPs were not detected.

Double-stranded DNA (dsDNA) bands were observed for the complete beta-tubulin fragments at the bottom of the gel (Figure 4.8A & B). When high concentrations of single-stranded DNA were loaded, the denatured PCR products may reanneal to form double-stranded DNA being the predominant band (Fujita & Silver, 1994; Humphries *et al.*, 1997). It is unlikely that these bands were heteroduplexes since DNA used for amplification was from a single species. Heteroduplexes are formed when single-stranded DNA from different sources reanneal to form DNA molecules with mismatched base pairs (Humphries *et al.*, 1997; Axton & Hanson, 1998). The dsDNA bands illustrated different migration positions for the various *Fusarium* species (Figure 4.8A & B).

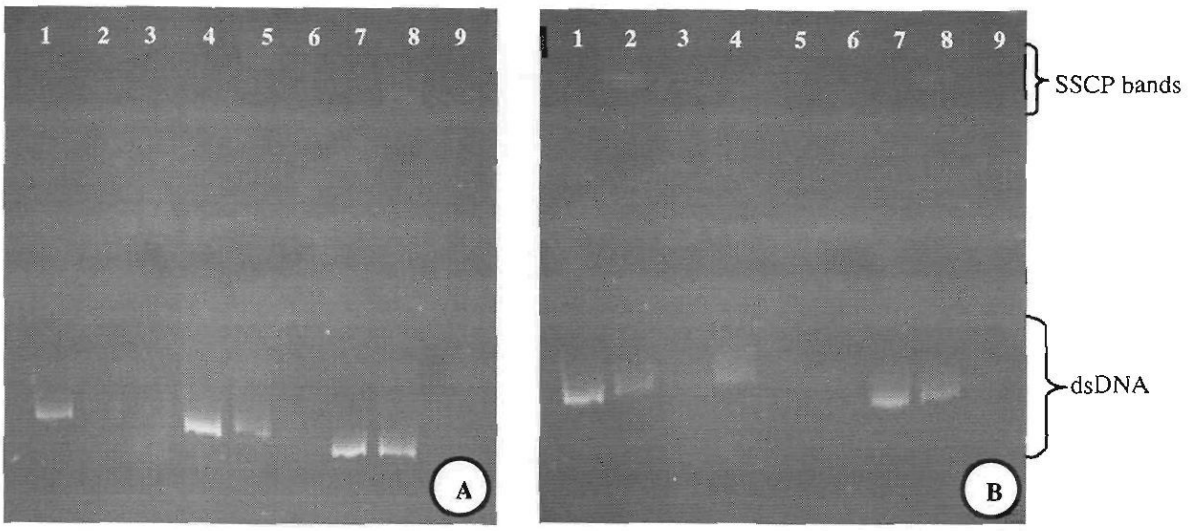

Figure 4.8: SSCP analyses of the complete and restricted beta-tubulin fragments of six *Fusarium* species on a 6% (w/v) non-denaturing polyacrylamide gel. Electrophoresis was carried out at 500V for 3.5 hours. Each species also had a control. (A) Lane 1 – *F. proliferatum* (control); lane 2 – *F. proliferatum* (650bp BT fragments); lane 3 – *F. proliferatum* (restricted BT fragments); lane 4 – *F. oxysporum* (control); lane 5 - *F. oxysporum* (650bp BT fragments); lane 6 - *F. oxysporum* (restricted BT fragments); lane 7 – *F. moniliforme* (control); lane 8 – *F. moniliforme* (650bp BT fragments); and lane 9 – *F. moniliforme* (restricted BT fragments). (B) Lane 1 – *F. subglutinans* (control); lane 2 – *F. subglutinans* (650bp BT fragments); lane 3 – *F. subglutinans* (restricted BT fragments); lane 4 – *F. verticillioides* (control); lane 5 - *F. verticillioides* (650bp BT fragments); lane 6 - *F. verticillioides* (restricted BT fragments); lane 7 – *F. nygamai* (control); lane 8 – *F. nygamai* (650 bp BT fragments); and lane 9 – *F. nygamai* (restricted BT fragments).

Pure DNA, as well as the re-extracted EF-1α fragments from the multiplex PCR of the various *Fusarium* species and strains, was amplified with the Alfie-1 primer set without the GC clamp (Table 3.2). The fragments were then subjected to a 6% (w/v) non-denaturing polyacrylamide gel. Electrophoresis was carried out at 500V for 4.5 hours.

SSCP analysis of the EF-1α fragments is illustrated in Figure 4.9 for five *F. verticillioides* strains (Lanes 2 to 6) and three *F. nygamai* strains (Lanes 7 to 9) on a 6% (w/v) non-denaturing polyacrylamide gel. Pure DNA was used for PCR amplification prior to SSCP analysis. The SSCP patterns for four of the *F. verticillioides* strains illustrated similar migration positions (Lanes 2 to 5). Two distinct bands were visible for each *F. verticillioides* strain. Thus, differentiation between the four *F. verticillioides* based on the SSCP patterns was not possible. Three of *F. nygamai* strains also produced two high intensity SSCP bands (Lanes 7 to 9). Two of the *F. nygamai* strains (Lanes 7 & 8) produced a faint third SSCP band while one *F. nygamai* strain (Lane 9) produced a third SSCP band of high intensity at a different migration level. Double-stranded DNA patterns for two *F. nygamai* strains were similar (Lanes 7 & 8). Migration position of the dsDNA band of one *F. nygamai* strain (Lane 9) varied from the dsDNA patterns of the other two *F. nygamai* strains (Lane 7 & 8). The migration positions of the dsDNA bands of the *F. nygamai* strains (Lanes 7 to 9) differed slightly from those of the *F. verticillioides* strains (Lanes 2 to 5). Differences in the SSCP patterns between the *F. verticillioides* strains and *F. nygamai* strains permitted differentiation between the two species. For this SSCP, no heteroduplex bands were seen.

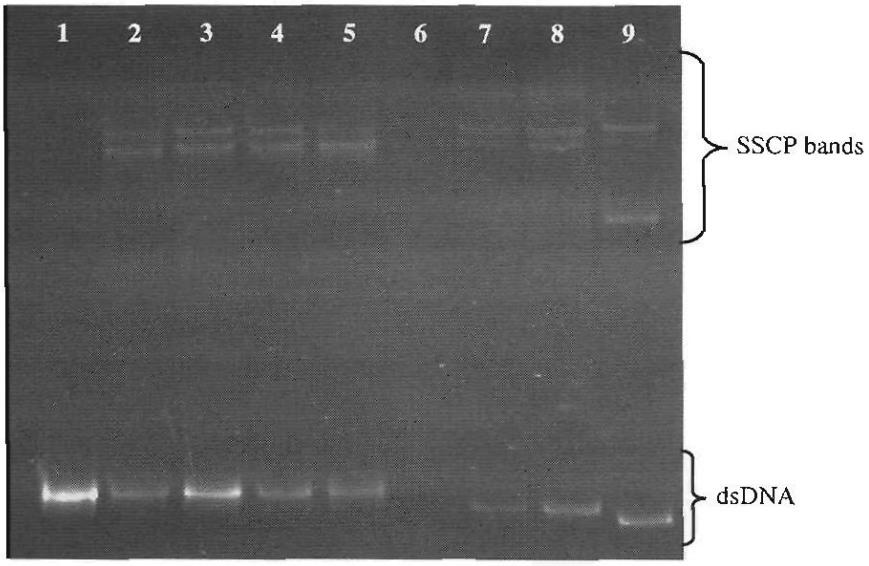

Figure 4.9: SSCP analysis of EF-1α fragments (500bp) on a 6% (w/v) non-denaturing polyacrylamide gel for five *F. verticillioides* and three *F. nygamai* strains. Pure DNA from samples was used for amplification with the Alfie-1 primer set (Table 3.2). Electrophoresis was carried out at 500V for 4.5 hours. Lane 1 – control; lane 2 to 6 represent the five *F. verticillioides* strains (MRC 8559, 8560, 826, 4317 & 4319); and lane 7 to 9 represent the three *F. nygamai* strains (MRC 3997, 8546 & 8547).

Figure 4.10 illustrates the SSCP patterns of the EF-1α fragments for the various *Fusarium* species on a 6% (w/v) non-denaturing polyacrylamide gel. Pure DNA was used for PCR amplification prior to SSCP analysis. Differences in the migration positions for the SSCP bands are clearly visible between the different species. *F. proliferatum* (Lane 2) and *F. subglutinans* (Lane 4) produced three distinct bands while the other species only produced two bands corresponding to the forward and reverse strands. The third SSCP band of *F. proliferatum* (Lane 2) and *F. subglutinans* (Lane 4) was located on different migration levels. Occurrence of the third band can correspond to: (i) various conformations of the two single strands of a single allele, (ii) the presence of two alleles or (iii) excess primers could have reannealed to the ssDNA after gel loading that lead to the third band (Hauser *et al.*, 1997; Wallace, 2002). Mobility shifts in the

dsDNA bands of *F. proliferatum* (Lane 2), *F. oxysporum* (Lane 3), *F. subglutinans* (Lane 4), *F. verticillioides* (Lane 5) and *F. nygamai* (Lane 6) were diverse. Differentiation between the *Fusarium* species, based on both the SSCP bands, was thus possible. Heteroduplex bands were not visible for this SSCP.

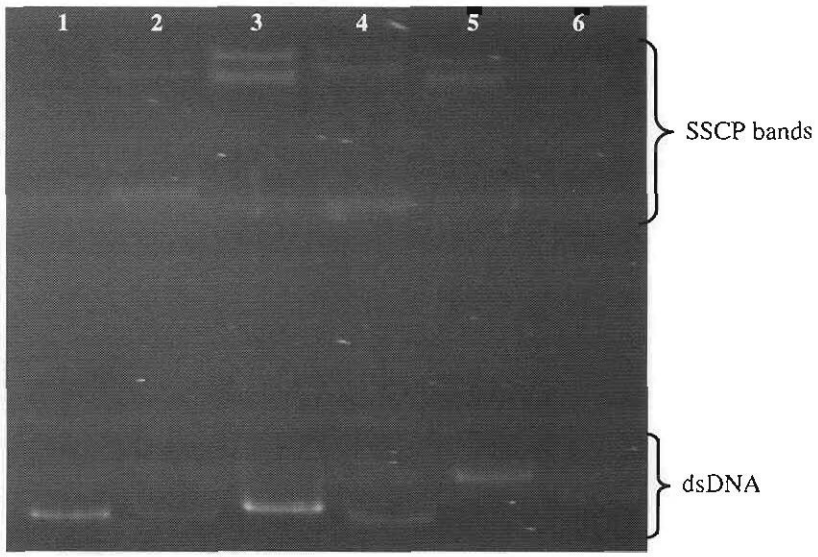

Figure 4.10: SSCP analysis of EF-1α fragments (500bp) on a 6% (w/v) non-denaturing polyacrylamide gel for five *Fusarium* species. Pure DNA was used for amplification with the Alfie-1 primer set (Table 3.2). Electrophoresis was carried out at 500V for 4.5 hours. Lane 1 – control; lane 2 – *F. proliferatum*; lane 3 – *F. oxysporum*; lane 4 – *F. subglutinans*; lane 5 – *F. verticillioides* (MRC 826); and lane 6 – *F. nygamai* (MRC 3997).

The EF-1α fragments were also subjected to an 8% (w/v) non-denaturing polyacrylamide gel. An increase in the gel concentration was made to test for improvement of the resolution of the fragments. Electrophoretic conditions were the same as for the 6% (w/v) gels.

SSCP patterns of the EF-1α fragments are illustrated in Figure 4.11 for the various *Fusarium* species and strains on an 8% (w/v) non-denaturing polyacrylamide gel. Pure DNA was used for amplification prior to SSCP analysis. The five *F. verticillioides* strains (Lanes 2 to 6)

demonstrated three closely related SSCP bands with no variation in migration shifts. SSCP bands of one of the *F. verticillioides* strains (Lane 2) were faint and unclear. Two of the *F. verticillioides* strains (Lanes 2 & 6) did either not produce a dsDNA band or bands were very faint. Migration positions of the dsDNA bands for four *F. verticillioides* strains (Lanes 3 to 6) differed slightly. Two of the *F. nygamai* strains (Lanes 7 & 8) produced two closely related SSCP bands. A third SSCP band was observed with one of the *F. nygamai* strains (Lane 9). A variation in the SSCP patterns of *F. proliferatum* (Lane 12), *F. oxysporum* (Lane 13), *F. subglutinans* (Lane 14) and *F. verticillioides* (Lane 15) was observed. *F. proliferatum* (Lane 12), *F. subglutinans* (Lane 14) and *F. oxysporum* (Lane 13) also generated a low intensity third and fourth SSCP bands. There was a clear difference in the migration positions of the dsDNA bands of *F. proliferatum* (Lane 12), *F. oxysporum* (Lane 13), *F. subglutinans* (Lane 14) and *F. verticillioides* (Lane 15). Differentiation between the latter four *Fusarium* species, based on the SSCP bands, was thus possible. Heteroduplex bands were not evident for this SSCP.

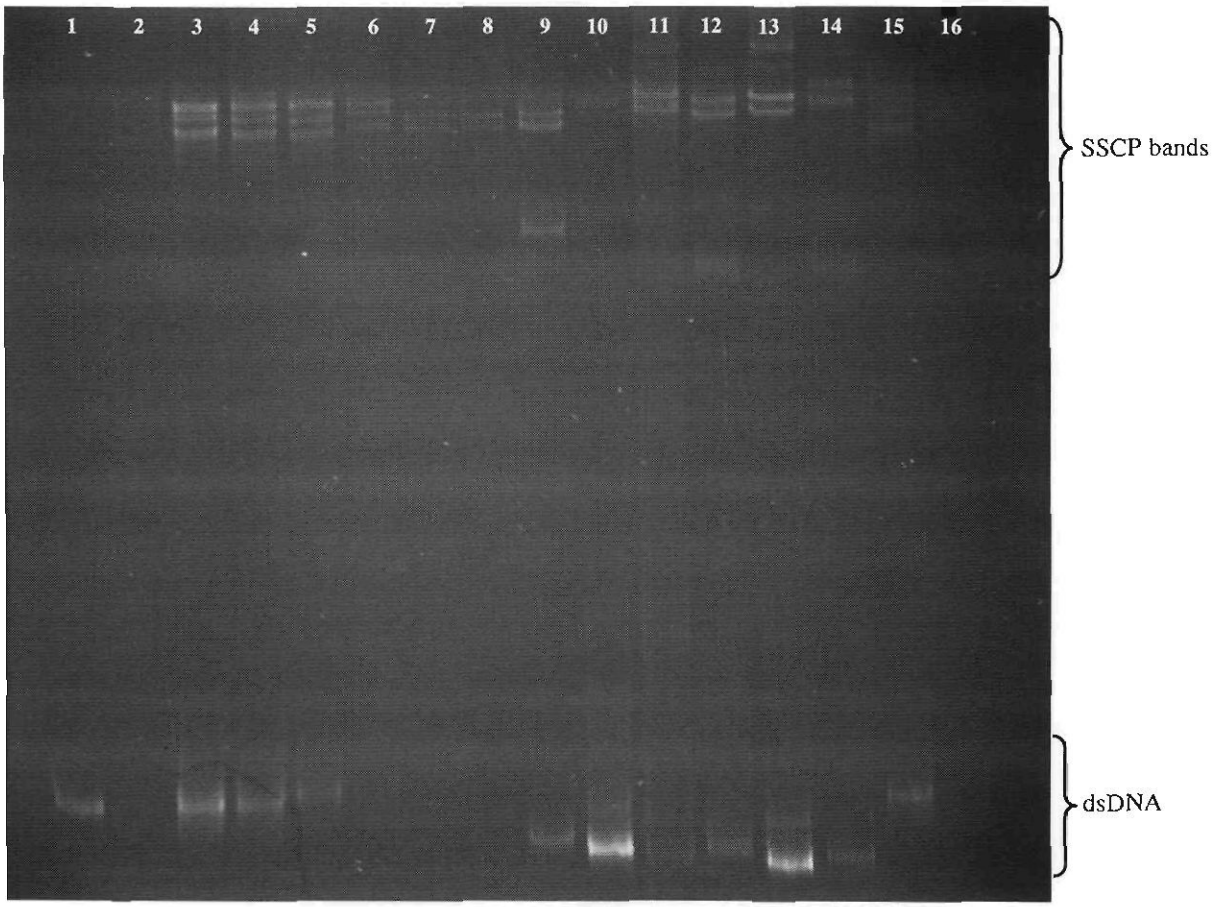

Figure 4.11: SSCP analysis of EF-1α fragments (500bp) on an 8% (w/v) non-denaturing polyacryalamide gel for several *Fusarium* species and strains. Pure DNA was used for amplification with the Alfie-1 primer set (Table 3.2). Electrophoresis was carried at 500V for 4.5 hours. Lane 1 – control, lane 2 to 6 represents the five *F. verticillioides* strains (MRC 8559, 8560, 826, 4317 & 4319); lane 7 to 9 represents the three *F. nygamai* strains (MRC 3997, 8546 & 8547); lane 10 – control; lane 11 – *Fusarium* mixture (*F. proliferatum, F. oxysporum, F. subglutinans, F. verticillioides* (MRC 826) and *F. nygamai* (MRC 3997)); lane 12 - *F. proliferatum*; lane 13 – *F. oxysporum*; lane 14 – *F. subglutinans*; lane 15 – *F. verticillioides* (MRC 826); and lane 16 – *F. nygamai* (MRC 3997).

