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Molecular characterization of *Fusarium* isolates from Ethiopia

by

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SUMMARY

The taxonomy of *Fusarium* species has long been complicated and controversial. This is mainly due to the application of differing taxonomic systems and the inherent morphological variability of some of these species. The controversy surrounding the morphology-based classification, as well as the limitations and advantages of the various tools used to circumvent this problem, are presented in Chapter 1. Emphasis in this case is on the *Fusarium oxysporum* species complex.

Very little is known regarding *Fusarium* species in Ethiopian agriculture. A diagnostic survey was thus undertaken, and the results showed that *F. oxysporum* and *F. solani* are the two species most commonly isolated from agricultural soils and plant tissues (Chapter 2). The phylogeny of these and seven other *Fusarium* spp., (*F. redolens*, *F. acutatum, F. equiseti, F. dlamini, F. avenaceum*, *F. lactis*, and an unidentified *Fusarium* sp.) encountered during the survey was further considered.

Species in the *F. solani-Haematonectria haematococc*a complex cause diseases in more than 87 genera of plants. The characterization of Ethiopian *F. solani* isolates was considered in Chapter 3. AFLPs grouped these isolates into two distinct clusters. The separation of these clusters was also supported by the presence of fixed β-tubulin nucleotide sites. EF-1α sequence analyses showed that all the Ethiopian isolates belong to one of the three clades previously designated for this species complex.

SSR markers provide a powerful tool for population genetic studies. Nine SSR markers were developed for the study of the *F. oxysporum* complex (Chapter 4). These SSR markers generate a high degree of polymorphism, which should be sufficient for population genetic studies of this important species complex.

The *F. oxysporum* species complex contains three phylogenetic clades that are designated based on DNA sequence information. In a study presented in Chapter 5, Ethiopian isolates of *F. oxysporum* were characterized. AFLPs, SSRs and DNA sequence analyses resolved these isolates into three concordant groups. Most of the Ethiopian isolates were nested in one of these three groups. This and the high degree of genetic similarity observed among the Ethiopian isolates using AFLPs and SSRs suggested

that the Ethiopian *F. oxysporum* isolates are largely clonal. The three groups that emerged from this study were also concordant with the three clades previously designated for this species complex.

F. oxysporum and *F. redolens* are very similar species that have been treated as one in the past. Differentiation between them, based on morphology, is difficult due to the presence of isolates with intermediate forms. In this study, species-specific primers were developed for accurate identification of *F. redolens* isolates by PCR (Chapter 6). A PCR-RFLP technique was also developed in this study to distinguish among the three clades of *F. oxysporum* that were described previously.

Accurate identification and knowledge of the genetic diversity of pathogenic *Fusarium* species is important in the management of the diseases that they cause. I believe that studies presented in this thesis have contributed to a better understanding of *Fusarium* species that are commonly found in agricultural soils and certain plants in Ethiopia. Clearly, there is much that will still need to be done; but it is my hope that the work presented in this thesis will form a foundation and provide a stimulus for further studies of these important fungi in Ethiopia.

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PREFACE

Fusarium is a diverse and widely distributed fungal genus. It consists of species that cause diseases in a wide variety of plants. This makes it one of the most important fungal genera in terms of agriculture and forestry. However, the significance of *Fusarium* species in Ethiopian agriculture has not been well studied. The aims of studies presented in this thesis were thus to expand the knowledge of *Fusarium* species occurring in Ethiopia, particularly from those occurring in the soil, and thereby to develop the molecular characterization of *Fusarium* species.

Fusarium species are commonly identified based on their micro- and macroscopic features. But these features are mostly unstable, and render the taxonomy of the group problematic. The presence of different taxonomic systems for the genus also contributes to this problem. A number of molecular tools have been used to circumvent these limitations, and also to characterize *Fusarium* isolates in terms of their genetic diversity, population biology and phylogeny. In the studies presented here, *Fusarium* strains isolated from agricultural soils and plant tissues from Ethiopia were characterized using different DNA-based tools.

F. oxysporum is one of the species with inherent morphological instability. It also represents one of the most important fungal pathogens in terms of agriculture and forestry. While the inherent instability of *F. oxysporum* has enabled the species to occupy a wide range of ecological niches, this has rendered its taxonomy complicated and controversial. Chapter 1 of this thesis provides a review of the literature, specifically considering tools that are used in the taxonomy and phylogeny of this species complex. Advantages and limitations of the morphology, vegetative compatibility, protein and DNA-based methods commonly used to study *F. oxysporum* are discussed in this chapter. The focus on *F. oxysporum* in this review was based on the finding that *F. oxysporum* was one of the two species that were most commonly isolated from agricultural soils and plant tissues from Ethiopia during a diagnostic survey (Chapter 2 of this thesis). The universal nature of most of the taxonomic tools applied to the genus *Fusarium* also warrants an adequate assessment of these tools by focusing on this important species of *Fusarium*.

The study presented in Chapter 2 deals with the results of a survey that was made to identify which *Fusarium* species most commonly occur in agricultural soils and plant tissues from Ethiopia. It discusses the identification and phylogenetic relationships of nine *Fusarium* spp., which were commonly encountered in this study.

F. solani is a complex species comprised of more than 25 phylogenetic and seven biological species. It affects more than 87 plant genera. *F. solani* was one of the two *Fusarium* spp. most commonly isolated from agricultural soils and plant tissues in this study. Chapter 3 of this thesis is dedicated to the characterization of these isolates using Amplified Fragment Length Polymorphism (AFLP) and DNA sequence analyses. This chapter discusses the genetic diversity among Ethiopian *F. solani* isolates and their phylogenetic position in the *Haematonectria haematococca-F. solani* species complex.

Simple Sequence Repeats (SSRs, also known as microsatellites) have contributed to the study of different fungal species including some *Fusarium* spp. Because of their hypervariability SSRs provide a high degree of resolution at the sub-species level. However, there have not been such markers for the study of *F. oxysporum*. Chapter 4 treats the development of SSR markers for the study of the *F. oxysporum* species complex.

In Chapter 5, the genetic diversity of the *F. oxysporum* isolates collected from Ethiopia is assessed. Isolates representing different *formae speciales* are also included in this study for comparative purposes. Three DNA-based techniques, namely, AFLPs, SSRs and sequence analyses, were used. This chapter also assesses how these techniques compare in the study of genetic diversity and phylogeny of *F. oxysporum* isolates.

Morphological differentiation between *F. oxysporum* and *F. redolens* is difficult due to the presence of intermediate forms. Currently, these species are distinguished based on the restriction patterns of their Internal Transcribed Spacer (ITS) region. This involves amplification of the ITS and digestion using three different restriction enzymes. A simpler and cheaper method, which is based only on the presence or absence of a PCR amplification product, was developed in a study presented in Chapter 6. This chapter deals with the development of this new technique to distinguish between these two species. It also describes a PCR-RFLP technique developed in this study as a simple and cheap tool for grouping *F. oxysporum* isolates into the three clades previously designated for this species based on DNA sequence information.

Studies presented in this thesis represent the most extensive series of investigations thus far, undertaken on *Fusarium* species from Ethiopia. Results illustrate that there is a great deal more to be found concerning these important fungi in the country. It is hoped that the studies presented here will form a foundation for additional work on the topic.

CHAPTER I

LITERATURE REVIEW:

Taxonomic Tools Used in the Classification of Species in the *Fusarium oxysporum* Complex

Taxonomic Tools Used in the Classification of Species in the *Fusarium oxysporum* Complex

1.0. INTRODUCTION

Fusarium is a diverse and widely distributed fungal genus with mainly saprophytic members (Snyder and Hansen, 1981). However, the genus is best known for its plant pathogenic species, which are of immense importance to agriculture and forestry (Toussoun, 1981). Some *Fusarium* species also have medical and/or veterinary importance (Rebel, 1981; Marasas *et al.*, 1984; Baran *et al.*, 1997; Krcmery *et al.*, 1997; Pereiro *et al.*, 2001; Yera *et al.*, 2003).

The taxonomy of *Fusarium* is controversial. This is largely because of the lack of morphological stability in isolates and the absence of a universal species concept applied to species within the genus (Szécsi and Dobrovolszky, 1985). Researchers in the field can be categorised into three groups based on the taxonomic system they suggest (Nelson, 1991). Some "splitters," who emphasize differences and, consequently, recognize more species; others are "lumpers," who underscore similarities and recognize fewer species; and still others adopt a middle course between these two extremes.

Wollenweber and Reinking are representative of the "splitters." They classified approximately 1,000 isolates into 16 sections, 65 species, 55 varieties and 22 forms (Wollenweber and Reinking, 1935). These isolates had previously been described based on the structure of their sporodochia on plant materials (Wollenweber and Reinking, 1935; Burgess *et al.*, 1994). However, they found many of them to be synonymous due to the inherent variability in *Fusarium* species (Wollenweber and Reinking, 1935; Nelson, 1991; Burgess *et al.*, 1994).