Figure 4.12 illustrates the SSCP patterns of the EF-1α fragments for the various *Fusarium* species and strains on an 8% (w/v) non-denaturing polyacrylamide gel. The EF-1α fragments that were re-extracted from the multiplex PCR were used for amplification prior to SSCP analysis. For

most of the *Fusarium* strains and species, one high intensity SSCP band was observed (Lanes 1 to 9 & 12 to 14). Only *F. proliferatum* (Lane 10) and *F. oxysporum* (Lane 11) produced two closely related high intensity SSCP bands. One of the *F. nygamai* strains (Lane 8) and *F. proliferatum* (Lane 10) produced a second and third SSCP band, respectively. These bands were also present in Figure 4.11, Lanes 9 & 12.

Multiple low intensity SSCP and/or non-specific bands were observed for all of the *Fusarium* strain and species (Figure 4.12, Lanes 1 to 14). A clear band in the upper part of the gel was visible for the five *F. verticillioides* strains (Lanes 1 to 5), three *F. nygamai* strains (Lanes 6 to 8), *Fusarium* mix (Lane 9), *F. proliferatum* (Lane 10) and *F. oxysporum* (Lane 11). Three non-specific bands in the center of the gel were observed for one of the *F. verticillioides* strains (MRC 826) (Lanes 3 & 13) and *F. subglutinans* (Lane 12). Faint non-specific bands were also visible for the *Fusarium* mix (Lane 9). These bands did not appear on the previous SSCP gel (Figure 4.11) where DNA was directly amplified with the Alfie-1 primer set prior to SSCP gel electrophoresis. Migration shifts in the dsDNA bands differed noticeably between the different *Fusarium* species (Figure 4.12, Lanes 10 to 14) and the three *F. nygamai* strains (Lanes 6 to 8). Migration positions of the dsDNA bands for the *F. verticillioides* strains (Lanes 1 to 5) were similar.

The PCR-SSCP method described in the present study can be used as a potential application tool to detect and differentiate between various *Fusarium* species. Elongation factor-1α gene proved to be a better means for discriminating between the *Fusarium* species and strains employed. However, from the results presented in Figure 4.9 to 4.12, it is evident that pure DNA should be used for amplification prior to SSCP analysis. Fragments derived from multiplex PCR assays

should not be used for SSCP analysis since multiple non-specific bands are obtained which may impede interpretation of the results.

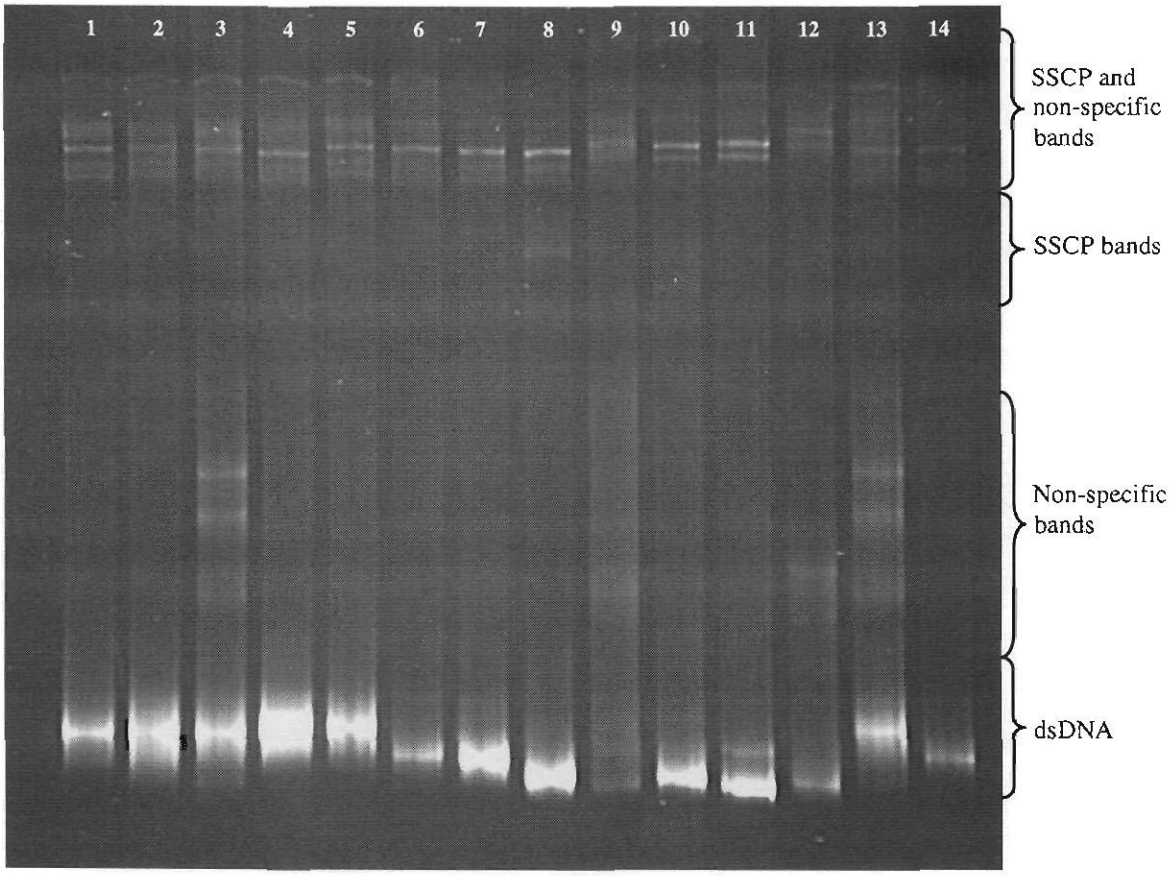

Figure 4.12: SSCP analysis of EF-1α fragments (500bp) on an 8% (w/v) non-denaturing polyacrylamide gel for several *Fusarium* species and strains. Re-extracted EF-1α fragments from the multiplex PCR was used for amplification with the Alfie-1 primer set (Table 3.2). Electrophoresis was carried at 500V for 4.5 hours. Lane 1 to 5 represents the five *F. verticillioides* strains (MRC 8559, 8560, 826, 4317 & 4319); lane 6 to 8 represents the three *F. nygamai* strains (MRC 3997, 8546 & 8547); lane 9 – *Fusarium* mixture (*F. proliferatum*, *F. oxysporum*, *F. subglutinans*, *F. verticillioides* (MRC 826) and *F. nygamai* (MRC 3997)); lane 10 – *F. proliferatum*; lane 11 - *F. oxysporum*; lane 12 – *F. subglutinans*; lane 13 – *F. verticillioides* (MRC 826); and lane 14 – *F. nygamai* (MRC 3997).

4.5 Detection limits of fungal genomic DNA

Detection limits were established through the construction of serial dilutions of purified DNA as template. *F. verticillioides* (MRC 826) and *F. subglutinans* (ARC 7365) were used to determine the detection limits for the EF-1α and FUM primer sets as described in Section 3.8.1.

4.5.1 Detection limits for individual *Fusarium* species DNA using conventional PCR

Ten nanograms (10ng) of *F. verticillioides* DNA as template, the product of the EF-1α primer set was detectable on a 1.5% (w/v) agarose gel stained with ethidium bromide (Figure 4.13A). Genomic DNA of *F. subglutinans* was detected up to 1ng for the EF-1 primer set (Figure 4.13B). From these data, the detection limit for the EF-1α primer set for *F. verticillioides* (Figure 4.13A) and *F. subglutinans* (Figure 4.13B) was estimated to be 10 and 1ng of DNA template per reaction, respectively. Bezuidenhout *et al.* (2006a) reported a detection limit of 1ng of *Fusarium* DNA with the EF-1α primer set. Thus, the detection limits for the EF-1α primer set in the present study are consistent with the findings of Bezuidenhout *et al.* (2006a). Primer dimers were constantly visible for both *Fusarium* species.

Amplification products with the FUM primer set were detectable up to 0.1ng DNA for *F. verticillioides* on a 1.5% (w/v) agarose gel (Figure 4.13C). Genomic DNA of *F. subglutinans* was detected up to 10ng with the FUM primer set (Figure 4.13D). Thus, the detection limit for the FUM primer set for *F. verticillioides* (Figure 4.13C) and *F. subglutinans* (Figure 4.13D) was estimated to be 0.1 and 10ng of DNA per reaction, respectively. Bluhm *et al.* (2002) reported a detection limit between 0.1 and 1ng of *F. verticillioides* template per reaction for the FUM primer set. The detection limits for the FUM primer set in the present study are consistent with the finding of Bluhm *et al.* (2002). Primer dimers were also a concern for both *Fusarium* species (Figure 4.13C & D).

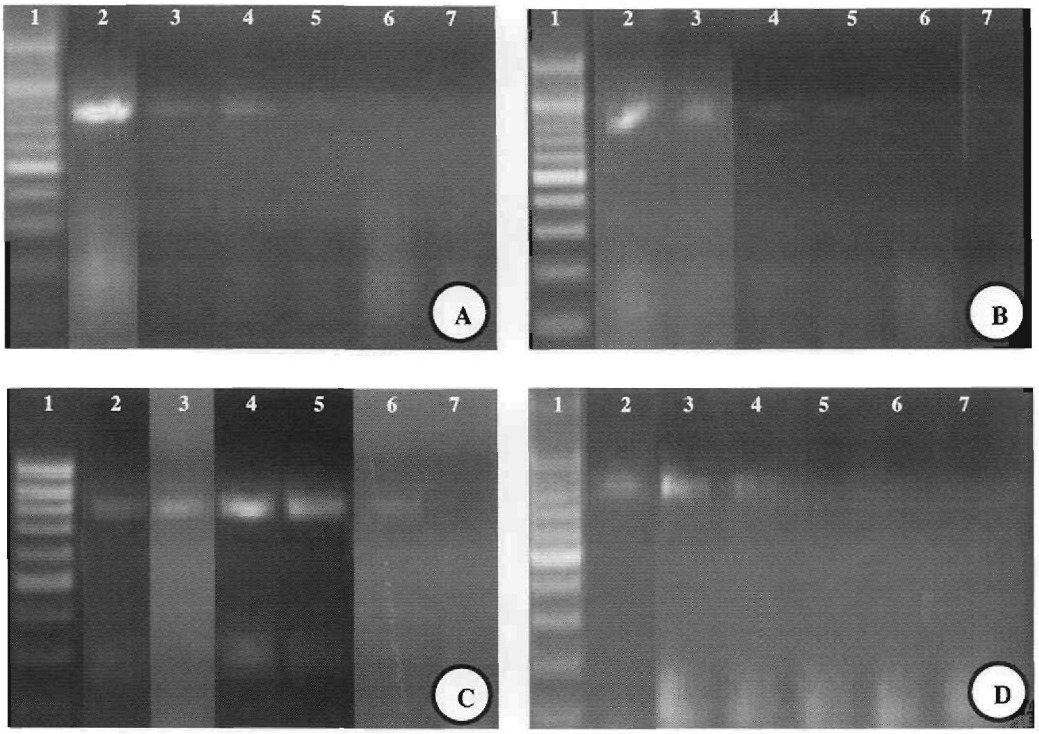

Figure 4.13: Combined 1.5% (w/v) agarose gels illustrating the detection limits for individual *Fusarium* species with serial dilutions of genomic DNA with the EF-1α and FUM primer sets. The dilution series ranged from 100 to 0.01ng DNA per reaction volume. Lane 1 contains a 100bp molecular marker (O'GeneRuler, Fermentas Life Sciences, US). (A) EF-1α primer set with DNA of *F. verticillioides* (MRC 826) as template. Lane 2 – 100ng DNA; lane 3 – 50ng DNA; lane 4 – 10ng DNA; lane 5 – 1ng DNA; lane 6 – 0.1ng DNA; and lane 7 – 0.01ng DNA. (B) EF-1α primer set with DNA of *F. subglutinans* (ARC 7365) as template. (C) FUM primer set with DNA of *F. verticillioides* (MRC 826) as template. (D) FUM primer set with DNA of *F. subglutinans* (ARC 7365) as template. The order of the amplified products for (B), (C) and (D) is the same as in (A).

4.5.2 Detection limits of *Fusarium* species DNA in the presence of non-*Fusairum* species DNA by conventional PCR

Detection limits were established through the construction of serial dilutions of purified DNA as template. *F. verticillioides* (MRC 826) and *F. subglutinans* (ARC 7365) were used to determine the detection limits for the EF-1α and FUM primer sets in the presence of non-*Fusarium* speices as described in Section 3.8.2.

Genomic DNA of *F. verticillioides* was detected up to 0.01ng with the EF-1α primer set in the presence of non-*Fusarium* DNA (Figure 4.14A). Similar results were obtained from *F. subglutinans* (Figure 4.14B). PCR products obtained with the EF-1α primer set for *F. verticillioides* and *F. subglutinans* in the presence of non-*Fusarium* species were ~450bp in length. This band was detected even if 0.01ng of *F. verticillioides* (Figure 4.14A) or *F. subglutinans* (Figure 4.14B) was used. The intensity was similar for all the samples tested irrespective of test DNA concentrations. Amplified EF-1α fragments for the individual *Fusarium* species were 700bp. Thus, unkown products were amplified with the EF-1α primer set in the presence of non-*Fusarium* spp. Sequence analysis will be necessary to determine the identity of the fragment. An 800bp band was visible for the 100ng dilution series of *F. verticillioides* (Figure 4.14A, Lane 2).

Genomic DNA of *F. verticillioides* was detected up to 10ng with the FUM primer set (Figure 4.14C) in the presence of non-*Fusarium* DNA. DNA of *F. subglutinans* was detected up to 1ng per reaction (Figure 4.14D). Therefore, the detection limit for *F. verticillioides* (Figure 4.14C) and *F. subglutinans* (Figure 4.14D) with the FUM primer set was estimated to be 10 and 1ng of DNA per reaction, respectively. Faint primer dimer bands were visible for both *Fusarium* species with the FUM primer set (Figure 4.14C & D).

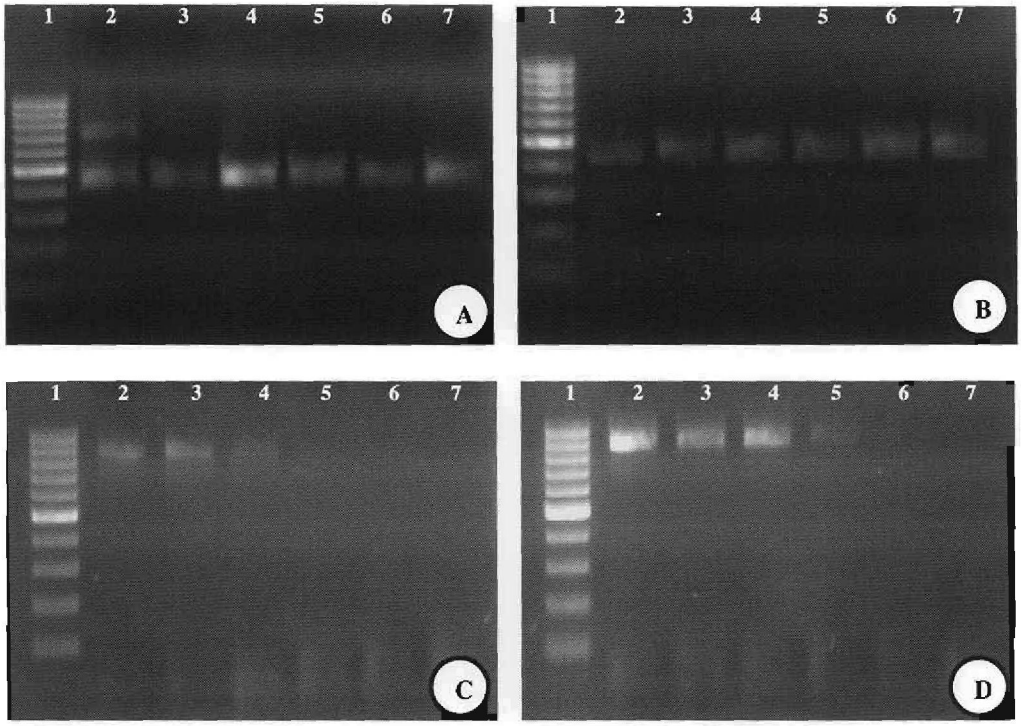

Figure 4.14: 1.5% (w/v) Agarose gels illustrating the detection limits of *F. verticillioides* and *F. subglutinans* in the presence of non-*Fusarium* species with the EF-1α and FUM primer sets. A dilution series was constructed ranging from 100 to 0.01ng DNA per reaction volume. DNA concentrations of the non-*Fusarium* species were constantly a 100ng per reaction volume. Lane 1 contains a 100bp molecular marker (O'GeneRuler, Fermentas Life Sciences, US). (A) EF-1α primer set with DNA of *F. verticillioides* (MRC 826) as template. Lane 2 – 100ng DNA; lane 3 – 50ng DNA; lane 4 – 10ng DNA; lane 5 – 1ng DNA; lane 6 – 0.1ng DNA; and lane 7 – 0.01ng DNA. (B) EF-1α primer set with DNA of *F. subglutinans* (ARC 7365) as template. (C) FUM primer set with DNA of *F. verticillioides* (MRC 826) as template. (D) FUM primer set with DNA of *F. subglutinans* (ARC 7365) as template. The order of the amplified products for (B), (C) and (D) is the same as in (A).

4.5.3 Quantitative detection of individual *Fusarium* species DNA

Real-time PCR was performed on *F. verticillioides* (MRC 826) and *F. subglutinans* (ARC 7365) using the EF-1α and FUM primer sets. Conditions were used as described in Section 3.8.3.

Genomic DNA of *F. verticillioides* and *F. subglutinans* was detected up to 10pg DNA per reaction with the EF-1α primer set (Figure 4.15A & B). Thus, the detection limit for *F. verticillioides* and *F. subglutinans* using real-time PCR with the EF-1α primer set was estimated to be 10pg DNA per reaction. Quantitative detection of *Fusarium* was a 1000 × more sensitive than the conventional PCR method. Amplified fragments were electrophoresed on a 1.5% (w/v) agarose gel. Elongation factor-1α fragments for *F. verticillioides* were visible up to 10ng DNA (Figure 4.16A, Lane 4), while the EF-1α fragments for *F. subglutinans* were visible up to 1ng DNA (Figure 4.16B, Lane 5). Bands indicating the presence of non-specific products for the 100ng, 50ng and 10ng dilution series of *F. verticillioides* were evident (Figure 4.16A, Lanes 2 to 4). Faint non-specific bands for the 100ng, 50ng and 10ng dilution series of *F. subglutinans* were also noticed (Figure 4.16B, Lanes 2 to 4). However, the presence of these non-specific products is not visible on the melt curve profiles for the two *Fusarium* species (Appendix B, Figure B3 & B4). Additional peaks present on the melt curve profiles illustrate the presence of primer dimers. From these profiles, it also evident that the 1ng, 0.1ng and 0.01ng dilutions series produced the largest primer dimer peaks. Smaller primer dimer peaks are visible for the 100ng, 50ng and 10ng dilution series. Non-specific products could have been detected on the melt curve profiles if the number of melting cycles was increased.

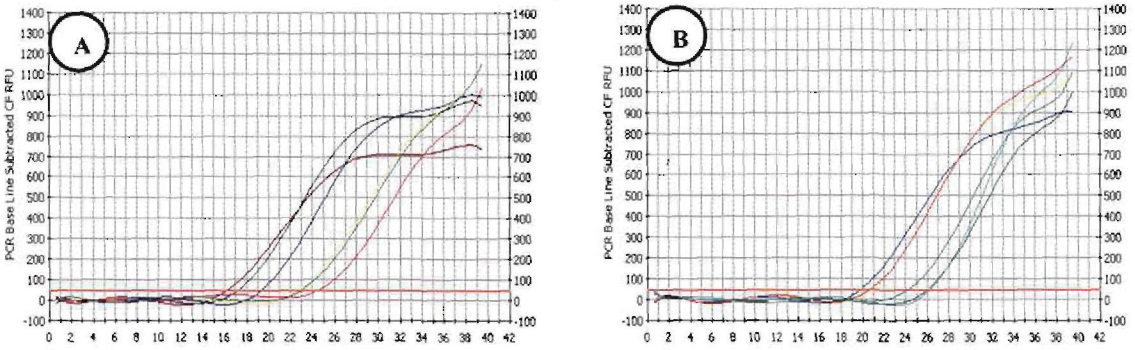

Figure 4.15: Real-time PCR assay of the EF-1α primer set for *F. verticillioides* (MRC 826) and *F. subglutinans* (ARC 7365) in a 10-fold dilution series. The detection limit was estimated to be 10pg DNA template per reaction volume. The colored lines indicate the concentration values for each dilution. (A) DNA of *F. verticillioides* (MRC 826) as template: 100ng (▬); 50ng (▬); 10ng (▬); 1ng (▬); 0.1ng (▬); and 0.01ng (▬). (B) DNA of *F. subglutinans* (ARC 7365) as template: 100ng (▬); 50ng (▬); 10ng (▬); 1ng (▬); 0.1ng (▬); and 0.01ng (▬).

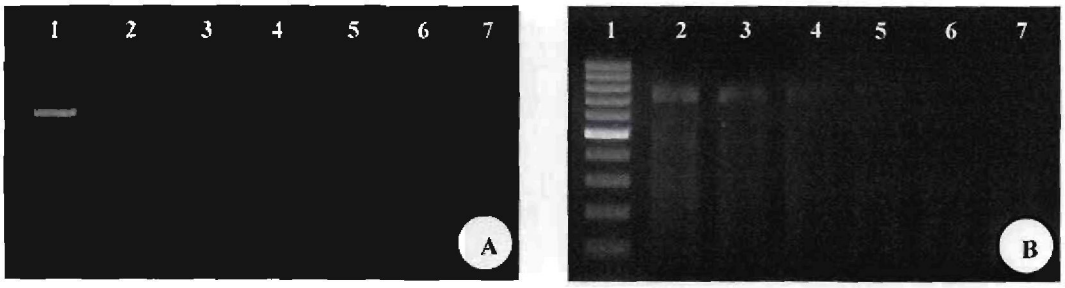

Figure 4.16: 1.5% (w/v) Agarose gels illustrating quantitative detection for (A) *F. verticillioides* and (B) *F. subglutinans* with serial dilutions of genomic DNA with the EF-1α primer set. The dilution series ranged from 100 to 0.01ng DNA per reaction volume. Lane 1 contains a 100bp molecular marker (O'GeneRuler, Fermentas Life Sciences, US). (A) EF-1α primer set with DNA of *F. verticillioides* (MRC 826) as template. Lane 2 – 100ng DNA; lane 3 – 50ng DNA; lane 4 – 10ng DNA; lane 5 – 1ng DNA; lane 6 – 0.1ng DNA; and lane 7 – 0.01ng DNA. (B) EF-1α primer set with DNA of *F. subglutinans* (ARC 7365) as template. The order of the amplified products is the same as in (A).

Genomic DNA of *F. verticillioides* and *F. subglutinans* was detected up to 10pg DNA per reaction with the FUM primer set (Figure 4.17A & B). Thus, quantitative detection for *F. verticillioides* and *F. subglutinans* with the FUM primer set was estimated to be 10pg DNA per reaction. Limits of detection with the FUM primer set using real-time PCR was a 1000 × more sensitive than the conventional PCR method. Amplified fragments were electrophoresed on a 1.5% (w/v) agarose gel. FUM fragments for *F. verticillioides* were visible up to 1ng DNA (Figure 4.18A, Lane 5), while the FUM fragments for *F. subglutinans* were visible up to 0.1ng DNA (Figure 4.18B, Lane 6). Non-specific bands (~500bp) corresponding to the 100 to 1ng and 100 to 0.1ng dilution series were evident for *F. verticillioides* (Figure 4.18A, Lanes 2 to 5) and *F. subglutinans* (Figure 4.18B, Lanes 2 to 6), respectively. However, the presence of these non-specific products is not visible on the melt curve profiles for the two *Fusarium* species (Appendix B, Figure B5 & B6). Additional peaks present on the melt curve profiles illustrate the presence of primer dimers. From these profiles, it also evident that the 1ng, 0.1ng and 0.01ng dilutions series produced the largest primer dimer peaks. Smaller primer dimer peaks are visible for the 100ng, 50ng and 10ng dilution series.

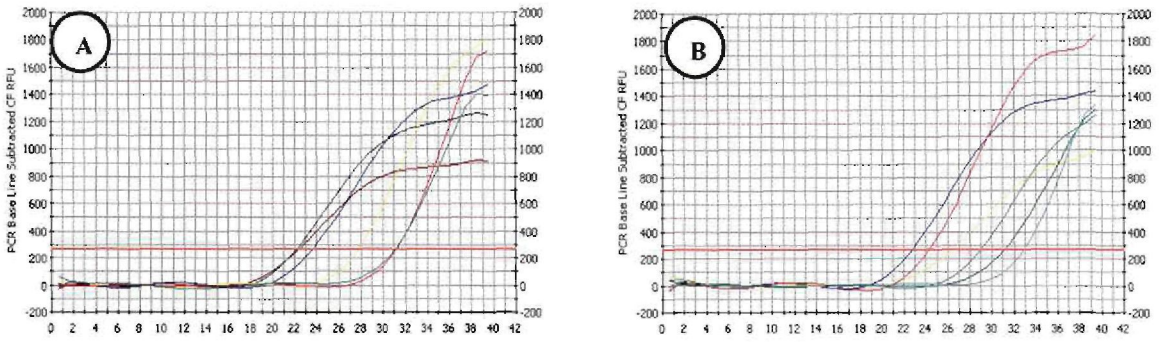

Figure 4.17: Real-time PCR assay of the FUM primer set for *F. verticillioides* (MRC 826) and *F. subglutinans* (ARC 7365) in a 10-fold dilution series. The detection limit was estimated to be 10pg DNA template per reaction volume. The colored lines indicate the concentration values for each dilution. (A) DNA of *F. verticillioides* (MRC 826) as template: 100ng ( ); 50ng ( ); 10ng ( ); 1ng ( ); 0.1ng ( ); and 0.01ng ( ). (B) DNA of *F. subglutinans* (ARC 7365) as template: 100ng ( ); 50ng ( ); 10ng ( ); 1ng ( ); 0.1ng ( ); and 0.01ng ( ).

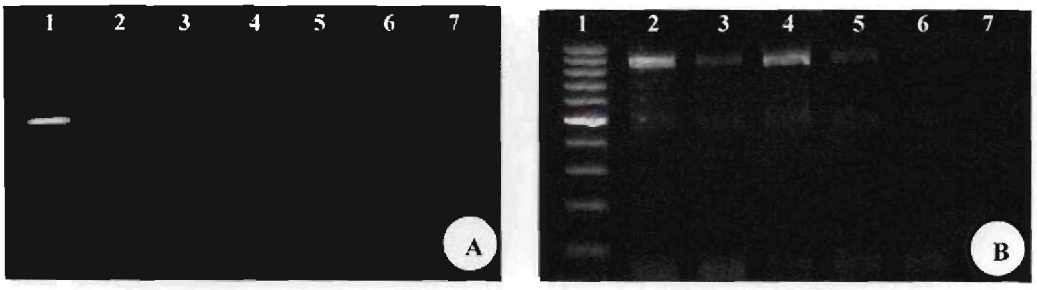

Figure 4.18: 1.5% (w/v) Agarose gels illustrating quantitative detection for (A) *F. verticillioides* and (B) *F. subglutinans* with serial dilutions of genomic DNA with the FUM primer set. The dilution series ranged from 100 to 0.01ng DNA per reaction volume. Lane 1 contains a 100bp molecular marker (O'GeneRuler, Fermentas Life Sciences, US). (A) FUM primer set with DNA of *F. verticillioides* (MRC 826) as template. Lane 2 – 100ng DNA; lane 3 – 50ng DNA; lane 4 – 10ng DNA; lane 5 – 1ng DNA; lane 6 – 0.1ng DNA; and lane 7 – 0.01ng DNA. (B) FUM primer set with DNA of *F. subglutinans* (ARC 7365) as template. The order of the amplified products is the same as in (A).

4.5.4 Quantitative detection of *Fusarium* species DNA in the presence of non-*Fusairum* species DNA

Genomic DNA for *F. verticillioides*, in the presence of non-*Fusarium* species, was detected up to 10pg per reaction (Figure 4.19A) when the EF-1α primer set was used. Similar results were obtained for *F. subglutinans* (Figure 4.19B). In Figure 4.19, it is also evident that the entire dilution series for *F. verticillioides* and *F. subglutians* have similar Ct values. Ct values range between 16.4 and 18.4. Amplified EF-1α fragments were electrophoresed on a 1.5% (w/v) agarose gel. Elongation factor-1α fragments for both *F. verticillioides* and *F. subglutinans* were visible up to 10pg DNA (Figure 4.20A & B). Non-specific products and primer dimers were absent on the agarose gels (Figure 4.20A & B). These results concur with their melt curve profiles (Appendix B, Figure B7 & B8). Clear, sharp melting peaks are visible and additional peaks due to non-specific products and primer dimers are absent. PCR product obtained with the EF-1α primer set for *F. verticillioides* and *F. subglutinans* in the presence of non-*Fusarium* spp. were ~450bp in length. This product was visible enven if 10pg of DNA was used (Figure 4.20A & B). The intensity of bands indicating the amplification products was similar for all the the samples irrespective of their DNA concentrations. Amplified EF-1α fragments for the individual *Fusarium* species were 700bp. Thus, unkown products were amplified with the EF-1α primer set in the presence of non-*Fusarium* species. Sequence analysis will be necessary to determine the identity of the fragment. Similar results were obtained using conventional PCR (Section 4.5.2).