A later group of researchers, Snyder, Hansen and colleagues, are representative of "lumpers." After intensive studies of monosporic cultures, Snyder and Hansen (1945) produced the "nine species system," in which they limited the number of species to nine, primarily based on the morphology of the macroconidia and cultural variations. Their important contribution was the use of single-spore cultures and recognition of the need to exclude degenerate cultural variants from taxonomic considerations (Nelson, 1991; Burgess *et al.*, 1994).

Gordon, Booth, Nelson and colleagues represent the intermediate group between "splitters" and "lumpers." Booth (1971), using the nature of conidiogenous cells as primary taxonomic characteristic, reduced the number of species proposed by Wollenweber and Reinking, but less extremely so than Snyder and Hansen. To date, however, there is still no general agreement as to which system is most appropriate for the classification of *Fusarium* species. The call by Nelson

(1991) for development of a practical system, which makes use of the information contained in the different historical taxonomic systems as well as that gleaned from current research, has not yet been answered.

Similarly, the taxonomy of *Fusarium oxysporum* Schlecht. (emend. Snyd. and Hans.) within this genus, and the composition of its members has been extensively debated. Wollenweber (1913) placed *F. oxysporum* in the Section *Elegans* together with six other *Fusarium* species and forms. However, Snyder and Hansen (1940) recognized the six taxa as synonymous with *F. oxysporum*. Currently, *F. oxysporum* is regarded as a complex of morphologically similar fungi with multiple phylogenetic origins consisting of at least three well-supported clades (O'Donnell *et al.*, 1998; Baayen *et al.*, 2000a).

Morphologically, *F. oxysporum* can be confused with *F. subglutinans* and *F. solani* (Booth, 1971; Nelson *et al.*, 1981). However, the presence of chlamydospores and the short microconidia-bearing monophialides in *F. oxysporum*, distinguish it from *F. subglutinans* and *F. solani*, respectively (Booth, 1971; Nelson *et al.*, 1981). *F. oxysporum* mutates frequently either to become more mycelial, or pionnotal, where aerial mycelium is depressed (Waite and Stover, 1960). This can present a further difficulty to identify isolates, especially to the less experienced researcher**.**

F. oxysporum is one of the most common soil-borne fusaria (Burgess, 1981). Most of its members appear to be vigorous saprophytes (Burgess, 1981; Correll *et al.*, 1986; Katan and Katan, 1988; Gordon *et al.*, 1989; Gordon and Okamoto, 1990). Some, however, are very pathogenic, causing wilts, crown rots and root rots in a wide variety of crops, and often limiting crop production (Nelson *et al.*, 1981; Correll *et al.*, 1986; Martyn and Bruton, 1989; Kim, *et al.* 1991b). As a species, it probably causes more economic damage to agricultural crops than any other pathogen (Correll, 1991). *F. oxysporum* is also implicated in post-harvest rots of some tropical crops such as apio (*Arracacia xanthorrhiza*), yams (*Discorea* spp.), and taro (*Colocasia esculenta*) (Stover, 1981).

Although pathogenic members of *F. oxysporum* cause destructive vascular wilts in a wide variety of crops, individual pathogenic strains within the species have limited host ranges. Snyder and Hansen (1940) subdivided the species into *formae speciales* based on the ability of the strains to cause disease in a particular host or group of hosts. Some of the *formae speciales* are further subdivided into races based on pathogenicity to a set of differential cultivars within the same plant species. Over 122 *formae speciales* and races have been described (Armstrong and Armstrong, 1981).

Given the differences among *formae speciales* and races in terms of their pathogenicity to crop species, the ability to distinguish between them assumes economic as well as scientific importance. However, their identification by pathogenicity alone is an expensive and labour intensive task (Ploetz and Correll, 1988). Moreover, results of pathogenicity tests are often inconclusive as they are affected by a range of factors, such as host genetic composition (Armstrong and Armstrong, 1981), host growth condition (Correll, 1991), stage of host (Hart and Endo, 1981), and mode of inoculation (Kraft and Haglund, 1978). Classification solely based on virulence also precludes the classification of non-pathogenic strains, which make up a significant component of the species complex (Correll, 1991).

Shortcomings in the taxonomy of *F. oxysporum* have prompted the search for alternative techniques that are quick and reliable, and that can be used either to complement or to replace those based on pathogenicity. The various techniques that have been developed can be grouped into vegetative compatibility tests, protein-based techniques, and DNA-based techniques. Examples of each category are described below, and their relative merits and limitations (Table 1) are discussed.

2.0. VEGETATIVE COMPATIBILITY

Vegetative compatibility refers to the ability of individual fungal isolates to undergo hyphal fusion and form a stable heterokaryon (Caten and Jinks, 1966). Heterokaryosis can serve as a first step in a parasexual life cycle where genetic materials are exchanged between individual fungal strains; or a state in which haploid fungi get the benefits of functional diploidy where the heterokaryon is more vigorous than the contributing individuals (Caten and Jinks, 1966; Leslie, 1993).

In *Fusarium*, classification based on vegetative compatibility is facilitated by the use of *nit* mutants, which arise when isolates are grown on media containing potassium chlorate (Puhalla, 1985). Such *nit* mutants are classified phenotypically based on their growth on a basal medium containing one of several nitrogen sources. When paired on a minimal medium with nitrate as the only source of nitrogen, phenotypically distinct *nit* mutants produce a zone of wild-type growth. This results from nutritional complementation in the heterokaryotic cells, which can form only between vegetatively compatible *nit* mutants.

Isolates belonging to the same VCG often share common biological, physiological and pathological attributes (Caten and Jinks, 1966). Based on this observation, Puhalla (1985) hypothesized that

strains in the same *forma specialis* or race fall in one or a few VCGs. Such correlations have been reported in several studies. Initially, Puhalla (1985) studied 21 strains of *F. oxysporum*, and found correlation between VCGs and the 12 *formae speciales* that the isolates represented. Then, Bosland and Williams (1987) identified three VCGs among 103 strains of *F. oxysporum* isolated from crucifers, and reported complete correspondence among *formae speciales*, electrophoretic types (see below), and VCGs. Other authors have also reported similar findings (Katan *et al.*, 1989; Elmer *et al.*, 1994; Alves-Santos *et al.*, 1999). VCGs were also found to differentiate between races (Correll *et al.*, 1986; Fernandez *et al.*, 1994), or even isolates of the same race from different geographical regions (Katan and Katan, 1988). Such results supported the theory that, in the absence of sexual reproduction and meiotic recombination, genes that determine vegetative compatibility and genes for virulence became fixed, giving rise to distinct VCGs characterized by virulence (Katan *et al.*, 1989).

The major shortcoming of vegetative compatibility in the characterisation of isolates of *F. oxysporum* is the lack of correspondence with pathogenicity. For example, specific VCGs may encompass more than one race (Zuniga *et al.*, 1977; Correll *et al.*, 1985; Elmer and Stephens, 1986; Ploetz and Correll, 1988; Jacobson and Gordon, 1991; Fiely *et al.*, 1995; Woo *et al.*, 1996); and a particular race may represent more than one VCG (Zuniga *et al.*, 1977; Jacobson and Gordon, 1988; Ploetz and Correll, 1988; Larkin *et al.*, 1990; Elias and Schneider, 1991; Jacobson and Gordon, 1991; Roebroeck and Mes, 1992; Fernandez *et al.*, 1994; Fiely *et al.*, 1995; Woo *et al.*, 1996). Pathogenic and non-pathogenic strains may share the same VCG, adding to the lack of correspondence between VCGs and virulence groups (Puhalla, 1984; Elmer and Stephens, 1986; Appel and Gordon, 1994; Fiely *et al.*, 1995). Moreover, 'bridging' isolates that form heterokaryons with testers of more than one VCG (Katan and Katan, 1999; Vakalounakis and Fragkiadakis, 1999; Rosewich *et al.*, 2003; Vakalounakis *et al.*, 2004), and vegetatively self-incompatible isolates (Correll *et al.*, 1986; Correll *et al.*, 1987; Jacobson and Gordon, 1988; Correll *et al.*, 1989; Aloi and Baayen, 1993; Mes *et al.*, 1994; Woudt *et al.*, 1995; Harveson and Rush, 1997), are not amenable to classification based on vegetative compatibility. The difficulty in recovering *nit* mutants, and the phenomenon of weak heterokaryon reactions between *nit* mutants of some strains, also limit the use of VCGs (Elmer and Stephens, 1989; Gordon and Okamoto, 1991; Woudt *et al.*, 1995; Elena and Pappas, 2002).

The use of vegetative compatibility for grouping isolates of *F. oxysporum* has proven superior to virulence because it provides a means of characterizing sub-specific groups based on the genetics of the fungus itself, rather than its reaction with differential hosts. Vegetative compatibility also allows

the characterization of non-pathogenic isolates. However, vegetative compatibility does not provide an adequate solution, as the correspondence with *formae speciales* or races is not as close as originally assumed. Also, VCGs do not provide information about the genetic relatedness between strains that are not compatible as they provide only qualitative measures (Jacobson and Gordon, 1991). Therefore, the use of vegetative compatibility in the characterisation of isolates of *F. oxysporum* is limited.