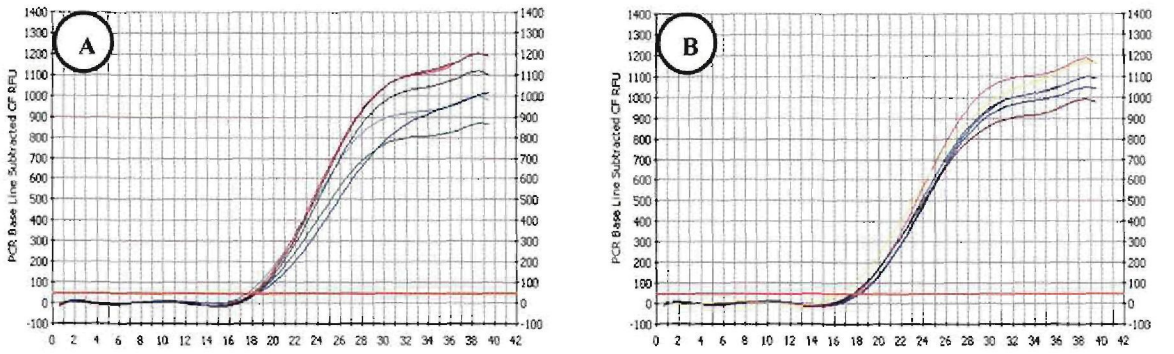

Figure 4.19: Real-time PCR assay of the EF-1α primer set for *F. verticillioides* (MRC 826) and *F. subglutinans* (ARC 7365) in the presence of non-*Fusarium* species. A 10-fold dilution series ranging from 100 to 0.01ng DNA was constructed. The detection limit was estimated to be 10pg DNA template per reaction volume. DNA concentrations of the non-*Fusarium* species were constantly a 100ng per reaction volume. The colored lines indicate the concentration values for each dilution. (A) DNA of *F. verticillioides* (MRC 826) as template: 100ng ( ); 50ng ( ); 10ng ( ); 1ng ( ); 0.1ng ( ); and 0.01ng ( ). (B) DNA of *F. subglutinans* (ARC 7365) as template: 100ng ( ); 50ng ( ); 10ng ( ); 1ng ( ); 0.1ng ( ); and 0.01ng ( ).

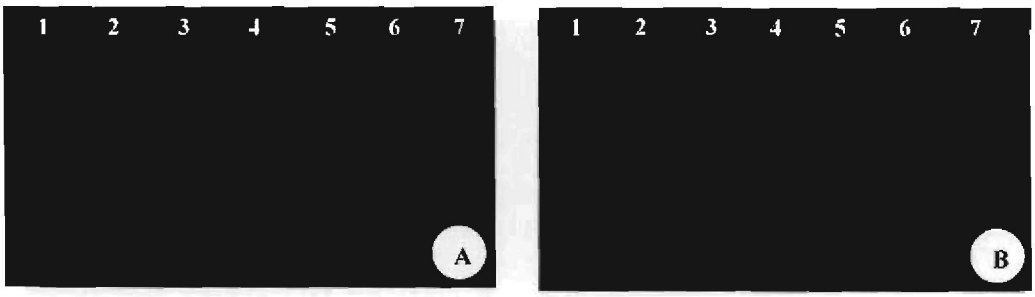

Figure 4.20: 1.5% (w/v) Agarose gels illustrating quantitative detection of (A) *F. verticillioides* and (B) *F. subglutinans* in the presence of non-*Fusarium* species with serial dilutions of genomic DNA with the EF-1α primer set. The dilution series ranged from 100 to 0.01ng DNA per reaction volume. DNA concentrations of the non-*Fusarium* spp. were constantly a 100ng per reaction volume. Lane 1 contains a 100bp molecular marker (O'GeneRuler, Fermentas Life Sciences, US). (A) EF-1α primer set with DNA of *F. verticillioides* (MRC 826) as template. Lane 2 – 100ng DNA; lane 3 – 50ng DNA; lane 4 – 10ng DNA; lane 5 – 1ng DNA; lane 6 – 0.1ng DNA; and lane 7 – 0.01ng DNA. (B) EF-1α primer set with DNA of *F. subglutinans* (ARC 7365) as template. The order of the amplified products is the same as in (A).

Genomic DNA for *F. verticillioides*, in the presence of non-*Fusarium* species, was detected up to 10pg per reaction (Figure 4.21A) when the FUM primer set was used. Similar results were obtained for *F. subglutinans* (Figure 4.21B). In Figure 4.21B, it is clear that the entire dilution series for *F. subglutinans* have similar Ct values. Ct values range between 32 and 33.2. Amplified FUM fragments were electrophoresed on a 1.5% (w/v) agarose gel. However, the PCR products were not visible on the gel (results not shown). It could thus not be conclusively established what the size of the amplification product was and whether it is of the expected size. The melt curve profile of *F. verticillioides* illustrated non-specific peaks for the 50 and 0.01ng dilution series (Appendix B, Figure B9). A non-specific peak for the 50ng dilution series on the melt curve profile of *F. subglutinans* was also observed (Appendix B, Figure B10). These non-specific products were not noticeable on the agarose gel (results not shown).

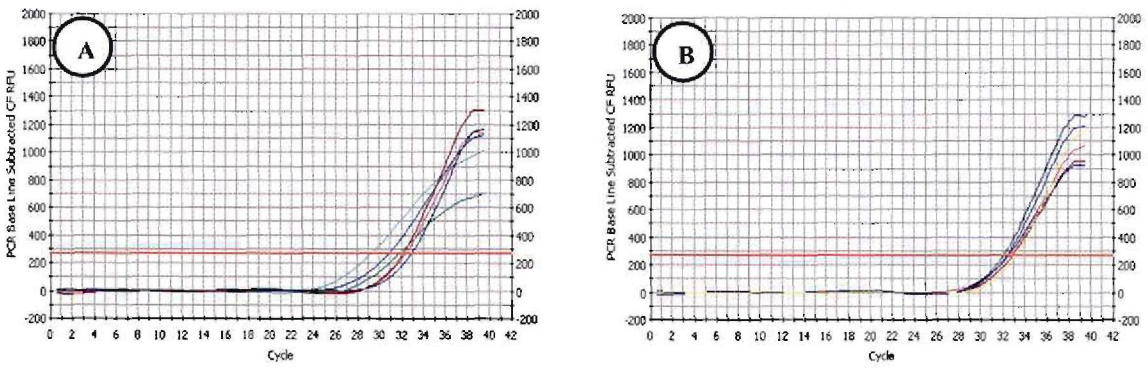

Figure 4.21: Real-time PCR assay of the FUM primer set for *F. verticillioides* (MRC 826) and *F. subglutinans* (ARC 7365) in the presence of non-*Fusarium* species. A 10-fold dilution series ranging from 100 to 0.01ng DNA was constructed. The detection limit was estimated to be 10pg DNA template per reaction volume. DNA concentrations of the non-*Fusarium* species were constantly a 100ng per reaction volume. The colored lines indicate the concentration values for each dilution. (A) DNA of *F. verticillioides* (MRC 826) as template: 100ng (▬); 50ng (▬); 10ng (▬); 1ng (▬); 0.1ng (▬); and 0.01ng (▬). (B) DNA of *F. subglutinans* (ARC 7365) as template: 100ng (▬); 50ng (▬); 10ng (▬); 1ng (▬); 0.1ng (▬); and 0.01ng (▬).

Quantitative PCR provides a more sensitive and accurate method for the detection of fumonisin and non-fumonisin producing *Fusarium* species. With conventional PCR, the detection of *F. verticillioides* and *F. subglutinans* with the EF-1α and FUM primer sets was possible at DNA concentrations between 100 and 1ng. Real-time PCR increased this detection limit for both species and primer sets to 10pg DNA per reaction. Therefore, quantitative PCR assays should rather be used for the detection of *Fusarium* on contaminated food and feed samples.

4.6 Sequence & Phylogenetic analysis

Identities of the amplified sequences were confirmed by Blastn searches (http://www.ncbi.nlm.nih.gov/BLAST) through GenBank. Identities of the amplified beta-tubulin and EF-1α sequences are illustrated in Tables 4.1 & 4.2, respectively. Blastn searches confirmed that the amplified sequences were in fact partial regions of the beta-tubulin and EF-1α genes of *Fusarium* species.

DNA sequence analysis of beta-tubulin sequences for several *Fusarium* species and strains indicated differences when compared to morphological classification. According to the GenBank database, *F. proliferatum* (MRC 8550) was identified as *F. oxysporum* f. sp. *phaseoli* using the beta-tubulin sequence (Table 4.1). Similar results of inconsistent identities were observed for *F. subglutinans* (PPRI 7383) and *F. verticillioides* (PPRI 7899, 17-13, 17-45 & 17-69) (Table 4.1). It is unlikely that differences in identities for beta-tubulin gene of these strains were due to background noise. Overall background noise for the beta-tubulin sequences was very low. Sequence similarities for the beta-tubulin sequences were higher than 98% and the Expected (E) values were below 0. High sequence similarities or an exact match can be considered very close to definite species identification. Low E values usually indicate great similarities between the input sequence and the match (http://www.ncbi.nlm.nih.gov/).

Sequence results for the EF-1α gene also illustrated inconsistent identities for *F. subglutinans* (PPRI 7383) and *F. verticillioides* (PPRI 7899, 17-45 & 17-69) (Table 4.2). High background noise was observed for all of the EF-1α sequences. This could have affected Blastn searches resulting in conflicting results for some of the *Fusarium* species. Sequences similarities for the EF-1α sequences were higher than 97% with the exception of *F. nygamai* (MRC 8546) that showed a 91% similarity (Table 4.2). E values were also below 0.

GenBank identities for *F. oxysporum* (PPRI 7376), *F. verticillioides* (MRC 826, PPRI 7877, PPRI 7897, PPRI 7899, 17-65 & 17-69) and *F. nygamai* (MRC 8546) were similar for both protein-coding genes. However, it was noticed that one of the *F. verticillioides* isolates (17-45) was identified as *F. denticulatum* (Table 4.1) with the beta-tubulin gene sequence while the EF-1α sequence identified this isolate as *F. subglutinans* (Table 4.2). GenBank identity of *F. proliferatum* (MRC 8550) and *F. verticillioides* (17-13) based on EF-1α could not be conducted since sequencing of the fragment failed more than twice.

The conflict between morphological and molecular identification of *Fusarium* species, as observed in this study, is not uncommon. It could be explained by morphological resemblances when grown on potato dextrose agar (Nelson *et al.,* 1983; Leslie & Summerell, 2006). This aspect is further discussed in Chapter 5.

Table 4.1: GenBank identification of the amplified beta-tubulin gene sequences for *Fusarium* species and strains used in this study.

Fusarium species	Nucleotides used	Background noise		GenBank ID	% Similarity	Sequence ID	E value
		Yes	No				
Fusarium proliferatum (MRC 8550)	140-350	✔		*Fusarium oxysporum* f. sp. *phaseoli* gi|148910831|EF450110.1	100	342/342	3e-178
Fusarium subglutinans (PPRI 7383)	90-500	✔		*Fusarium proliferatum* NRRL 31071 gi|15637118|AF291055.1	99	414/415	0.0
Fusarium oxysporum (PPRI 7376)	100-480	✔		*Fusarium oxysporum* f. sp. *phaseoli* gi|148910831|EF450110.1	100	383/383	0.0
Fusarium verticillioides (MRC 826)	100-490		✔	*Gibberella moniliformis* strain FV 4773 gi|41056301|AY435443.1	99	400/401	0.0
Fusarium verticillioides (PPRI 7877)	130-500		✔	*Gibberella moniliformis* strain FV 4773 gi|41056301|AY435443.1	100	414/414	0.0
Fusarium verticillioides (PPRI 7899)	100-480		✔	*Fusarium proliferatum* MUCL 31970 gi|14582744|AF336910.1	100	421/421	0.0
Fusarium verticillioides (PPRI 7897)	120-470		✔	*Fusarium verticillioides* gi|1808748|U34413.1	100	451/451	0.0
Fusarium verticillioides (17-13)	80-460		✔	*Fusarium nygamai* gi|1808710|U34426.1	98	414/421	0.0
Fusarium verticillioides (17-45)	80-450		✔	*Fusarium denticulatum* gi|3320157|U61550.1	98	453/461	0.0
Fusarium verticillioides (17-65)	100-480		✔	*Gibberella moniliformis* strain FV 5146 gi|41056300|AY435442.1	100	428/428	0.0
Fusarium verticillioides (17-69)	100-500		✔	*Fusarium proliferatum* MUCL 31970 gi|14582744|AF336910.1	100	446/446	0.0
Fusarium nygamai (MRC 8546)	100-500	✔		*Fusarium nygamai* gi|1808710|U34426.1	99	410/411	0.0

Table 4.2: GenBank identification of the amplified EF-1α gene sequences for the *Fusarium* species and strains used in this study.

Fusarium species	Nucleotides used	Background noise		GenBank ID	% Similarity	Sequence ID	E value
		Yes	No				
Fusarium subglutinans (PPRI 7383)	180-480	✔		*Fusarium proliferatum* isolate UR01 gi|159492097|EU220408.1	100	204/204	7e-102
Fusarium oxysporum (PPRI 7376)	120-420	✔		*Fusarium oxysporum* isolate DAOM238716 gi|155573851|EF521169.1	100	196/196	2e-97
Fusarium verticillioides (MRC 826)	250-500	✔		*Gibberella moniliformis* isolate MRC 826 gi|159492098|EU220409.1	98	84/85	4e-35
Fusarium verticillioides (PPRI 7877)	140-420	✔		*Gibberella fujikuroi* var. *moniliformis* gi|121491378|AM404157.1	99	363/366	0.0
Fusarium verticillioides (PPRI 7899)	130-450	✔		*Fusarium proliferatum* strain NRRL 43667 gi|149798487|EF452998.1	99	362/363	0.0
Fusarium verticillioides (PPRI 7897)	130-440	✔		*Gibberella moniliformis* isolate MRC 826 gi|159492098|EU220409.1	98	336/341	3e-172
Fusarium verticillioides (17-45)	120-360	✔		*Fusarium subglutinans* strain F2 gi|156510755|EU091074.1	97	231/236	3e-111
Fusarium verticillioides (17-65)	130-400	✔		*Gibberella moniliformis* strain NRRL 43697 gi|149798535|EF453022.1	99	447/450	0.0
Fusarium verticillioides (17-69)	120-490	✔		*Fusarium proliferatum* strain NRRL 43667 gi|149798487|EF452998.1	99	339/340	4e-176
Fusarium nygamai (MRC 8546)	110-350	✔		*Fusarium nygamai* strain FRC M1374 gi|37812626|AY337445.1	91	232/253	1e-105

The beta-tubulin and EF-1α sequences were used to demonstrate potential evolutionary relationships between the *Fusarium* species and isolates from *morogo*. Blastn search results of the various reference cultures (MRC 826, 8546 & 8550) were used and the five closest related sequences were selected and included in the alignments. Mega 4 (version 4.0.2) software was used for the construction of neighbor-joining phylogenetic gene trees. These two gene trees were then compared to each other for topology and similarities.

Figure 4.22 and 4.23 illustrate the neighbor-joining phylogenetic trees of 28 taxa for the beta-tubulin and EF-1α genes, respectively. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test are shown next to the branches (Felsenstein, 1985).

Phylogenetic relationships between the *Fusarium* species and strains for the beta-tubulin gene are illustrated in Figure 4.22. The gene tree is divided into two major clusters (Cluster A and B). Cluster A consists of the three reference strains *F. verticillioides* (MRC 826), *F. nygamai* (MRC 8546) and *F. proliferatum* (MRC 8550), as well as *F. subglutinans* (PPRI 7383). *F. verticillioides* (MRC 826) was grouped together with the *G. moniliformis* subcluster and had a bootstrap support of 78%. *F. nygamai* (MRC 8546) was also grouped into the correct subcluster with bootstrap support of 93%. However, *F. proliferatum* (MRC 8550) and *F. subglutinans* (PPRI 7383) were clustered within the *F. oxysporum* and *F. proliferatum* subclusters, respectively. High bootstrap values were observed for these two clusters (77% and 96%, respectively). However, the grouping of these two *Fusarium* species may be morphologically incorrect. This aspect was, however, not further pursued but should be done.

Cluster B consist of *F. verticillioides* strains isolated form *morogo* and three *F. oxysporum* strains obtained from GenBank. Phylogenetic relationship for this cluster is supported by a 100%

bootstrap support. Two *F. verticillioides* strains (PPRI 7899 & 17-69) were clustered together with *F. oxysporum* showing a bootstrap value of 70%. The rest of the isolates formed two smaller subclusters with bootstrap support of 83% and 65%.

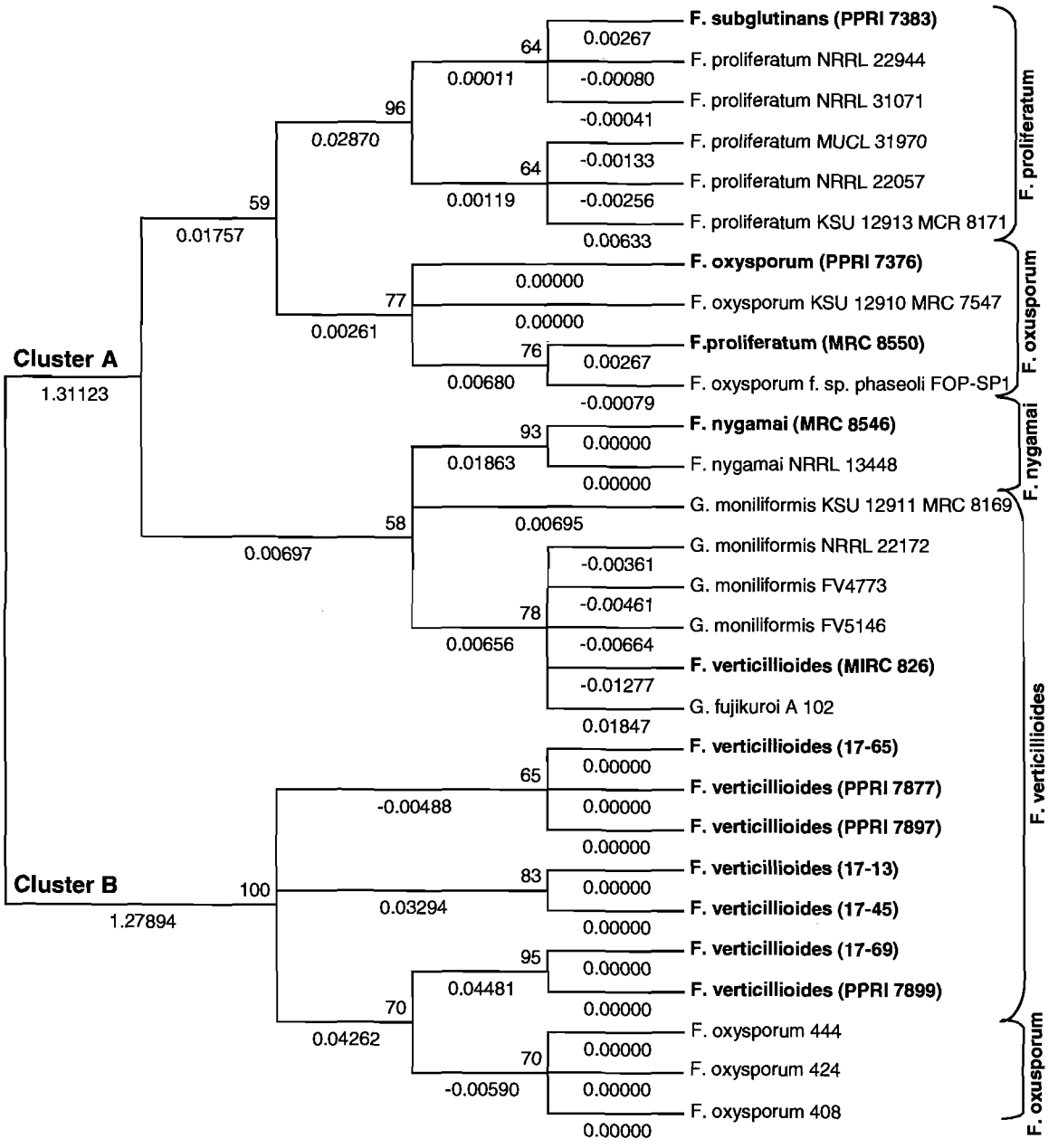

Figure 4.22 Neighbor-Joining phylogenetic tree for the beta-tubulin gene from Table 4.1. GenBank identities for the *Fusarium* species and strains employed in this study were used in the construction of the tree. Only bootstrap values higher than 50% are shown.

Phylogenetic relationships between the various *Fusarium* species and strains for the EF-1α gene are shown in Figure 4.23. The gene tree is divided into four main clusters: *F. verticillioides* (A); *F. nygamai* (B); *F. proliferatum* (C) and *F. oxysporum* (D). Reference strains *F. verticillioides* (MRC 826) and *F. nygamai* (MRC 8546) were grouped into the correct clusters with an overall support of 99 and 89%, respectively. Three of the *F. verticillioides* strains (PPRI 7897, PPRI 7877 & 17-65) were grouped within the *G. fujikuroi/moniliformis* cluster. One of the *F. verticillioides* strains (17-45) was not grouped within any of the clusters. According to its position within the dendogram, this strain was closer related to *F. verticillioides* and/or *F. nygamai*. Three of the *F. verticillioides* strains (PPRI 7383, PPRI 7899 & 17-69) were grouped within the *F. proliferatum* cluster (C) with bootstrap support of 99%. These molecular identities were in conflict with the morphological identities. *F. oxysporum* (PPRI 7376) was grouped correctly within the *F. oxysporum* cluster (D) and there was a 100% bootstrap support for this relationship.

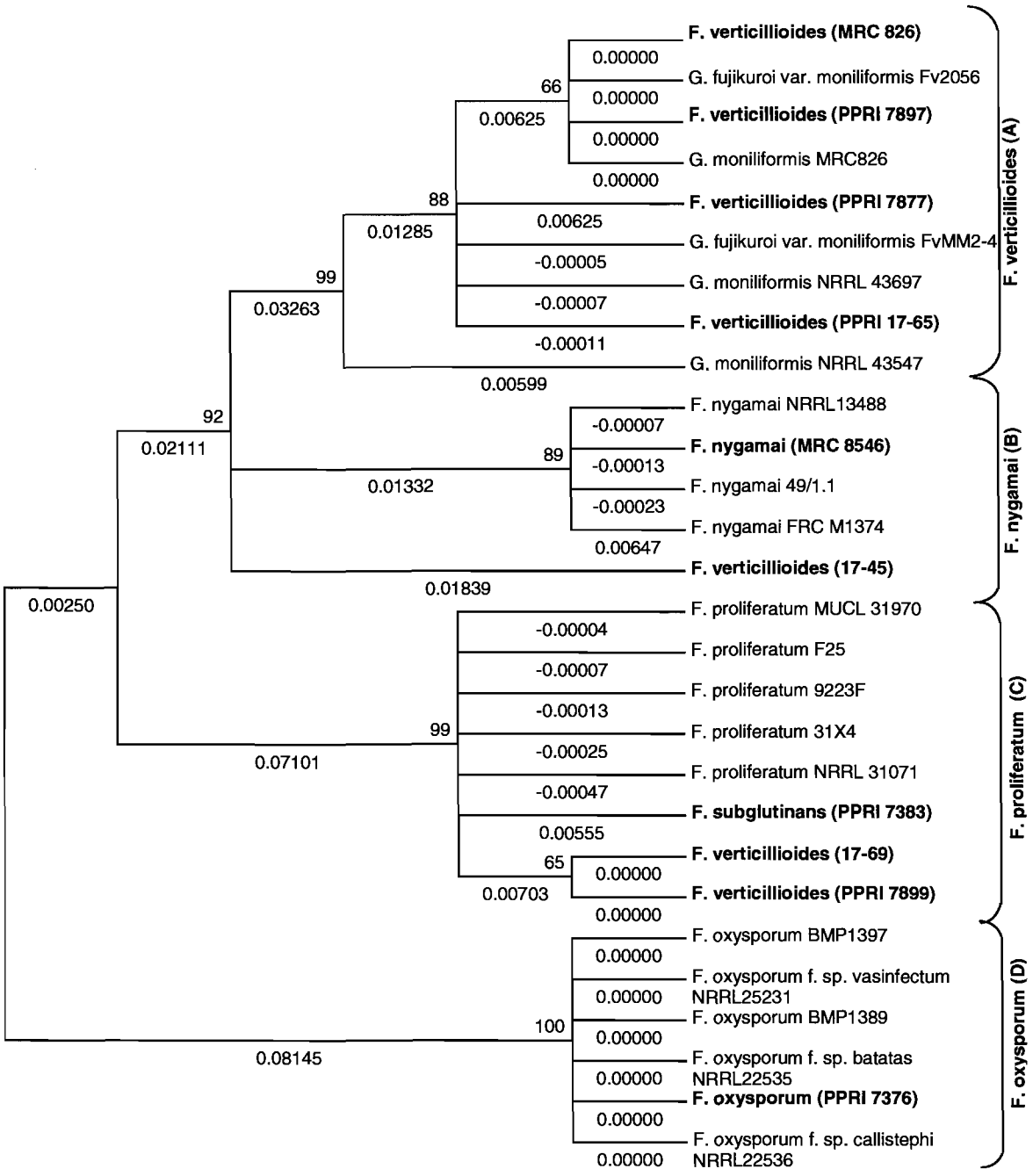

Figure 4.23 Neighbor-Joining phylogenetic tree for the EF-1α gene from Table 4.2. GenBank identities for the *Fusarium* species and strains employed in this study were used in the construction of the tree. Only bootstrap values higher than 50% are shown.

Phylogenetic relationships between the *Fusarium* species and strains were more accurate with the EF-1α gene than with the beta-tubulin gene. *Fusarium* species and strains were grouped into the correct cluster and bootstrap values for the EF-1α gene were also higher than for the beta-tubulin gene. The EF-1α gene phylogenetic marker was thus more consistent than beta-tubulin.

4.7 Summary

In the present study, a multiplex PCR, PCR-DGGE, PCR-SSCP, and real-time PCR were evaluated for rapid detection and identification of *Fusarium* species. PCR-DGGE and SSCP were also evaluated for differentiating between the various *Fusarium* species and strains. For these applications, DNA of a consistent and good quality is required.

Although the quantity was lower, DNA isolated using the E.Z.N.A Fungal DNA Mini Kit was consistently of better quality compared to the CTAB-PVP method evaluated. The EF-1α, beta-tubulin and FUM primer sets were specifically designed for *Fusarium* (O'Donnell & Cigelnik, 1997; Bluhm *et al.,* 2002; Geiser *et al.,* 2004) while the 18S primer set was a universal fungal primer set and was thus suitable to act as a contol. Elongation factor-1α and beta-tubulin primer sets permitted the detection and identification of *Fusarium* while the FUM primer set identified potential fumonisin producing *Fusarium* species.

The EF-1α, FUM and 18S primer sets were selected for multiplex PCR since they provided fragments of various sizes. Multiplex PCR analysis with the EF-1α, FUM and 18S primer sets resulted in the amplification of only the 18S fragments. Therefore, multiplex PCR was performed with the EF-1α and FUM primer sets at equal concentrations. Two fragments (700 and 800bp) were consistently obtained for *F. verticillioides* and *F. nygamai.* On the other hand, *F. proliferatum, F. oxysporum* and *F. subglutinans* DNA yielded only the EF-1α fragment (700bp).

DGGE analysis of the various *Fusarium* species and strains using the EF-1α and 18S fragments was not viable since the migration positions for the *Fusarium* species and strains were similar. Multiple banding patterns were also evident for single strains and species. In contrast to DGGE, SSCP analysis of PCR amplified EF-1α fragments generated from DNA obtained from pure cultures, permitted clear differentiation between the *Fusarium* species. SSCP patterns for the beta-tubulin fragments were indistinct or absent and could therefore not be used to discriminate between the *Fusarium* species and strains. PCR-SSCP with EF-1α fragments reamplified after multiplex PCR resulted in multiple non-specific bands which impeded interpretation of the results. This demonstrated that DNA from pure cultures should be used in SSCP analysis.

Real-time PCR was a more sensitive method for detecting fungal DNA than the conventional PCR method. *Fusarium* DNA was detected up to 10pg in the absence and presence of non-*Fusarium* DNA (various fungal and *morogo* DNA) using quantitative PCR, while conventional PCR detected DNA at higher concentrations (~10ng).

Sequence analysis showed that the beta-tubulin and EF-1α sequences permitted identification of the *Fusarium* species and strains up to species level. However, DNA sequence identities (beta-tubulin and EF-1α) for some of the *Fusarium* species and strains were different from morphological classification. The EF-1α gene sequences provided a better resolution of phylogenetic relationships between the *Fusarium* species and strains than the beta-tubulin gene sequences.

CHAPTER 5

DISCUSSION

5.1 DNA isolation

In this study, the CTAB-PVP method and E.Z.N.A Fungal DNA Mini Kit (PeQLab, Germany) was evaluated for the isolation of genomic DNA. The quantity of DNA isolated with the CTAB-PVP method exceeded the amount of DNA isolated with the Fungal DNA Mini Kit. DNA yield for the CTAB-PVP method and Fungal DNA Mini Kit ranged between 80 and 300ng/µl, and 9 and 150ng/µl, respectively. However, the quality of DNA isolated with the CTAB-PVP method was not as good as DNA isolated with the Fungal DNA Mini Kit. Excessive amounts of RNA were also isolated with the CTAB-PVP method. This corresponded to the high 260:280 ratios. DNA isolated with the Fungal DNA Mini Kit showed no RNA contamination and/or DNA shearing. Amplification with DNA isolated with the CTAB-PVP method was problematic. Various PCR conditions were tested using this DNA. The PCRs worked inconsistently and were poorly reproducible. The CTAB-PVP method was also more time consuming than the Fungal DNA Mini Kit. Preparation of reagents for the CTAB-PVP method required the handling of hazardous and dangerous chemicals whilst the Fungal DNA Mini Kit was safer and user friendly. DNA isolated with the Fungal DNA Mini Kit was used for further analysis because it was sufficient and of better quality.

Various studies made use of a kit or the CTAB-PVP method for isolation of DNA from fungi (O'Donnell *et al.,* 1998a; Steenkamp *et al.,* 1999; Gomes *et al.,* 2003; Jurado *et al.,* 2005; Yergeau *et al.,* 2005; Oros-Sichler *et al.,* 2006). Steenkamp *et al.* (1999) isolated fungal DNA, by means of the CTAB-PVP method, to differentiate *Fusarium subglutinans* f. sp. *pini* by histone

gene sequence data. Yergeau *et al.* (2005) used a kit for isolation of DNA from 19 *Fusarium* isolates. DNA was then used in a PCR-DGGE approach to assess the diversity of *Fusarium* in asparagus. Both DNA isolation methods described in the above studies demonstrated that DNA was of sufficient purity and quality for subsequent PCR amplification reactions, sequencing and profiling techniques. Main advantages of a DNA extraction kit is that it is less time consuming, it does not require the preparation of reagents, good quality DNA is guaranteed and it is safe to use.