3.0. PROTEIN-BASED TECHNIQUES

Several protein-based techniques have been employed in the taxonomy of *F. oxysporum*. Broadly, these can be grouped into total protein profiles, isozyme analyses and immunoserology. Total protein profile refers to the electrophoretic banding patterns of total soluble proteins, which are revealed using general protein staining techniques. Isozyme analysis is the detection of specific enzymes using enzyme-specific staining systems. Immunoserology involves the detection of antigen-antibody interactions, where antibodies are raised in an animal model against a protein extract from an isolate. These can then be made to cross-react with protein extracts from other isolates.

3.1. Total Protein Profiles

Total protein gel profiles that arise as a result of differences in the electrophoretic mobility of proteins reflect differences in the gene sequences encoding these proteins. Thus, if protein patterns of two individuals differ, it is assumed that these differences are genetically based and heritable. After Chang *et al.* (1962) demonstrated that protein extracts obtained from different species of *Neurospora* had distinct electrophoretic patterns, the technique was increasingly used in fungal taxonomy (Clare and Zentmeyer, 1966; Durbin, 1966; Gill and Powell, 1968a; 1968b). In 1967, disc electrophoresis was suggested to be useful in characterising *formae speciales* and races of *Fusarium* species (Macko *et al.*, 1967). At the same time, however, Hall (1967) showed that culturally and pathogenically distinct isolates of *F. solani* produced essentially identical protein patterns. Since then, several studies have reported on the merits and limitations of the technique in the taxonomy of *F. oxysporum*. These reports are reviewed in this section.

Glynn and Reid (1969) reported that protein profiles do not correlate with virulence. They employed acrylamide gel disc electrophoresis to study isolates of *F. oxysporum*, other *Fusarium* species, *Veritcillium albo-artum* and a *Graphium* sp. Variation in the number and position of electrophoretic bands was evident among 24 pathogenically distinct *formae speciales* of the 33 *F. oxysporum* isolates studied. However, this difference was neither consistent nor correlated with individual isolates or *formae speciales*. This was in line with the findings of Hall (1967), and Gill and Powell (1968b), who also demonstrated the inability of gel electrophoresis to differentiate biotypes or races of their respective test organisms. Moreover, the difference in protein patterns among the four species was within the range of variation of each species. These studies indicated that electrophoretic patterns of total soluble proteins have little or no significance in delimiting sub-species groups, at least in these fungal species.

Later on, however, Sayed *et al.* (1976) reported that electrophoretic patterns of proteins are useful not only in differentiating among *formae speciales*, but also in studying relationships among them. They subjected protein extracts from *F. o. udum, F. o. vasinfectum* and *F. o. ciceri* to electrophoresis in glass tubes packed with polyacrylamide gel. Sayed *et al*. (1976) observed that *F. o. udum* and *F. o. vasinfectum* had more bands in common than between either of them and *F. o. ciceri,* suggesting a closer relationship between *F. o. udum* and *F. o. vasinfectum*. A drawback of their study, however, was that only single isolates representing each of the *formae speciales* were studied. They did not show whether or not there were variations within each of the *formae speciales*, and how these variations would have affected differentiation among the *formae speciales*.

Proteins do not reveal much variation at the sub-species level. Firstly, introns, which house most of the variability in the genes, are spliced out during transcription. Secondly, third base differences in codons do not necessarily result in changes in amino acid residues due to degeneracy of the genetic code. Hence, identical amino acid sequences may not imply identical DNA sequences. Thirdly, proteins with different amino acid sequences may not necessarily differ in their electrophoretic mobility due to the neutrality of some amino acids in an electric field. As such, similar bands may have different amino acid sequences. Lastly, variation in the number and position of electrophoretic protein bands is to be expected under different cultural conditions (*i.e.,* age, temperature, growth medium, etc.) (Glynn and Reid, 1969). As a result, total protein profiles have limited applicability in the molecular taxonomy of isolates of *F. oxysporum*.

3.2. Isozymes

Isozymes are molecular forms of an enzyme, which usually have similar enzymatic properties, but different amino acid sequences (Bonde *et al.*, 1993). In starch gel electrophoresis (Micales *et al.*, 1986), which is the most commonly employed technique, mycelial extracts are absorbed on chromatography wicks, inserted into a sample slot in a horizontal starch gel, and electrophoresed. Horizontal slices of the gel are then stained separately using various staining systems. The technique allows comparison of several loci coding for the respective enzymes. Co-dominance of enzyme expression is also an advantage of the technique. Other advantages of isozyme analysis using starch gel electrophoresis are that it is faster than immunological techniques and less expensive than tests involving PCR and species-specific primers (Bonde *et al.*, 1993).

Isozymes have been reported to enable differentiation among virulence groups in *Fusarium*. An example is the work of Bosland and Williams (1987), who studied 103 isolates from crucifers using 18 enzymes, four buffer systems, and 36 stains. Based on seven polymorphic loci, they differentiated the isolates into three pathotypes that fully corresponded with VCGs. Races 1 and 2 of *F. o. lycopersici* were also differentiated. Similarly, De Granada *et al.* (1999) demonstrated that electrophoresis of arylesterases enable differentiation of isolates of *F. o. lycopersici* and *F. o. dianthi* from non-pathogenic *F. oxysporum* isolates. They also differentiated among races 1, 2, 4, and 8 of *F. o. dianthi*. De Granada *et al.* (1999) concluded that arylesterase electrophoresis is a useful tool in differentiating between and among populations of *Fusarium* species.

Other studies, however, demonstrated the inadequacy of isozyme analyses. A case in point is the work of Skovgaard and Rosendahl (1998), who analysed intracellular (obtained from ground mycelium) and extracellular (extracted from culture medium) isozymes from 24 isolates of *F. oxysporum*. Based on UPGMA (Unweighted Pair-Group Method Using Arithmetic means) analysis of Electrophoretic Phenotypes (EPs) of intracellular isozymes, they grouped the *F. oxysporum* isolates into three main clusters. However, these clusters did not correlate with groups based on host or geographical origin. Moreover, the two *F. solani* and *F. o. redolens* isolates, which were included in the study, grouped with other isolates of *F. oxysporum,* separate from the three main clusters. There was also no congruence between the three main clusters and groups based on EPs of extracellular isozymes. Similar results were reported in other studies also (Katan *et al.*, 1991; Elias and Schneider, 1992).

The findings of Mohammadi *et al.* (2004) also reveal the inadequacy of isozyme analyses in *Fusarium* characterisation, although the authors concluded otherwise. They studied 13 isolates of *F. o. melonis* and nine isolates from eight other *formae speciales*. Using six enzyme systems, they recognized 10 putative loci and 110 EPs. At a 35% similarity index, phenetic analysis of EPs using UPGMA grouped the isolates into three major groups, where Group I contained six Iranian isolates each from different *formae speciales*. This indicates the lack of differentiation among these *formae speciales*. Group II, which further sub-divided into IIA and IIB at 42.5% similarity index, contained *F. o. melonis* isolates from USA and Iran, respectively. However, an isolate from Iran (I-17) grouped with USA isolates. A USA isolate (B-35) also grouped with Iranian isolates. Moreover, Group II represented all the four races in *F. o. melonis*, and three VCGs. This shows that although *F. o. melonis* isolates from Iran and USA grouped separately from other *formae speciales*, resolution corresponding to race, VCG or geographical origin was not achieved. Iranian isolates belonging to *F. o. capsici* and *F. o. tuberosi*, together with two *F. o. melonis* isolates from France, constituted Group III. This is also indicative of the inadequacy of the technique to differentiate between *formae speciales*. However, the authors concluded that isozyme polymorphism in the isolates they studied was highly correlated with VCG and geographical origin, and to a lesser extent with races.

The only two 'successful' applications of isozyme analyses in the taxonomy of *F. oxysporum* in the literature are found in the works of Bosland and Williams (1987) and that of De Granada *et al.* (1999). However, only three *formae speciales* were included in these studies. Compared to the large number of *formae speciales* that have been described in the *F. oxysporum* species complex, the ability to differentiate among the three *formae speciales* can only be considered limited at best. Moreover, the fact that these *formae speciales* were represented in both studies only by one or a very few isolates renders the studies to be preliminary at best. Used alone, isozyme analyses do not appear to differentiate *F. oxysporum* isolates based on VCG, virulence or geographical origin. The small number of available reports on the use of isozymes is also a reflection of this fact.