5.2 Amplification of fungal DNA

5.2.1 Amplification of fungal DNA with individual primer sets

Selected primer sets yielded amplification products of various lengths. These were of predicted sizes as indicated in Table 3.2. The EF-1α, beta-tubulin and FUM primer sets were designed specifically for *Fusarium* DNA (O'Donnell & Cigelnik, 1997; Bluhm *et al.,* 2002; Geiser *et al.,* 2004), while the 18S primer set was selected to act as a positive control in the present study since it will amplify any fungal DNA. Optimized PCR conditions were needed for the EF-1α and beta-tubulin primer sets to yield high quantities of amplification product. Primer sets for the protein-coding genes, i.e. the EF-1α and beta-tubulin primer sets permitted the detection and identification of the various *Fusarium* species and strains. The FUM primer set was group-specific for fumonisin producing *Fusarium* species. However, this primer set could not amplify FUM fragment from some of the potential fumonisin producing *Fusarium* species employed in this study.

Protein-coding genes, EF-1α and beta-tubulin, were used in this study mainly because of its high discriminating power at species level (O'Donnell *et al.,* 1998b). These genes have been used by previous studies as a genetic marker for phylogenetic studies (Baldauf & Palmer, 1993; Baldauf & Doolittle, 1997; O'Donnell *et al.,* 1998a; O'Donnell *et al.,* 1998b; O'Donnell *et al.,* 2000; Yli-

Mattila *et al.,* 2004; Yergeau *et al.,* 2005). Elongation factor-1α gene has high phylogenetic value since the gene is consistently single copy in *Fusarium,* and it has a high level of sequence polymorphism between closely related species (Geiser *et al.,* 2004). O'Donnell *et al.* (1998b) demonstrated that the EF-1α gene possessed 50% more phylogenetic information than the mtSSU rDNA.

The beta-tubulin gene also presents several advantages when used for phylogenetic studies: (i) insertions and deletions are much less common, making sequence alignments less ambiguous; and (ii) the gene has been studied in several ascomycetaccous fungi (Thon & Royse, 1999). A primary drawback to the use of beta-tubulin sequences for phylogenetic studies in many eukaryotes is the presence of multiple beta-tubulin gene copies (Thon & Royse, 1999). However, the presence of multiple β-tubulin genes in fungi is less frequent than in other organisms (Thon & Royse, 1999).

Amplification of ribosomal RNA (rRNA) or rDNA gene sequences has also been shown to be an appropriate method for taxonomic studies in *Fusarium* (Abd-Elsalam *et al.,* 2003; Yli-Mattila *et al.,* 2004; Kosiak *et al.,* 2005) and other fungi (Kowalchuk *et al.,* 1997; de Souza *et al.,* 2004). Ribosomal gene sequences are useful for phylogenetic studies since it is a conserved region (Ercolini, 2004) and the different regions of the rDNA repeat unit evolve at very different rates (Hillis & Dixon, 1991). However, there do appear to be criticisms against the usage of rDNA markers for fungal phylogenetic studies. O'Donnell & Cigelnik, (1997) and O'Donnell *et al.* (1998a) have demonstrated that many fusaria within the *Gibberella* group possess non-orthologous copies (independent of speciation events) of the ITS2 region which can lead to incorrect phylogenetic analysis. Also, identification by means of the 18S rDNA gene is limited to genus or family level (Anderson & Cairney, 2004). A study conducted by O'Donnell *et al.*

(1998b) comparing phylogenetic markers in *Fusarium* have shown that the EF-1α gene possesses more phylogenetic information than 18S rDNA. The same study showed that combined EF-1α and 18S rDNA provided better declaration of relationships among and within families than other loci, such as beta-tubulin and calmodulin genes, ITS regions and the 5'-end of the 28S rDNA. In this study, it was attempted to use the 18S rDNA gene as an internal control in the multiplex PCR, and potentially as phylogenetic marker in PCR-DGGE analysis to discriminate between the various *Fusarium* species.

Several studies used FUM1 primer sets for detection of fumonisin positive *Fusarium* species in environmental samples (Bluhm *et al.,* 2002; Bluhm *et al.,* 2004; Bezuidenhout *et al.,* 2006a; Bezuidenhout *et al.,* 2006b). Only a single study was found that investigated genetic variability of FUM1 gene fragments among geographically isolated *F. verticillioides* strains. In this study, da Silva *et al.* (2007) demonstrated that, although strains isolated from sorghum produced less fumonisins than strains from corn, the sequence variation in the gene segment that they selected was virtually non-existent. They proposed a more detailed study using other fragments of this gene to investigate this issue. This is an approach that should be considered in future studies.

5.2.2 Multiplex PCR Analysis

The combination of the EF-1α, 18S and FUM primer sets in multiplex PCR resulted in the production of only the 18S fragment. Although concentrations between the three primer sets were adjusted as described by Bezuidenhout *et al.* (2006a), one fragment (only the 18S rDNA) was continuously obtained. Therefore, the multiplex PCRs were performed with only the EF-1α and FUM primer sets at equal concentrations. This resulted in the production of EF-1α and FUM bands for the *F. verticillioides* and *F. nygamai* strains. *F. proliferatum*, *F. oxysporum* and *F. subglutinans* yielded only the EF-1α band. According to literature (Rheeder *et al.,* 2002), the

latter three species are also fumonisin producers. Bluhm *et al.* (2002) also did not detect PCR product with the FUM primer set for *F. subglutinans* and *F. oxysporum*, but obtained FUM product for *F. proliferatum*.

The non-amplification of the FUM gene of potential fumonisin producers in the present study may be a function of the primer set specificity. It could also be due to preferential amplification of EF-1α during multiplex PCR. This should be further investigated in follow up studies.

In multiplex PCR, it is important that primer sets are well designed and compatible with one another (Edwards & Gibbs, 1994). Target sequences, the relative sizes of the fragments, and the optimization of PCR technique should also be well selected to accommodate the amplification of multiple fragments (Edwards & Gibbs, 1994). The EF-1α and FUM primer sets used in this study targeted sequences that are specific for the EF-1α and FUM genes of two *Fusarium* species. However, modifications to the FUM primer set are necessary to detect additional fumonisin producing species such as *F. proliferatum*, *F. oxysporum* and *F. subglutinans*.

PCR amplification efficiency of the multiplex PCR was lower compared to PCR with the individual primer sets although optimized PCR conditions were used. However, the multiplex PCR assay in this study was able to detect the *Fusarium* genus as well as recognizing some fumonisin producing species. A key advantage of combining multiple primers in a single reaction is that the presence or absence of a genus-specific band serves as an internal control for species-specific or group-specific identification of toxigenic *Fusarium* species (Bluhm *et al.*, 2002).

5.3 Denaturing gradient gel electrophoresis of the *Fusarium* species and strains

Differentiation between the various *Fusarium* species and strains using the EF-1α and 18S rDNA regions was not possible with the PCR-DGGE approach described in this study. EF-1α and 18S rDNA PCR amplified fragments of the various *Fusarium* species and strains migrated to the same position and in some cases multiple bands were obtained for a single species making it difficult to identify the band of interest. In a study conducted by Yergeau *et al.* (2005), differentiation between various *Fusarium* species, mostly isolated from asparagus fields worldwide, was possible with their PCR-DGGE method using the EF-1α region. Most of the *Fusarium* isolates used in their study showed only a single band on the DGGE gels. However, previous DGGE studies also reported multiple banding patterns and it was hypothesized that heteroduplexes, multiple gene copies, primer degeneracy or multiple operons were potential causes for this phenomenon (Nicolaisen & Ramsing, 2002; Salles *et al.,* 2002). Yergeau *et al.* (2005) also noticed similar band migration positions for some species despite the high sensitivity of their method. This was also reported in other PCR-DGGE studies (Kowalchuk *et al.,* 2002; Salles *et al.,* 2002).

A study conducted by Kowalchuk *et al.* (1997) demonstrated that the 18S rDNA region was not sufficient in discriminating between strains of a single species and distantly related species using PCR-DGGE. Some species and strains showed similar migration behaviours resulting in similar banding patterns. Van Elsas *et al.* (2000) also reported similar banding patterns with the 18S rDNA region for related fungal species. These results correspond to the DGGE results obtained in the present study using the same gene fragments. Most of the *Fusarium* isolates migrated to similar positions on the polyacrylamide gel, except for *F. proliferatum* which could be easily distinguished from the other strains and species. This suggests that the intraspecies variation within the 18S rDNA fragment was minimal. Thus, taxonomic resolution using 18S rDNA and

DGGE analysis might not always be sufficient to identify and differentiate fungal species and strains.

DGGE has a theoretical resolution of 1bp difference between two fragments (Myers *et al.,* 1985; Myers *et al.,* 1987). However, the separation between the two fragments will mainly depend on the length and sequence of the products. It was demonstrated by Jackson *et al.* (2000) that multiple nucleotide differences do not always produce differences in electrophoretic mobility. Also, fragments obtained from two different species can migrate to similar positions even if their sequences are dissimilar (Muyzer *et al.,* 1993; Sekiguchi *et al.,* 2001). This possibly explains the results of the present study in which different band positions for the various *Fusarium* species and strains were similar.

5.4 Single-strand conformation polymorphism of the *Fusarium* species and strains

PCR-SSCP results for this study showed differences in migration positions for the various *Fusarium* species on a 6% (w/v) and 8% (w/v) polyacrylamide gel using the EF-1α region. These differences indicate that there exist nucleotide sequence polymorphisms within the EF-1α region for the various *Fusarium* species. Simple patterns with two bands and complex ones with three or four bands were generated. In the simple patterns, each band corresponds to a single strand of the PCR product, allowing easy interpretation (Hauser *et al.,* 1997). Patterns with three or four bands for the EF-1α region may correspond to (i) various conformations of the two single strands of a single allele, (ii) the presence of two alleles or (iii) excess primers could have reannealed to the ssDNA after gel loading that lead to the third or fourth band (Hauser *et al.,* 1997; Han & Robinson, 2003; Wallace, 2002). The optimal fragment size for SSCP is between 150 and 200bp (Humphries *et al.,* 1997). Although larger fragments (500bp) were used in this study, the

technique maintained its sensitivity and single base changes created different SSCP patterns for the different *Fusarium* species.

SSCP analysis with the re-extracted and reamplified EF-1α fragments from the multiplex PCR produced additional bands compared to SSCP results where pure DNA was used for amplification. The additional bands may be the result of contamination, non-specific products and/or primer dimers obtained through multiplex PCR and a second round of amplification prior to SSCP analysis. In a review conducted by Kanagawa (2003), mechanisms responsible for PCR bias and artifact formation, as well as suitable methods for the elimination of these problems, were discussed. Elnifro (2000) also suggested that multiplex PCR should also avoid the use of nested primers requiring a second round of amplification. This is major contributor to false-positive results due to carry over contamination. Cycling conditions also play an important role in multitemplate reactions. Generally, extension times should be increased with the number of regions amplified (Chamberlain *et al.,* 1991). However, long extension and annealing times could contribute to the formation of non-specific products (Chamberlain *et al.,* 1991). Cycling conditions should therefore be carefully optimized.

PCR bias can result in incorrect interpretation of data and artifacts can show non-existent microorganisms. It is thus essential to eliminate PCR bias and artifacts to obtain reliable data. In this study, 35 cycles of extension and annealing, and a final extension of 10 minutes were used in the multiplex PCR. The latter, as well as the amplification of the EF-1α fragments prior to SSCP analysis, could have contributed to the formation of spurious amplification products. Although the multiplex PCR-SSCP was a quick method under the described conditions it was not successful in distinguishing between the *Fusarium* species and strains. It demonstrated potential

for use and further careful optimization of this process is thus essential to discriminate between various *Fusarium* species directly from environmental samples.

SSCP analysis for the complete beta-tubulin fragments (650bp) was absent or indistinct for the various *Fusarium* species and strains on a 6% (w/v) polyacrylamide gel. The beta-tubulin fragments were restricted with an enzyme (KpnI) to determine if the sensitivity of the analysis will improve with shorter fragments (350bp). However, SSCP patterns were still absent and/or vague. Because of poor results obtained for the beta-tubulin fragments on a 6% (w/v) polyacrylamide gel, it was not viable to apply the fragments to an 8% (w/v) polyacrylamide gel. Therefore, the beta-tubulin gene was not used in any further experiments.

The PCR-SSCP approach developed in this study using the EF-1α region permitted clear differentiation between the *Fusarium* species and strains tested. The EF-1α gene was a better phylogenetic marker than the beta-tubulin and 18S rDNA regions. This supports previous findings (Geiser *et al.*, 2004; Yergeau *et al.*, 2005) that demonstrated a high level of sequence polymorphism for the EF-1α gene among various *Fusarium* species.

5.5 Detection limits of fungal genomic DNA

5.5.1 Detection limits for individual *Fusarium* species DNA using conventional PCR

Limits of detection for *F. verticillioides* and *F. subglutinans* DNA with the EF-1α primer set were estimated to be 10ng and 1ng DNA per reaction volume, respectively. Detection limits for the EF-1α primer set in the present study were consistent with the findings of Bezuidenhout *et al.* (2006a) which demonstrated a detection limit of 10ng of DNA. Amplification products with this primer set were 700bp in length. Amplification products with the FUM primer set for *F. verticillioides* and *F. subglutinans* were detected up to 0.1 and 10ng DNA, respectively.

Detection limits for the FUM primer set were consistent with the findings of Bluhm *et al.* (2002) who reported a detection limit between 0.1 and 1ng of DNA. Amplification products with the FUM primer set were 800bp in length.

5.5.2 Detection limits of *Fusarium* species DNA in the presence of non-*Fusarium* species by conventional PCR

Amplification products for the EF-1α primer set were detected in all the reactions irrespective of the DNA concentrations. Products were smaller in size (450bp) compared to the EF-1α products (700bp) obtained with the individual *Fusarium* species. Also, the intensity of the products was similar. Thus, unidentified products were generated and may be PCR artefacts. The products should be subjected to sequence analysis to determine the identities of the fragments. The latter was not performed since the objective of this study was to determine the detection limits for the EF-1α and FUM primer sets.

Detection limits for *F. verticillioides* and *F. subglutinans* DNA with the FUM primer set in the presence of non-*Fusarium* DNA (fungal and *morogo* DNA) was estimated to be 10 and 1ng DNA, respectively. Sizes of the fragments were as expected. Bluhm *et al.* (2002) was able to detect fungal genomic DNA in the presence of maize genomic DNA. Maize genomic DNA and *F. graminearum* or *F. verticillioides* DNA were mixed at a 100:1 mass ratio of maize genomic DNA/fungal genomic DNA. Both *Fusairum* species were detected by multiplex PCR analysis using the ITS primer set and the TRI6 or FUM5 primer set, respectively. The present study, as well as Bluhm *et al.* (2002), demonstrated that *Fusarium* DNA is still detectable in the presence of additional fungal and plant material even though the detection sensitivity is lower.

Previously, Bezuidenhout *et al.* (2006b) demonstrated that detection of EF-1α and FUM genes are negatively affected by the presence of plant DNA. Prakitchaiwattana *et al.* (2004) demonstrated that large amounts of plant DNA relative to fungal DNA could interfere with the kinetics of PCR reactions. In the present study, it may thus be possible that interference from the plant DNA resulted in the amplification of non-specific fragments.