One of the major disadvantages of isozyme analysis is that it can differentiate only between isozymes that have different net charges or large differences in shape, and that make up an estimated third of the total possible isozymes (Micales *et al.*, 1986; Bonde *et al.*, 1993). Different types of enzymes exhibit different degrees of variation due to the intensity of selection pressure. Hence, a study that uses non-regulatory enzymes (*e.g.*, esterases and phosphatases), which are under less selective pressure and tend to vary highly, may detect a disproportionately high level of intra-specific variation (Bonde *et al.*, 1993). This reduces the value of such enzymes as a tool for

assessing differences or relatedness between isolates. Moreover, since they are proteins, the use of isozyme analysis has similar limitations to that of total protein profiles (see above). For these reasons, isozyme analysis has proven inadequate in the taxonomy of *F. oxysporum*.

3.3. Immunoserology

Immunoserology involves the production of antibodies against a specific antigen (a protein extract), and measuring the interaction of the antibodies with antigens from other sources. In the taxonomy of *Fusarium* species, antibodies raised against a protein extract from a specific isolate are used to test cross-reactivity of this antigen (the homologous antigen) to protein extracts from other isolates (heterologous antigens). The degree of cross-reaction obtained with the heterologous antigens relative to that obtained with the homologous antigen is used as a measure of genetic relatedness. Antigen-antibody interactions are commonly measured using the agar diffusion or precipitin test where they are observed as precipitin lines (Link and Willcox, 1933).

Early attempts to produce sufficient titres of antibodies against *Fusarium* species were unsuccessful (Nelson, 1933; Madhosingh, 1964). Later, Link and Wilcox (1933) produced sufficient titres against several species of *Fusarium*, but could not differentiate amongst them using the precipitin test. In 1957, Tempel succeeded in differentiating between *F. o. lupini* and *F. o. pisi.* However, his further attempts to differentiate other strains of *Fusarium* failed (Madhosingh, 1964).

After a serological comparison using the agar diffusion method, Madhosingh (1964) reported that it was possible to differentiate among *F. oxysporum, F. solani* and *F. moniliforme*. This author also reported that a higher number and intensity of antigen-antibody precipitin lines formed on the agar between *F. oxysporum* and *F. moniliforme* isolates. Based on this, Madhosingh (1964) concluded that *F. oxysporum* and *F. moniliforme* were more closely related to each other than either of them were to *F. solani*. The study employed only single isolates as representatives of each of the three species. As a result, the degree of intra-species variation, and how this variation would influence differentiation among the species was not determined. Similarly, Sayed *et al.* (1976) reported that it was possible to differentiate among *F. o. udum, F. o. vasinfectum*, and *F. o. ciceri* using immunoserology. However, their study also suffered from the same shortcomings as that of Madhosingh (1964). They used only single isolates to represent *formae speciales*; did not show the extent of variation in each of these *formae speciales*; and, as a result, failed to show whether the

variation observed among the *formae speciales* was sufficiently larger than variation within each of the *formae speciales* studied.

A more successful use of serotaxonomy with *Fusarium* species and strains was made by Iannnelli *et al.* (1982) who examined 19 strains of *F. oxysporum*, and an isolate each of *F. moniliforme* and *F. xylarioides*. The authors reported clear and consistent differences, not only among species, but also among *formae speciales* and even races. They ascribed the success of their study to the protein extraction method used. Radiolabelling of the antibodies also made the method more specific and sensitive than the agar diffusion method using unlabeled antibodies. Although this finding was reported in 1982, it still remains the only-report on the use of radio-double diffusion in the taxonomy of *F. oxysporum*. More studies including larger number of *formae speciales* and races need to be conducted in order to confirm the usefulness of the method.

There are only a few reports on the use of immunoserology in the taxonomy of *F. oxysporum*. However, the available reports indicate that serology can be used as a tool, although it can be expected to have similar drawbacks as total protein profiles because proteins are also pivotal in immunoserology. Moreover, the degree of reactivity between antibodies and antigens depends on the affinity and specificity of the antibodies as well as how accurately the methods can measure these properties. This is a significant limitation in the applicability of the method.

4.0. DNA-BASED TECHNIQUES

The use of DNA polymorphisms as a trait has added a new dimension to the recognition and classification of genetic variability in fungi. In theory, DNA-based techniques such as Random Amplified Polymorphic DNA (RAPD), Restriction Fragment Length Polymorphism (RFLP), Amplified Fragment Length Polymorphism (AFLP) and DNA sequence analyses, overcome all the limitations associated with the identification of sub-species taxa using morphology, pathogenicity, vegetative compatibility, and protein-based methods (Table 1). If correlated to pathogenicity, they could also be useful in identifying virulence groups (Woo *et al.*, 1996). DNA-based techniques are also valuable for use in phylogenetic studies.

For the purpose of this review, the DNA-based techniques are broadly grouped into RAPD, RFLP, AFLP, electrophoretic karyotyping, Simple Sequence Repeats (SSRs) and DNA sequence analyses.

A description of these tools and a review of their use in the taxonomy of *F. oxysporum* isolates are presented in the following sections.

4.1. Random Amplified Polymorphic DNA (RAPD)

RAPD (Williams *et al.*, 1990) or the Arbitrarily Primed PCR (AP-PCR, Welsh and McClelland, 1990), and its variation, DNA Amplified Fingerprinting (DAF), use single primers of arbitrary sequence to generate DNA fragments. DAF differs from RAPD in that it uses shorter primers (usually, 8-12 nucleotides) with a 5'-prime mini-hairpin structure to help minimize interactions within and between the termini of amplification products during PCR (Cateno-Anollés *et al.*, 1991).

RAPD has several advantages as a means of characterizing genetic variability. These include speed, low cost, low DNA concentration, lack of radioactivity and there is no need for prior sequence information about the target DNA. It is also applicable to large numbers of isolates and enables analysis of variation at more than one locus (Bentley *et al.*, 1995). As a result, this technique has been used extensively in the molecular characterization of *F. oxysporum* isolates.

Several studies have revealed correspondence between virulence groups and/or VCGs on one hand, and RAPD Finger Print Groups (FPGs) on the other. For instance, Wang *et al*. (2001) studied 24 isolates belonging to 13 *formae speciales* using RAPDs. Seven RAPD primers selected from an initial set of 132, revealed RAPD fingerprints unique to each *forma specialis*. Probes developed from *forma specialis*-specific RAPD bands showed different specificity to the 13 *formae speciales* after Southern hybridisation (Southern, 1975) with RAPD fingerprints. Based on these findings, the authors concluded that markers based on differences in fingerprints are potentially useful for the identification of *formae speciales* without the need for pathogenicity tests. Similar studies showed that RAPD FPGs correspond with VCGs (Grajal-Martin *et al.*, 1993; Bentley *et al.*, 1995; Tantaoui *et al.*, 1996; Nelson *et al.*, 1997; Mes *et al.*, 1999), *formae speciales* (Chiocchetti *et al.*, 1999; Hernandez *et al.*, 1999; Vakalounakis and Fragkiadakis, 1999; Pasquali *et al.*, 2003), and/or races (Assigbetse *et al.*, 1994; Manulis *et al.*, 1994; de Haan *et al.*, 2000). However *formae speciales* in these studies were represented by only one to three isolates.

Unsuitability of RAPD to differentiate isolates based on pathogenicity or geographical origin has also been reported. An example is the work of Cramer *et al.* (2003) who studied 34 isolates including *F. o. phaseoli, F. o. betae*, non-pathogenic *F. oxysporum* and an isolate of *F. solani*.

UPGMA analysis of the data, generated using twelve 10-mer primers, revealed that only *F. o. phaseoli* isolates collected from the same geographical area clustered together. However, this group also contained members of races 1 and 4. No other grouping by race or geographical area was evident. In fact some *F. o. betae* isolates appeared to be more closely related to non-pathogenic *F. oxysporum*, and the out-group *F. solani*. Cramer *et al.* (2003) concluded that RAPD analysis is not suitable for such a study. The inadequacy of RAPDs to differentiate isolates based on pathogenicity or geographical origin has also been reported in other studies (Woo *et al.*, 1996; Vakalounakis *et al.*, 2004).

Like RAPD, DAF has been reported to be inadequate for use in the taxonomy of *F. oxysporum*. Bentley *et al.* (1998) used DAF to differentiate among VCGs, although this was based only on single representative isolates. VCGs 01213 and 01216 produced identical genotypes no matter which primer was employed. For the majority of the ten primers used, identical genotypes were obtained for VCGs 0120 and 0125, VCGs 0124 and 0125, and VCGs 0129 and 01211. The authors attributed this to the presence of 'bridging' isolates between these pairs of VCGs. Among the 133 isolates of unknown VCG, several produced a fingerprint typical of an existing VCG although the isolates were not compatible with testers of those VCGs. Bentley *et al*. (1998) suggested that finding new testers for such VCGs may solve the problem. Results of this study indicate that although there is some correlation between DAFs and VCGs, the former technique cannot replace the latter.

The use of RAPD markers in phylogenetic analysis was evaluated. O'Donnell *et al*. (1999) employed two 10-mer and one 15-mer primer for RAPD analysis of 10 *F. oxysporum* isolates. The tree inferred from the dataset generated with the 15-mer provided a better resolution than those inferred from the individual and combined data generated with the 10-mers. RAPD analysis revealed the underlying phylogenetic structure as accurately as DNA sequence data (see below). However, it failed to reveal sister relationships among isolates within one of the clades. This clade received 90-95% bootstrap support in trees inferred from sequence data, whereas there was less than 50% bootstrap support for it in RAPD trees. The authors concluded that it would be better to use longer primers (15- to 20-mer) for phylogenetic studies to reduce the homoplasy associated with shorter RAPD primers. They also suggested that trees inferred from RAPD analysis should be treated cautiously, especially when 10-mers are used.

The RAPD technique has several limitations when used for grouping *F. oxysporum* isolates. The technique may distinguish between *formae speciales* and fail to differentiate between VCGs and/or

races (Hernandez *et al.*, 1999; Vakalounakis and Fragkiadakis, 1999). It may distinguish between VCGs and fail to reveal races (Grajal-Martin *et al.*, 1993; Bentley *et al.*, 1995). It may also fail to differentiate between pathogenic and non-pathogenic isolates (Woo *et al.*, 1996). Moreover, in order to reach an effective RAPD system, it is necessary to screen a large number of primers since not all primers can resolve isolates (Wang *et al.*, 2001). The high homoplasy in data generated with RAPD primers, the lack of co-dominance, and its low repeatability, also pose limitations in the use of the technique.

DAF has a number of advantages over RAPD as a molecular tool. The low repeatability of RAPDs is of minor importance in DAFs. This is because the high primer-template ratio used in DAF accommodates for rare amplification events that arise from primer-template mismatches during early cycles of the PCR. The complex fingerprint obtained with DAF also ensures that the absence of a particular amplification product does not affect the probability of amplification of other products (Bentley *et al.*, 1998). However, unlike RAPD, DAF has not been used extensively in studies of the taxonomy and phylogeny of *F. oxysporum*. Consequently, correspondence between DAFs and pathogenicity groups and/or VCGs cannot be assessed adequately. However, from its similarity with RAPD, DAF is likely to have many of the same limitations as RAPDs.

4.2. Restriction Fragment Length Polymorphisms (RFLPs)

RFLPs represent differences in the size of DNA fragments produced when two or more DNA templates are digested with sequence-specific endonucleases. Commonly, these fragments are resolved by electrophoresis on agarose gels containing ethidium bromide, and subsequently visualized by UV illumination. Alternatively, the fragments can be probed using labeled nucleic acids in Southern analyses (Southern, 1975). Polymorphisms can be generated by a gain or loss in restriction sites resulting from nucleotide substitution or from the rearrangement of DNA sequences (Taylor, 1986). RFLP markers are ideally suited to genetic diversity studies because most are selectively neutral, produce more polymorphisms compared to other types of markers such as isozymes, they are reproducible and co-dominant (Koenig *et al.*, 1997).

RFLPs can be broadly divided into two groups according to the type of template used. These are total DNA RFLPs and RFLPs of PCR-amplified products (PCR-RFLPs). Total DNA RFLPs are sub-divided into genomic DNA (gDNA) RFLPs and mitochondrial DNA (mtDNA) RFLPs depending on the type of template and/or probe used. The term ‘mtDNA RFLP’ has been used in

the literature in connection with RFLPs involving mtDNA templates (*e.g.*, Jacobson and Gordon, 1991; Kim *et al.*, 1991a), or where mtDNA fragments are used as probes (*e.g.*, Kistler *et al.*, 1987; Jacobson and Gordon, 1990; Gordon and Okamoto, 1992; Kim *et al.*, 1992; 1993b). For the sake of convenience, RFLP analyses involving mtDNA templates and/or mtDNA-based probes are treated in the mtDNA RFLPs section. RFLP analyses involving hybridisation with SSR-containing probes are discussed in section 4.6.

4.2.1. Total DNA RFLPs

Total DNA RFLP involves the digestion of total DNA with restriction endonucleases, which produce a complex pattern of bands that are commonly revealed by Southern analysis. Multicopy sequence repeats such as those found in the nuclear ribosomal RNA coding DNA (rDNA) produce RFLP bands that help distinguish between genotypes.

Several studies have reported that RFLP groups correspond well with VCGs or pathogenicity groups. An example is found in the work of Woudt *et al.* (1995) who studied 53 pathogenic and 26 non-pathogenic *F. oxysporum* isolates recovered from cyclamen. Southern blots of *Eco*RV-digested gDNA were hybridised with a plasmid that carries a [α-^{32}P]-dCTP-labeled 6.0-kb *Eco*RV DNA fragment obtained from *F. o. niveum*. Fingerprints of the pathogenic strains differed from the non-pathogenic isolates, and grouped into one of three distinct FPGs that fully corresponded with VCGs. The hybridisation patterns within a FPG were identical, with very few, if any, bands shared between FPGs. Similar results have also been reported where FPGs fully corresponded with *formae speciales* (Namiki *et al.*, 1994), or VCGs (Zuniga *et al.*, 1977; Manicom *et al.*, 1987; 1993; Mes *et al.*, 1994; Woo *et al.*, 1996), although either the restriction enzyme or the enzyme-probe combination used was different in each case.

Other reports, however, indicated the lack of correspondence between RFLP FPGs and VCGs and/or pathogenicity groups. Similar fingerprints were shared by isolates belonging to different VCGs (Jacobson and Gordon, 1990; Gordon and Okamoto, 1992; Fernandez *et al.*, 1994; Baayen *et al.*, 1998) or races (Zuniga *et al.*, 1977; Fernandez *et al.*, 1994; Mes *et al.*, 1994). Some pathogenic and non-pathogenic isolates also shared the same fingerprint generated with a particular restriction enzyme or enzyme-probe combination (Woo *et al.*, 1996). This shows that RFLP FPGs of total DNA depend on the restriction enzyme and/or enzyme-probe combination used, and that FPGs may

not always correspond with VCGs and/or pathogenicity groups. This can be a limitation to the applicability of RFLPs in the taxonomy of *F. oxysporum* isolates.

4.2.2. Mitochondrial DNA RFLPs (mtDNA RFLPs)

mtDNA RFLP involves separation of the mtDNA component from the total DNA extract using bisbenzimide-CsCl density gradient ultracentrifugation (Garber and Yoder, 1984), and restriction of the mtDNA template with endonucleases. Alternatively, it may involve restriction of gDNA and visualization of restriction fragments by Southern hybridization with labeled mtDNA fragments. As compared with nuclear DNA, mtDNA is small in size and thus relatively simple. It evolves at a faster rate than nuclear DNA because of the lesser functional constraint in mtDNA (Gordon and Okamoto, 1992). Therefore, mtDNA RFLP is more appropriate for differentiation at sub-specific levels (Jacobson and Gordon, 1990). It is also useful in the study of genetic diversity within *F. oxysporum* because the available mtDNA-based probes represent approximately 95% of the mitochondrial genome of the species (Gordon and Okamoto, 1992).

The only report where RFLP groups based on mtDNA templates corresponded with *formae speciales* was made by Kistler *et al*. (1987). They analysed the restriction pattern of mtDNA from 25 crucifer-infecting isolates using the endonucleases *Eco*RI, *Bgl*II and *Hind*III. Each enzyme revealed three FPGs corresponding to *F. o. conglutinans, F. o. raphani* and *F. o. matthioli.* However, none of the endonucleases differentiated between races within a *forma specialis*.

It appears that mtDNA RFLP FPGs and sub-species taxa based on pathogenicity are usually incongruent. For instance, Jacobson and Gordon (1991) studied the RFLP patterns of mtDNA templates from 176 strains of *F. o. melonis* encompassing eight VCGs. Using three endonucleases they obtained seven distinct FPGs, six of which uniquely corresponded to six of the VCGs. The seventh FPG was shared between two VCGs. Similarly, Kim *et al.* (1992) detected six FPGs among 50 *F. o. niveum* isolates. Races 1 and 2 fell into three and two, respectively, of the RFLP FPGs. Furthermore, cluster analysis of the binomial data generated with the restriction enzymes placed some of the FPGs in the same cluster with 90% similarity. Other studies have also demonstrated such a lack of correspondence between mtDNA FPGs and VCGs, pathogenicity or geographical groups (Jacobson and Gordon, 1991; Kim *et al.*, 1991a; Gordon and Okamoto, 1992).