5.5.3 Quantitative detection of individual *Fusarium* species DNA

Real-time PCR could detect 10pg DNA in the absence and presence of non-*Fusarium* DNA (fungal and *morogo* DNA) with the EF-1α FUM primer sets. Amplification products for *F. verticillioides* and *F. subglutinans* with the EF-1α primer set were visible up to 10 and 1ng DNA on a 1.5% (w/v) agarose gel, respectively. FUM products for *F. verticillioides* and *F. subglutinans* were evident up to 1 and 0.1ng DNA on a 1.5% (w/v) agarose gel, respectively. Bluhm *et al.* (2004) used a multiplex real-time PCR method to detect genomic DNA from *F. graminearum* and *F. verticillioides* with the TRI6 and FUM1 primer sets. They demonstrated that the detection limit for both *F. graminearum* and *F. verticillioides* was 5pg DNA per reaction.

Melt curve profiles for both primer sets and *Fusarium* species in the present study illustrated additional peaks indicative of primer dimers. On the 1.5% (w/v) agarose gels the latter were visible for both *F. verticillioides* and *F. subglutinans*. Occurrence of primer dimers may be an indication of excess primer in the PCR reaction. If these PCR products were to be used for sequence analysis, excess primer may hinder this process. PCR conditions for this technique should be optimized to eliminate excess primer in PCR reactions and also to increase the sensitivity of detection limits for the EF-1α and FUM primer sets.

5.5.4 Quantitative detection of *Fusarium* species DNA in the presence of non-*Fusarium* species DNA

Results for both *F. verticillioides* and *F. subglutinans* with the EF-1α primer set in the presence of non-*Fusarium* DNA (fungal and *morogo* DNA) were similar to results for the individual *Fusarium* species. *Fusarium* DNA was detected for all dilutions. Amplification products were confirmed on a 1.5% (w/v) agarose gel illustrating the ~450bp fragments for the entire dilution series. Melt curve profiles for both *Fusarium* species illustrated clear, sharp peaks exclusive of additional peaks corresponding to primer dimmers and/or non-specific products. Amplification with the FUM primer set in the presence of non-*Fusarium* DNA was also detected up to 10pg DNA. However, amplification products were not evident on the 1.5% (w/v) agarose gel. It could thus not be determined if the region of interest was amplified and whether the fragments were of expected sizes. The melt curve profiles for *F. verticillioides* and *F. subglutinans* illustrated non-specific peaks; however, these were also not evident on the 1.5% (w/v) agarose gels.

The results presented here demonstrate that real-time PCR was more sensitive than the conventional PCR method detecting small amounts of DNA. Accurate and sensitive detection of pathogenic fungi from environmental and clinical samples is vital in order to recognize the pathogen and implementing regulations involving control and quarantine. Fungal pathogens are not only responsible for serious damages to agricultural crops (Krstanović *et al.,* 2005; Yergeau *et al.,* 2005; Jurado *et al.,* 2006), but they also cause severe infections which can be life threatening, especially in immunocompromised patients (Nucci & Anaissie, 2002; Dornbusch *et al.,* 2005; Jensen *et al.,* 2004). It is thus important that rapid, reliable and accurate methods be developed for early detection and quantification of fungal pathogens to decrease the risk of fungal infections and yield losses. In this study, it was demonstrated that real-time PCR was a

more sensitive method for detecting small amount of fungal DNA in the absence and presence of non-*Fusarium* DNA, than conventional PCR.

5.6 Sequence & Phylogenetic analysis

Blastn results of the beta-tubulin and EF-1α sequences used in this study confirmed that the sequences were partial regions of the beta-tubulin and EF-1α gene. However, sequence analysis of the *Fusarium* species and strains showed some conflict between morphological and molecular identities. Differences in morphological classification were evident for *F. proliferatum* (MRC 8550), *F. subglutinans* (PPRI 7383) and *F. verticillioides* (PPRI 7899, 17-13 & 17-45). This conflict could be explained by morphological resemblances when grown on potato dextrose agar (Nelson *et al.,* 1983; Leslie & Summerell, 2006). PDA cultures of *F. proliferatum* and *F. oxysporum* often appear similar, but these species could be distinguished by very careful consideration of morphological characteristics such as the presence of microconidia in chains, chlamydospores and micorconidia in false heads (Leslie & Summerell, 2006). *F. verticillioides* is similar to *F. proliferatum* and *F. nygamai*, but is distinguished from the latter two species by the morphology of the microconidia (Klaasen & Nelson, 1998; Leslie & Summerell, 2006). Blast results identified *F. subglutinans* as *F. proliferatum* with both the beta-tubulin and EF-1α sequences. According to literature, *F. subglutinans* is morphological similar to *F. bulbicola*, *F. circinatum*, *F. guttiforme*, *F. mangiferae*, *F. pseudocircinatum*, *F. sacchari* and *F. sterilihyphosum* (Nirenberg & O'Donnell, 1998; Britz *et al.,* 2002; Leslie & Summerell, 2006). No sources could be found that mention morphological resemblances between *F. subglutinans* and *F. proliferatum.* One of the *F. verticillioides* strains (17-45) yielded different GenBank identities with the beta-tubulin and EF-1α gene sequences. Beta-tubulin and EF-1α sequences identified this *F. verticillioides* strain as *F. denticulatam* and *F. subglutinans*, respectively.

Considerable background noise was observed for the EF-1α sequence which could have contributed to a different identity.

Incorrect morphological classifications can become a problem when large numbers of isolates are to be processed. Geiser *et al.* (2004) proposed that one could overcome these uncertainties when doubts exist by using sequence data of EF-1α of the unknown isolates and Blast these in the *Fusarium* sequence data base (http://www.fusarium.cbi-o.psu.edu/). If a conflict between the sequence and morphological identification exist, then the researcher is advised to carefully reanalyze the original culture for morphological characteristics for the species identified by the EF-1α sequences in guides such as Leslie & Summerell (2006). For example, Dornbusch *et al.* (2005) demonstrated in a clinical case that sequences obtained with EF-1α primers (EF-1 and EF-2) were more discriminatory than ITS primers. They were able to establish that a nasal infection in an immunocompromised individual was due to a single *Fusarium* species (*F. verticillioides*) and not a range as was suggested by ITS sequences. Their deductions were supported later, by microscopy results. Zhang *et al.* (2006) used EF-1α sequences analysis to demonstrate that *F. solani* species complex members that can cause infections in both humans and plants were common in the environment. It was demonstrated by Hageskal *et al.* (2006) that EF-1α sequences were useful to identify *Fusarium* species isolated from Norwegian drinking water sources. These latter authors only used the EF-1α sequence results and did not do any morphological identification.

The beta-tubulin and EF-1α sequences were also evaluated to resolve phylogenetic relationships between the *Fusarium* species used in this study based on the beta-tubulin and EF-1α gene sequences. Divergent topologies were observed between the beta-tubulin and EF-1α gene trees. Yergeau *et al.* (2005) used EF-1α sequence data of various fusaria isolates obtained from

asparagus to demonstrate that there is sufficient variation between the various species to allow for DGGE analysis. The authors (Yergeau *et al.,* 2005) also used an unrooted neighbor-joining tree to demonstrate phylogenetic relationships between their isolates. In their study, *F. verticillioides* and *F. nygamai* were also in the same subcluster. Healy *et al.* (2005), on the other hand, used EF-1α sequence data to verify their rep-PCR (a DNA fingerprinting technique) results. They demonstrated that the variations in DNA fingerprints for the various reference cultures and isolates could be explained by the clustering pattern of the dendogram as determined by the EF-1α sequences.

Yli-Mattila *et al.* (2004) demonstrated that the beta-tubulin sequences were able to safely distinguish *F. langsethiae* from *F. sporotrichioides*, but were not sufficient in resolving the phylogenetic relationship between these two species. In this study, it was demonstrated that the beta-tubulin gene sequences could not differentiate between the various *Fusarium* species and strains as efficiently as EF-1α. Neither could it resolve the phylogenetic relationships between these species and strains in the same manner as EF-1α. Examples presented in this section demonstrated that sequence identification and phylogenetic approaches used in the present study is frequently used in studies of the genus *Fusarium.*

In conclusion, the methods used and validity of results as obtained in this study could be discussed using recent literature. This study demonstrated the sensitivity of real-time PCR in detecting *Fusarium* species DNA, the potential of multiplex-PCR in differentiating fumonisin positive *Fusarium* strains from non-fumonisin producers and PCR-SSCP in differentiating between various *Fusarium* species.

CHAPTER 6

SUMMARY, CONCLUSION AND RECOMMENDATIONS

6.1 Summary

Fusarium is a well studied fungal group due to their importance in agriculture and human health. They have the ability to cause several plant diseases (Nelson *et al.,* 1981) and synthesize a variety of mycotoxins (Marasas *et al.,* 1984; Marasas, 1987; Leslie & Summerell, 2006). Mycotoxins can contaminate food and feed and is a major concern regarding health safety of commercially produced and subsistence crops (Nelson *et al.,* 1994; Sweeney & Dobson, 1998; Bennett & Klich, 2003). Identification of *Fusarium* species is based primarily on morphology and host plant association (Nelson *et al.,* 1983; Nelson, 1992; Leslie & Summerell, 2006) as well as sequence and molecular characters (Abdel-Satar *et al.,* 2003; Yergeau *et al.,* 2005; Jurado *et al.,* 2006; Fredlund *et al.,* 2008).

6.2 Conclusion

To evaluate multiplex PCR and DNA profiling methods (DGGE and SSCP) for the detection of mycotoxigenic *Fusarium* spp., six well-formulated objectives were set for this study. The trends and conclusions for each objective are briefly discussed below.

(i) Optimization of multiplex PCR with EF-1α, FUM and 18S primer sets

The potential of the multiplex PCR approach described in this study in detecting and identifying fumonisin producing *Fusarium* species was demonstrated. The EF-1α and FUM primer sets were specific for *Fusarium* and the amplified fragments were of expected sizes. Multiplex PCR with

the EF-1α, FUM and 18S primer sets resulted in preferential amplification of the 18S region. Further careful optimization of this process is thus required.

(ii) Optimization of PCR-DGGE methods for *Fusarium* diversity using 18S rDNA-and EF-1α fragments

The DGGE approach described in this study was not sufficient to distinguish between the various *Fusarium* strains and species. Similar banding patterns were observed for most of the *Fusarium* isolates. Different gel and electrophoretic conditions were tested for improvement of the resolution of the fragments. Despite these attempts, banding patterns were still similar. Multiple bands were also observed for a single strain and species. Further optimization of this method is thus necessary.

(iii) Evaluation of PCR-SSCP for assessing *Fusarium* diversity using beta-tubulin and EF-1α fragments

The PCR-SSCP method permitted clear differentiation between the various *Fusarium* strains and species using the EF-1α region. Although large EF-1α fragments (500bp) were used, the technique maintained its sensitivity and different banding patterns were observed between the *Fusarium* strains and species. In contrast, the large beta-tubulin region (650bp) yielded inconsistent results and could therefore not distinguish between the *Fusarium* species and strains. It could thus be concluded that the PCR-SSCP, based on the 500bp EF-1α fragment, has the potential to assess *Fusarium* diversity.

(iv) Comparison of the efficiency PCR-DGGE and PCR-SSCP as DNA profiling methods for differentiating between *Fusarium* spp.

The PCR-SSCP method described in this study was more sensitive differentiating between the various *Fusarium* species and strains than the described PCR-DGGE method. This PCR-SSCP technique therefore has the potential be used to assess *Fusarium* community composition from contaminated food and feed samples. However, this method needs to be further optimized for analyzing complex fungal communities in environmental samples.

(v) Detection limits of EF-1α and FUM DNA with real-time PCR

Detection of toxigenic *Fusarium* species with species-specific and group-specific primers is essential since *Fusarium* is among the most economically important pathogenic fungi. The real-time PCR method used in this study permitted the detection of 10pg of fungal DNA for individual *Fusarium* species and mixed template samples. This method was more sensitive than conventional PCR which detected fungal DNA up to 1ng per reaction volume. Therefore, real-time PCR should rather be used for rapid and sensitive detection of economical and medical important fungal species in environmental and clinical samples.

(vi) Sequence analysis of the beta-tubulin and EF-1α genes

Sequence data obtained in this study showed that the amplified sequences of the various *Fusarium* species and strains were partial regions of the beta-tubulin and EF-1α genes. Some of the isolates also showed variation between their GenBank and MRC/PPRI identities. Phylogenetic analysis of the EF-1α sequences was more accurate and reliable than the beta-tubulin sequences. The *Fusarium* species and strains were grouped within the appropriate cluster with sequence similarities above 88%.

6.3 Recommendations

Discussions and conclusions in the preceding chapter and section have lead to the following recommendations:

1) To obtain sufficient mycelia for DNA isolation and further molecular analysis *Fusarium* could be grown in selective broth for 24 hours as described by Bluhm *et al.* (2002). This incubation period could be shorter but it will have to be determined by conducting experiments.
2) The ITS region can be used as an alternative internal control for multiplex PCR. Multiplex PCR combining the FUM, Tri6, ITS/18S, and EF-1α genes can be developed and optimized. This approach will permit the detection and identification of fumonisin- and trichothecene producing *Fusarium* species in a single reaction. The ITS region may also be useful as a phylogenetic marker in PCR-DGGE and SSCP analysis to discriminate between various *Fusarium* species. These aspects should be investigated in follow-up experiments.
3) The optimum concentration for each primer set used in this study should be tested and determined to reduce excess primer in the PCR reaction.
4) PCR bias and artifacts in multiplex PCR can be eliminated by: (i) stopping the PCR reaction in advance; (ii) monitor PCR products in real time PCR to determine when best to stop the reaction; and (iii) PCR products from independent amplifications can be mixed (Kanagawa, 2003). These aspects should be carefully evaluated in future experiments. Furthermore, the multiplex PCR-SSCP and DGGE methods should also be further optimized to eliminate the production of PCR artifacts.
5) Smaller EF-1α and beta-tubulin fragments (150-200bp) should be tested for PCR-SSCP analysis. This may improve the detection of single-nucleotide base substitutions/polymorphisms that distinguish *Fusarium* species and strains. Different gel and

electrophoretic conditions should also be tested and optimized for each gene. Dilution and clean-up of PCR products prior to SSCP may also help to reduce the formation of PCR artifacts and double-stranded DNA.

6) rRNA-SSCP technique can be used for detection and identification of *Fusarium* species in contaminated samples. RNA may be more sensitive to base-change-induced mobility shifts than DNA (Fujita & Silver, 1994). This technique has the advantage over profiling methods such as DGGE that no PCR amplification is involved prior to sequencing. Thus, non-specific products and primer dimers obtained through amplification can be avoided (MacGregor & Amann, 2006).

Finally the aim of this study was the evaluation of multiplex PCR and DNA profiling methods (DGGE and SSCP) for the detection of mycotoxigenic *Fusarium* spp. was achieved using appropriate methods.

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APPENDIX A

DNA isolation methods

CTAB-PVP DNA Extraction Method

Freeze dried or fresh mycelium were transferred to 2ml microfuge tubes and resuspended in 600-800µl of CTAB extraction buffer (100mM Tris-Cl pH 8.4, 1.4M NaCl, 25mM EDTA, 2% CTAB). Tubes were incubated at 65°C for 30min to overnight. Following extraction, an equal volume of chloroform was added to each tube which was vortexed briefly and then spun for 10min at 12 300 × g. The upper phase was removed to a new 1.5ml tube and DNA was precipitated by the addition of 600µl of -20°C isopropanol. DNA was pelletized by spinning the tubes at 12 300 × g for 5min. The supernatant was discarded and the pellet was gently washed with 70% ethanol and resuspended in 100µl TE buffer (10mM Tris-Cl pH 8.0, 1mM EDTA pH 8.0). DNA samples were stored at 4°C until required.