Three other important factors also pose a problem in the applicability of mtDNA RFLPs. Firstly, the number and type of FPGs within a *forma specialis* or a VCG, depend on the number and type of restriction enzymes and probes used. For example, Kim *et al.* (1993a) detected five FPGs among 20 *F. o. melonis* isolates. However, among the five FPGs (FPGs A, B, C, D and F) previously reported by Jacobson and Gordon (1990), Kim *et al.* (1993a) could detect only four. Kim *et al.* (1993a) could not differentiate FPG A from FPG B, which were originally differentiated on the bases of *Hae*III restriction site (Jacobson and Gordon, 1990), an enzyme Kim *et al.* (1993a) did not include. However, an additional FPG was detected by Kim *et al.* (1993a) using *Hind*III, which was not used by Jacobson and Gordon (1990). This is because RFLPs are defined by the restriction site of the enzyme(s) used and the presence/absence of insertion/deletions (indels) that affect sizes of the restriction fragments generated. The second limitation of mtDNA RFLP arises from the difficulty to detect and separate mt-plasmids that may be co-isolated with mtDNAs of some strains (Kim *et al.*, 1991a). The last factor that poses a problem in applying mtDNA RFLP in the taxonomy of *F. oxysporum* is the inability of RFLPs to recognize alleles (Majer *et al.*, 1996).

4.2.3. RFLPs of Amplified Products (PCR-RFLPs)

PCR-RFLP involves PCR amplification of a specific DNA segment from two or more isolates, and RFLP analysis of the resulting amplicons. Commonly, amplicons are digested with different enzymes, and electrophoretic patterns obtained with each enzyme are assigned unique letters. A combination of these letters obtained with each of the enzymes defines a PCR-RFLP haplotype of the amplified region.

There are no reports where PCR-RFLP groups fully corresponded with VCGs or pathogenicity groups. However, different authors have reported varying degrees of correlation between VCGs and PCR-RFLP groups. For instance, Rosewich *et al.* (2003) reported a correlation between IGS haplotypes and VCGs among 57 *F. o. lycopersici* isolates representing five VCGs. Restriction analysis of the IGS region using *Eco*RI, *Rsa*I, and *Hae*III resulted in four haplotypes fully corresponding to four VCGs. However, VCGs 0032 and 0030 shared a single haplotype. Appel and Gordon (1995) also reported such a close correspondence in *F. o. melonis*.

The size of the IGS region of the rDNA amplified with the widely used primers CNL12 and CNS1 was reported to vary between isolates, ranging between 2.55 – 2.60 kb (Alves-Santos *et al.*, 1999). However, this variation was not correlated with VCGs, pathotypes, or geographical groups. IGS

haplotypes defined by restriction of the IGS with *Cla*I, *Xho*I, *Ava*I, *Acc*I and *Msp*I also did not show correlation with VCGs, pathotypes or geographical groups. This shows that PCR-RFLP groups do not always correspond to VCGs. It also suggests that a larger number of isolates and VCGs need to be studied in order to get a better picture of the correlation even in the *formae speciales* where correlations have been reported.

RFLPs have several limitations in addition to the lack of correspondence with VCGs, pathogenicity or geographical groups. Firstly, the number and types of FPGs are dependent on the number and type of restriction enzyme and/or restriction enzyme-probe combination used. Secondly, a single nucleotide substitution that results in a loss or gain of a restriction site, hence in varying fragment sizes between isolates, may be scored more than once resulting in an overestimation of variation during phenetic analysis. Thirdly, it is difficult to detect and separate mt-plasmids that may be co-isolated with mtDNAs of some strains. Lastly, the discriminating power of PCR-RFLP depends on the size and variability of the DNA segment amplified. For instance, conserved sequences found in the large and small subunit genes of the rDNA are more useful in the taxonomy of distantly related fungi (Bowman *et al.*, 1992; Bruns *et al.*, 1992), whereas spacer regions of the rDNA, which evolve more rapidly, are more appropriate in the study of intra-specific relationships (Baura *et al.*, 1992; Lee and Taylor, 1992; O'Donnell, 1992). These shortcomings limit the applicability of the method in the taxonomy of *F. oxysporum*.

4.3. Electrophoretic Karyotyping

Electrophoretic karyotyping is differentiation of strains based on the electrophoretic migration patterns of their chromosome-size DNAs. Typically the cell wall from germinating conidia is digested using enzymes, and protoplasts are embedded in 1% low-melting agarose, which is then poured into a plug mould. Plugs are loaded on agarose gels and chromosome-size DNAs are separated by pulsed-field gel electrophoresis (PFGE, Schwartz and Cantor, 1984), which also allows analysis of the number of chromosomes (Orbach *et al.*, 1988), and genomic organization (Hamer *et al.*, 1989).

Electrophoretic karyotyping has been of limited use in the taxonomy of *F. oxysporum*. This is because of the lack of correlation with VCGs, pathogenicity groups, and/or geographical origins. For instance, Kim *et al.* (1993a) detected six Electrophoretic Karyotypes (EKs) among seven geographically separated isolates representing three of the races and six of the mtDNA RFLP

groups reported previously (Kim *et al.*, 1992). Each isolate of race 1 and 2 had a different karyotype. There was also an overlap in EKs between races 1 and 2 showing the lack of association between EKs and races. Additionally, there was no association between EKs and mtDNA haplotypes. The lack of correspondence between pathotypes and EKs was also reported in *F. o. dianthi* (Migheli *et al.*, 1995). Previously, differences in EKs among various *Fusarium* species and isolates of the same *forma specialis* had led Migheli *et al.* (1993) to conclude that EKs may be useful in differentiating among *formae speciales* and races. However, their later finding showing that EKs do not correlate with races (Migheli *et al.*, 1995), suggests otherwise.

Little information is available on the use of EKs in the taxonomy of *F. oxysporum* isolates. However, since karyotypes show the number and structure of chromosomes of an organism, which is not expected to vary much at the sub-species level, their use in the taxonomy of *F. oxysporum* isolates can be expected to be limited. Inadequate electrophoretic resolution among the large size groups of chromosome-size DNAs may lead to underestimation of chromosome number unless extra-care is taken (Zolan, 1995). Up to three weeks of PFGE may be needed to separate some heavy molecular weight bands (Talbot *et al.*, 1991; Migheli *et al.*, 1995). Aneuploidy may result in inaccurate determination of chromosome numbers, especially in the absence of genomic maps, since single-copy probes cannot determine whether a segment or an entire chromosome has been duplicated (Zolan, 1995). These shortcomings limit the applicability of EKs in the taxonomy and phylogeny of *F. oxysporum* isolates.

4.4. Amplified Fragment Length Polymorphisms (AFLPs)

AFLP is a DNA fingerprinting technique based on the detection of genomic restriction fragments by PCR amplification (Vos *et al.*, 1995). gDNA is simultaneously digested with a six-base cutter (usually *Eco*RI) and a four-base cutter (commonly *Mse*I or *Msp*I) restriction enzymes. Adaptors are ligated to the ends of the restricted fragments, which are then amplified using adaptor-specific primers with one (+1) or no (+0) selective nucleotides at their 3'-ends. Fewer fragments are selectively amplified in a secondary PCR using adaptor-specific primers with two (+2) or three (+3) selective nucleotides.

AFLP analysis has several advantages. Prior sequence knowledge is not required, and specific co-amplification and detection of a large number of restriction fragments is possible (Vos *et al.*, 1995). It provides neutral (not subject to natural selection) markers and allows analysis of variation at more

than one locus (Majer *et al.*, 1996; Baayen *et al.*, 2000a). Since most AFLP fragments correspond to unique positions on the genome, they can be exploited as landmarks in genetic and physical maps (Vos *et al.*, 1995). Consequently, AFLP analysis has been used in the taxonomy and phylogeny of different fungi (Majer *et al.*, 1996; Rosendahl and Taylor, 1997; Baayen *et al.*, 2000a; Kiprop *et al.*, 2002; Abdel-Satar *et al.*, 2003). However, it has not been applied widely in the *F. oxysporum* species complex.

The only report where AFLP was used to differentiate among races was made by Sivaramakrishnan *et al*. (2002). Included in their study were single representatives of each of the four races of *F. o. ciceri*, and 39 other isolates of unknown race. Cluster analysis based on the similarity index data obtained with AFLP analysis, placed representatives of races 1 and 2 in clusters 1 and 2, respectively; and that of races 3 and 4 in cluster 3. The authors then concluded that all the other isolates in clusters 1 and 2 belong to races 1 and 2, respectively. This, however, seems unreasonable since only single isolates were used as representatives of each of the four races. The diversity within each of the races has not been demonstrated, and one cannot tell if there are no overlaps between races, and therefore, between clusters. Moreover, the authors also reported lack of clear groupings on the dendrogram based on UPGMA analysis of the same data matrix, which means that AFLP did not actually distinguish between races.

A major shortcoming of AFLPs is the lack of correspondence with vegetative compatibility, pathogenicity, and/or geographical origin. This was best demonstrated by Baayen *et al.* (2000b), who studied 89 strains representing eight *formae speciales*. Non-pathogenic strains, and a reference strain from each of the three main clades identified by O'Donnell *et al.* (1998) were also included. The relationship between AFLP-based groups, VCGs and *formae speciales* was complex. For instance, AFLP grouped together *F. o. gladioli* NRRL 28406 (VCG 0341) and *F. o. dianthi* NRRL 28401 (VCG 0021) separately from other VCGs in their respective *formae speciales*. Among the six VCGs in *F. o. gladioli*, three (0341, 0342, and 0343) were nested in clade 2, and three other (0340, 0344, and 0345), in clade 3. AFLP-based phylogeny also grouped pathogenic strains with non-pathogenic strains. Other studies also showed such a lack of correspondence between AFLPs and pathogenicity, geographic origin, and/or cultural characteristics (Bao *et al.*, 2002; Kiprop *et al.*, 2002; Abdel-Satar *et al.*, 2003). These results show that despite its advantages (see above), AFLP alone is inadequate in taxonomy and phylogeny of *F. oxysporum*.