E.Z.N.A Fungal DNA Mini Kit (PeQLab, Germany)

The fungal tissue was disrupted and then lysed by a specially formulated buffer containing detergent provided by the in the kit. Proteins, polysaccharides, and cellular debris were then precipitated. Contaminants were further removed by isopropanol precipitation of DNA. Binding conditions were adjusted and the sample was applied to a HiBind® DNA spincolumn. Two rapid wash steps removed trace contaminants such as residual polysaccharides. The pure DNA was then eluted in water or low ionic strength buffer. DNA samples were stored at 4°C until required.

APPENDIX B

Standard & melt curve graphs for real-time PCR

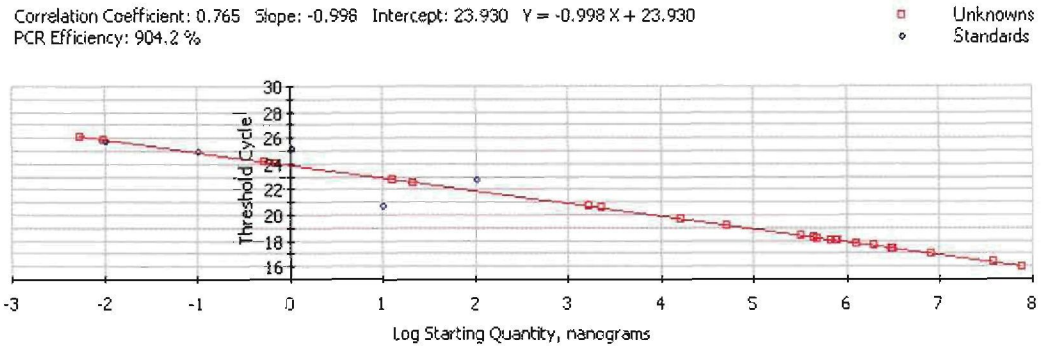

Figure B1. Standard curve for the EF-1 primer set showing the log DNA concentrations (ng) vs. the threshold cycle (Ct) for 10-fold dilutions of *Fusarium* DNA. The assay showed a non-linear relationship between the DNA concentrations and the Ct values with a low correlation coefficient (R^2=0.765). The PCR efficiency was >900% indicating handling and concentration errors.

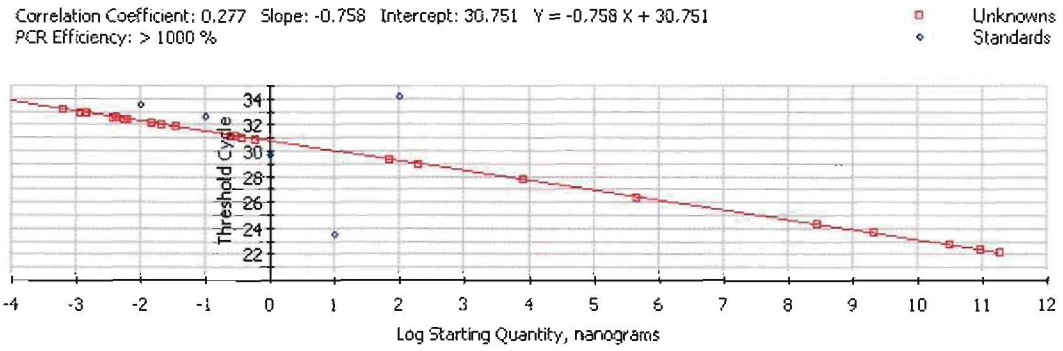

Figure B2. Standard curve for the FUM primer set showing the log DNA concentrations (ng) vs. the threshold cycle (Ct) for 10-fold dilutions of *Fusarium* DNA. The assay showed a non-linear relationship between the DNA concentrations and the Ct values with a very low correlation coefficient (R^2=0.277). The PCR efficiency was >1000% indicating handling and concentration errors.

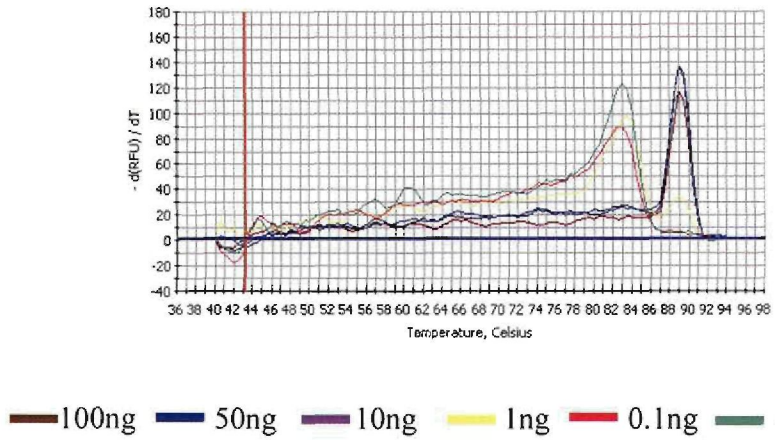

Figure B3. Melting curve profile for *F. verticillioides* with EF-1 primer set in the absence of additional fungal and plant DNA. The negative derivative of fluorescence with respect to temperature is plotted as -dF/dT versus temperature to obtain a graphical representation of the melting peaks (Filion *et al.*, 2003). The additional peaks of melting curves illustrated the presence of primer dimers.

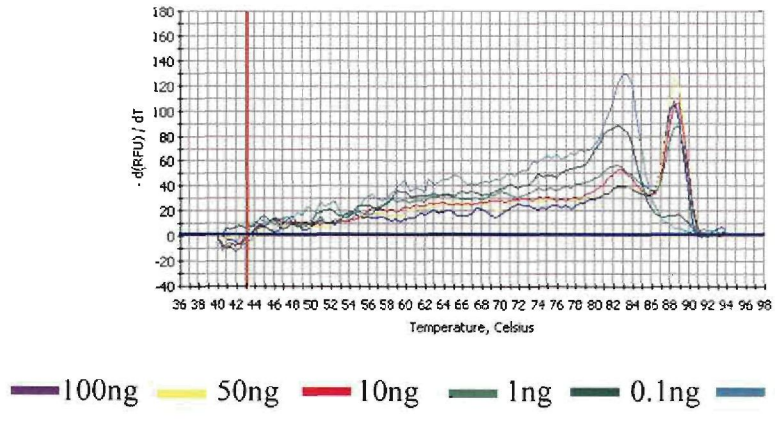

Figure B4. Melting curve profile for *F. subglutinans* with the EF-1 primer set in the absence of additional fungal and plant DNA. The negative derivative of fluorescence with respect to temperature is plotted as -dF/dT versus temperature to obtain a graphical representation of the melting peaks (Filion *et al.*, 2003). The additional peaks of melting curves illustrated the presence of primer dimers.

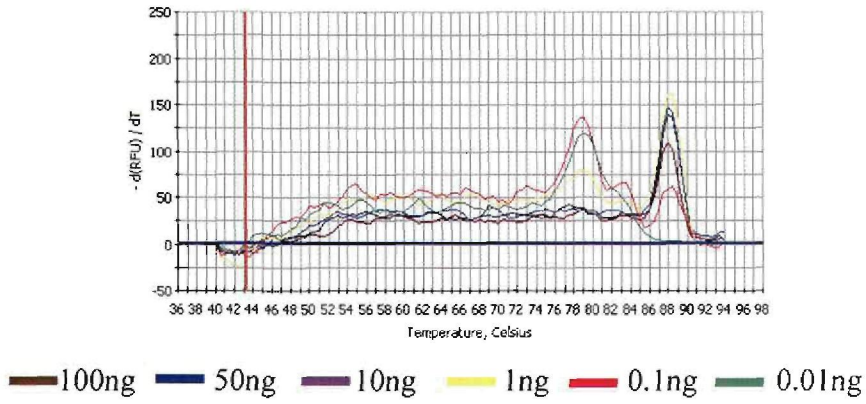

Figure B5. Melting curve profile for *F. verticillioides* with the FUM primer set in the absence of additional fungal and plant DNA. The negative derivative of fluorescence with respect to temperature is plotted as -dF/dT versus temperature to obtain a graphical representation of the melting peaks (Filion *et al.*, 2003). The additional peaks of melting curves illustrated the presence of non-specific products.

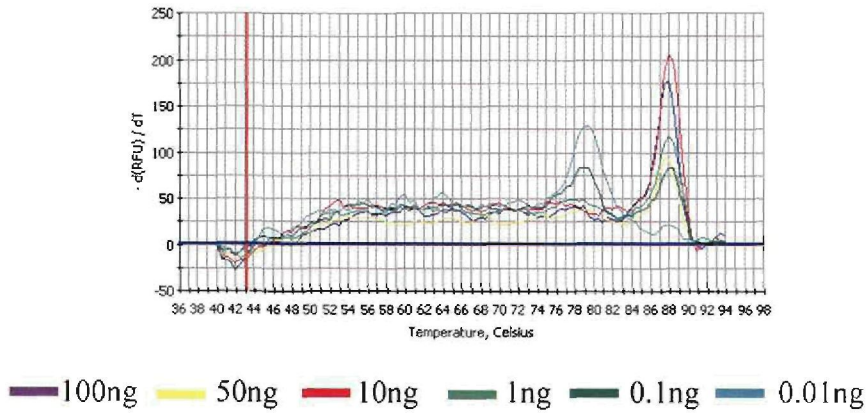

Figure B6. Melting curve profile *F. subglutinans* with the FUM primer set in the absence of additional fungal and plant DNA. The negative derivative of fluorescence with respect to temperature is plotted as -dF/dT versus temperature to obtain a graphical representation of the melting peaks (Filion *et al.*, 2003). The additional peaks of melting curves illustrated the presence of non-specific products.

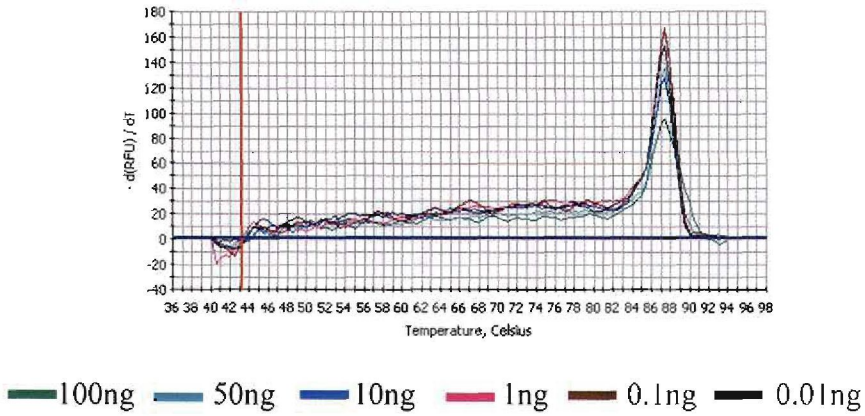

Figure B7. Melting curve profile *F. verticillioides* with the EF-1 primer set in the presence of additional fungal and plant DNA which stayed constantly a 100ng per reaction volume. The negative derivative of fluorescence with respect to temperature is plotted as -dF/dT versus temperature to obtain a graphical representation of the melting peaks (Filion *et al.*, 2003). Clear, sharp melting peaks were observed. Additional peaks due to non-specific products and primer dimers were absent.

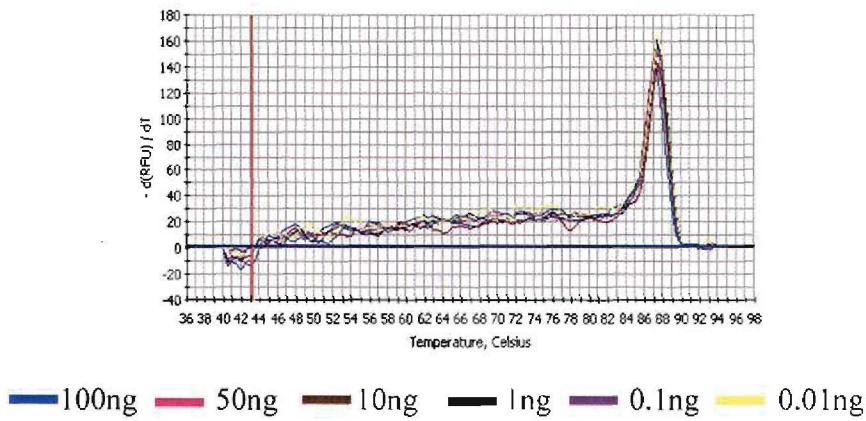

Figure B8. Melting curve profile for *F. subglutinans* with the EF-1 primer set in the presence of additional fungal and plant DNA which stayed constantly a 100ng per reaction volume. The negative derivative of fluorescence with respect to temperature is plotted as -dF/dT versus temperature to obtain a graphical representation of the melting peaks (Filion *et al.*, 2003). Clear, sharp melting peaks were observed. Additional peaks due to non-specific products and primer dimers were absent.

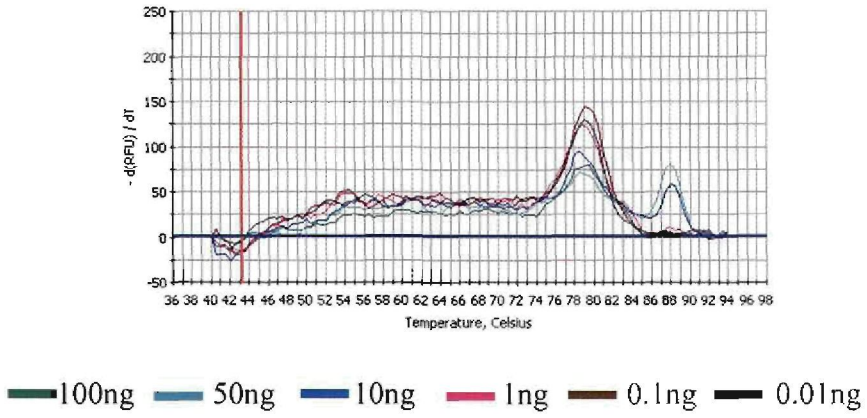

Figure B9. Melting curve profile for *F. verticillioides* with the FUM primer set in the presence of additional fungal and plant DNA which stayed constantly a 100ng per reaction volume. The negative derivative of fluorescence with respect to temperature is plotted as -dF/dT versus temperature to obtain a graphical representation of the melting peaks (Filion *et al.*, 2003). The additional peaks of melting curves illustrated the presence of non-specific products.

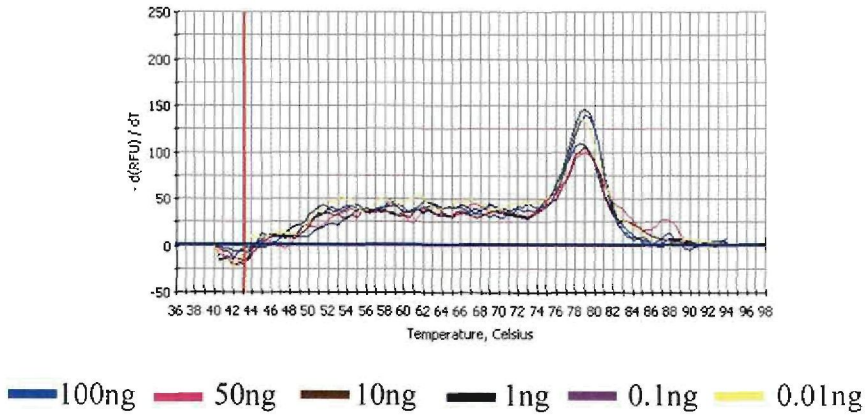

Figure B10. Melting curve profile for *F. subglutinans* with the FUM primer set in the presence of additional fungal and plant DNA which stayed constantly a 100ng per reaction volume. The negative derivative of fluorescence with respect to temperature is plotted as -dF/dT versus temperature to obtain a graphical representation of the melting peaks (Filion *et al.*, 2003). The additional peaks of melting curves illustrated the presence of non-specific products.