The use of AFLPs also has some technical limitations. Incomplete restriction of DNA samples can result in detection of differences in banding patterns, which do not reflect true DNA polymorphisms

(Vos *et al.*, 1995). The type of frequent cutter used can reduce the power of the technique. For example, when used with eukaryotic genomes, which are AT-rich, *Taq*I (recognition site TCGA) results in fewer fragments compared with *Mse*I (recognition site TTAA) (Vos *et al.*, 1995). Therefore, careful designing of primers and selection of appropriate restriction enzymes is crucial. Other limitations include the inability to recognize alleles, which leads to an overestimation of variation because allelic fragments are scored as independent, which they are not (Majer *et al.*, 1996). Loss of restriction sites due to point mutations, and changes of fragment sizes due to indels, result in an overestimation of variation. For instance, the loss of an *Mse*I-site adjacent to an *Eco*RI-site may lead to the disappearance of the *Eco*RI-*Mse*I fragment of that size with the simultaneous appearance of a larger fragment, assuming another *Mse*I-site is nearby (Majer *et al.*, 1996).

4.5. DNA Sequence Analysis

Nucleotide sequences from certain genes reflect phylogeny at various taxonomic levels. Consequently, sequence information from relatively conserved genes/DNA segments such as the Internal Transcribed Spacer (ITS), the Intergenic Spacer (IGS), the Nitrate Reductase-Coding Region (NIR), the mitochondrial Small Subunit (mtSSU), the translation Elongation Factor-1α (EF-1α), and β-tubulin partial genes, have been widely used in the taxonomy and phylogeny of *Fusarium* species. This is because their variability, which is harboured mainly in the introns, provides sufficient resolution at the sub-species level.

The classical example of a nucleotide sequence-based phylogeny in *F. oxysporum* was presented by O'Donnell *et al.* (1998), where isolates of *F. o. cubense* were demonstrated to have polyphyletic origins. The authors studied 47 *F. oxysporum* isolates representing 12 VCGs of *F. o. cubense* and 10 other *formae speciales*. One *F. inflexum*, and two unidentified *Fusarium* isolates were also included. The *F. o. cubense* isolates grouped into two separate clades, Clades 1 and 2. Several *formae speciales* also fell in one clade indicating that the clades did not correspond to *formae speciales*. Based on these sequence data, the 12 VCGs of *F. o. cubense* were separated into five clonal lineages, with lineages 1, 3 and 5 in Clade 2, and Lineages 2 and 4 in Clade 1. This showed that there was also no congruence between VCGs and clonal lineages. Similarly, Baayen *et al.* (2000a) used EF-1α and mtSSU partial gene sequences to study 101 *F. oxysporum* isolates representing eight *formae speciales* and 12 non-pathogenic strains, and found no correlation between clades and virulence. Sequence information from other regions of the DNA, such as the

NIR, ITS1, ITS2, and β-tubulin, has also not solved the problem of lack of correlation (Skovgaard and Rosendahl, 1998; Bao *et al.*, 2002).

These studies showed that nucleotide sequence analysis is useful to group isolates of *F. oxysporum* into sub-species taxa based on phylogeny. However, these phylogenetic groups do not always correspond with groups based on the traditional virulence-based classification. Also, phylogenetic groups may not correspond with VCGs. Because of this lack of correspondence, nucleotide sequence analysis cannot replace pathogenicity tests for grouping isolates into *formae speciales* and races. However, sequence-based phylogenetic groups are very useful because they show the evolutionary relationships among isolates within a *forma specialis* or a race, and also between *formae speciales* and/or races. Phylogenetic groups also help to determine relationships between pathogenic and non-pathogenic isolates, and study the diversity within each of these sub-species groups.

An important limitation of sequence-based phylogeny is that certain regions of the DNA are cladistically uninformative and even misleading. A case in point is the work of O'Donnell and Cigelnik (1997) who studied the phylogeny of *F. oxysporum* and the *Gibberella fujikuroi* species complexes. They found that all the isolates studied harboured two non-orthologous rDNA ITS2 types. Half of the species of the *G. fujikuroi* and *F. oxysporum* lineages studied possessed either type I or type II ITS2 sequences as the major ITS2 type. The divergence between the two ITS2 types was greater than that observed within each type. ITS2 gene trees were therefore discordant with trees inferred from the partial β-tubulin gene, mtSSU rDNA, nuclear 28S rDNA, and nuclear rDNA ITS regions.

4.6 Simple Sequence Repeats (SSRs)

SSRs, also known as microsatellites, are tandem repeats of nucleotide motifs that are usually less than 100 base pairs, and embedded in unique DNA stretches (Tautz, 1989). Primers that amplify SSR-containing regions are developed using one of several available techniques (Van Der Nest *et al.*, 2000; Zane *et al.*, 2002). Then, PCR products amplified from different isolates using such SSR primers are compared for size polymorphism. SSRs are advantageous in that they are hypervariable, abundantly found in eukaryotic genomes, analytically simple and readily transferable co-dominant markers (Tautz, 1989). For these reasons they have been widely used in the taxonomic and population genetic studies of various fungi (Dusabenyagasani *et al.*, 1998; Burgess *et al.*, 2001).

There are no SSR markers developed for the study of *F. oxysporum*. The only SSR-related study in *F. oxysporum* is a DNA fingerprinting involving hybridisation with SSR-containing probes (Barve *et al.*, 2001). Barve *et al.* (2001) reported that RFLP analyses involving hybridisation with various SSR-containing probes differentiated among the four races of *F. o. ciceri*. Race 3 always showed a distinct pattern and weaker hybridisation signals regardless of the type of enzyme-probe combination used. Races 1, 2 and 4 were differentiated using UPGMA analysis of hybridisation data of 21 enzyme-probe combinations. However, fingerprint profiles were dependent on both the endonuclease and SSR probe used. Probes, such as $(TG)_{10}$, $(AGT)_5$, and $(ATC)_5$, which have low GC content, and six base-recognizing endonucleases, as opposed to four- and five base-recognizing endonucleases, were the most informative for fingerprinting the races. A major drawback of this study was that only single isolates were used to represent each of the four races. Consequently, the variation within each race, and how it might affect the differentiation of the races was not shown. A limitation of the use of SSRs in *F. oxysporum*, in general, is the absence of markers that are developed for the study of this fungus. Developing SSR markers is also very demanding and costly because it traditionally involves the screening of big genomic libraries (Zane *et al.*, 2002).

5. SUMMARY AND CONCLUSIONS

One of the problems in the characterisation of isolates in the *F. oxysporum* species complex is the assumption that there is a link between pathogenicity to a specific host or a group of host species and sub-species taxa. In most cases this assumption is either incorrect or an over simplification of the actual situation. Non-pathogenic *F. oxysporum* isolates are genetically diverse and make up a significant component of the species complex. Moreover, many phylogenetic studies have shown that some pathogenic isolates are more closely related to non-pathogenic strains than to other pathogenic strains in the same *formae speciales* or races. This suggests that pathogenicity may be governed by a few genes, and that pathogenic genotypes may arise from non-pathogenic genotypes and *vice versa*, through mutations involving these genes. Consequently, the use of pathogenicity as a sole characteristic for grouping *F. oxysporum* isolates is flawed.

Vegetative compatibility tests, unlike pathogenicity tests, allow for the classification of non-pathogenic isolates, and are based on the genetics of the fungus itself rather than its interaction with host species. However, vegetative compatibility is also governed by a very small number of genes, and the presence of cross-compatible isolates indicates that vegetative incompatibility is not an absolute barrier for genetic exchange between VCGs. This is also reflected in the fact that members

of the same VCG may fall in different phylogenetic groups (clades) indicating the genetic heterogeneity in such VCGs. These facts highlight the flaw in the assumption that, genes for pathogenicity and vegetative compatibility have been fixed in the absence of sexual recombination, resulting in VCGs characterized by pathogenicity. Further evidence for this is the complex relationship between VCGs and pathogenicity groups.

The lack of sufficient morphological variation for sub-species classification, the presence of different pathogenicity and vegetative compatibility groups, and the realization that these groups lack congruence, has led researchers to seek other characteristics that can be used more reliably. However, as has already been discussed above and summarized in Table 1, various approaches that have been used have sometimes worked, although the same characteristics have not been useful at other times.

Electrophoretic analyses of soluble proteins and isozymes, and immunoserology, have contributed to some extent to the taxonomy and phylogeny of *F. oxysporum*. However, congruence has not been achieved between groups designated using these techniques and those based on VCGs and virulence. The use of protein-based techniques is also limited because proteins do not harbour sufficient variation for classification at the sub-species level. Similarities observed between different proteins with regard to amino acid sequences and electrophoretic mobility do not necessarily reflect similarities in nucleotide and amino acid sequences, respectively. Moreover, protein composition varies with cultural conditions. Consequently, analyses involving proteins do not provide sufficient resolution for distinguishing *formae speciales* and races.

DNA-based techniques such as RAPDs, RFLPs and AFLPs also have contributed significantly to the taxonomy and phylogeny of *F. oxysporum*. These techniques allow analysis of variation at more than one locus unlike single gene genealogies, which examine specific nuclear and/or mitochondrial genes. However, as with protein-based techniques, complete correspondence has not been achieved between DNA-based groups (specifically EKs, and haplotypes) and VCGs, *formae speciales* and races. RAPDs, RFLPs, and AFLPs are also plagued by high levels of homoplasy, they do not recognize alleles, and the groups they retrieve depend on the number and type of restriction enzymes, probes and primers used. RAPD is also known to have low repeatability.

All heritable information is potentially accessible using DNA sequencing. Consequently, DNA sequence analysis is expected to provide the solution to the problem associated with the taxonomy and phylogeny of *Fusarium* species in general, and *F. oxysporum* in particular. However, genome-

wide sequence comparison is not physically feasible or economically reasonable. Sequence information from certain regions of the DNA such as the ITS2 rDNA region can be misleading or inappropriate for use in the taxonomy and phylogeny of *F. oxysporum*. As such, the usefulness of DNA sequence analyses depends on the nature of the problem at hand, and how informative the particular segment of DNA is. As yet, congruence between sequence-based clades and pathogenicity groups appears to be far from perfect.

The grouping of isolates into *formae speciales* and races is a practical and important issue in terms of agriculture and forestry. Since no single technique used alone or in combination with others has given an adequate solution to characterising these components of *F. oxysporum* populations, it appears that there is no simple alternative to pathogenicity-based classification, despite its limitations. In fact, an adequate solution may never be achieved using any one method alone. A practical approach would be the use of a diversity of these techniques supported with pathogenicity tests, so that a more reliable system of classification is achieved.

Phylogeny of isolates can be assessed using both protein- and DNA-based methods, with the latter generally providing better resolution at the sub-species level. If based on appropriate number and types of genes, sequence analysis is a superior choice for phylogenetic studies in the *F. oxysporum* species complex. However, since each technique has some advantages over others, the use of a combination of techniques should allow the development of more reliable classification systems.

Table 1. A summary of advantages and limitations of tools used in the taxonomy and phylogeny of the *F. oxysporum* species complex.

Tool	Advantage	Disadvantage
Pathogenicity tests	- Have a practical significance in terms of agriculture and forestry	- Time consuming and expensive due to the large number of *formae speciales* and races available. - Inconclusive because results are dependent on the interaction among the host, the pathogen and the environment. - Tests preclude classification of non-pathogenic members.
Vegetative compatibility tests	- Not affected by environmental factors. - Allow classification of non-pathogenic members. - Provide information on genetic relatedness of compatible strains.	- VCGs may lack correspondence with pathogenicity groups. - Self-incompatible and 'bridging' isolates are not amenable to the test. - Do not provide information on genetic relatedness between VCGs.
Protein-based techniques	- Allow classification of non-pathogenic members. - Allow study of phylogeny. - Provide co-dominant markers.	- May lack correspondence with pathogenicity groups. - Are affected by culture conditions. - Lack resolution at sub-species levels. - Identical protein or isozyme profiles do not necessarily imply identical nucleotide sequence due to neutrality of some amino acids and the redundancy of the genetic code. - Only isozymes with different net charges or large differences in shape or size (a third of the total possible isozymes) can be differentiated. - Enzymes exhibit different degrees of variation due to the intensity of selection pressure.
DNA-based techniques	- Allow classification of non-pathogenic members. - Provide neutral markers. - Provide resolution at the sub-species level. - Widely used in phylogenetic studies. - RAPDs, RFLPs and AFLPs, allow multilocus analysis of variation. - RFLPs provide co-dominant markers.	- May lack correspondence with pathogenicity groups. - RAPDs and AFLPs are dominant markers, and retrieve high homoplasy. - RAPDs have low repeatability. - RFLP haplotypes depend on the number and type of restriction enzyme and/or probe used. - Inadequate resolution and aneuploidy can result in underestimation of chromosome number and hence erroneous karyotyping. - There are no SSR markers developed; and AFLPs have not been used widely. - Sequences from certain regions of the DNA are cladistically uninformative and misleading.

6.0. **REFERENCES**

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CHAPTER 2

A survey of *Fusarium* species from Ethiopia using morphology and DNA sequence information

ABSTRACT

The significance of *Fusarium* species in African agriculture and forestry has not been well investigated. Reports of these fungi from the horn of Africa, particularly Ethiopia, are few, relatively old, and mainly based on inventories of crop diseases. A diagnostic survey was thus conducted in 2000 and 2001 to determine which *Fusarium* spp. commonly occur in agricultural soils and plant tissues from Ethiopia. The isolates were identified using morphology and DNA-based approaches. The latter was accomplished by sequencing two protein-coding genes (EF-1α and β-tubulin) followed by similarity searches using public domain databases (www.ncbi.nih.gov and http://fusarium.cbio.psu.edu) and phylogenetic analyses. β-tubulin sequence information was not useful for diagnosing *F. equiseti* and *F. solani* due to lack of β-tubulin sequences for these species in the database. Nonetheless, the combined use of sequence similarity searches and phylogenetic analyses provided unambiguous identification for all the species studied. The results generally corresponded well with morphological identifications. A total of nine *Fusarium* spp. (*F. oxysporum, F. solani, F. redolens, F. avenaceum, F. equiseti, F. dlamini, F. acutatum, F. lactis* and an apparently un-described *Fusarium* sp.) were isolated from the soil and plant samples. Some of these species appeared to be associated with specific soil samples. For example, the highest number of *F. oxysporum* and *F. solani* isolates was recovered from 'tef' and barley farms, respectively. *F. solani* and *F. oxysporum* were also the most commonly isolated species from soil. *F. oxysporum* was commonly isolated from banana roots and may play a role in the wilt diseases reported by growers. The most common *Fusarium* spp. associated with wheat and barley stems were *F. oxysporum, F. solani* and *F. avenaceum*, and these are suspected to play a role in *Fusarium* head blight and other diseases of wheat in Ethiopia.

INTRODUCTION

Fusarium is a diverse genus with a cosmopolitan distribution (Nelson *et al.*, 1983). Most of its members are beneficial saprophytes (Snyder and Hansen, 1981). Some are useful biological control agents against certain plant pathogens (Lemanceau and Alabouvette, 1991; Mandeel and Baker, 1991; Postma and Rattink, 1992). Others have medical and/or veterinary significance (Marasas *et al.*, 1984; Gupta *et al.*, 2000; Pereiro *et al.*, 2001). However, the genus is best known for its plant pathogenic members that are responsible for important problems in agriculture and forestry (Toussoun, 1981).

The significance of *Fusarium* in African agriculture and forestry has not been well investigated. Most of the available information is from studies conducted in South Africa (*e.g.*, Fandohan *et al.*, 2003). Reports concerning *Fusarium* spp. from the horn of Africa are few and mainly based on surveys of diseases of various crops (Baker, 1970; 1972; Ebbels and Billington, 1972; Ibrahim and Hussein, 1974; Ofong, 1974; Ibrahim and Owen, 1981; Hillocks, 1983; Abdel-Rahim and Tawfig, 1984; Bekele, 1985; Mutitu *et al.*, 1988; Beniwal *et al.*, 1992; Kihurani and Skoglund, 1993; Hallmann and Sikora, 1994; Bekele and Karr, 1997; Mansuetus *et al.*, 1997; Kedera *et al.*, 1999; Lodwig *et al.*, 1999; Ahmed *et al.*, 2001; Hillocks and Kibani, 2002; Kiprop *et al.*, 2002). Also, a significant number of these reports were made in the 1990s and earlier. A few of these studies dealt with the biological control of *Fusarium* spp. (Mutitu *et al.*, 1988), or the use of *Fusarium* spp. in the biological control of certain weeds (Abdel-Rahim and Tawfig, 1984; Ahmed *et al.*, 2001) and nematodes (Hallmann and Sikora, 1994). The study conducted by Lodwig *et al.* (1999) was the only report that I encountered, which applied DNA-based characterisation of *Fusarium* spp. from the horn of Africa. Reports on the importance of *Fusarium* spp. in Ethiopian agriculture are even less common and have focused on inventories of diseases that affect cereals and pulses (Bekele, 1985; Beniwal *et al.*, 1992; Bekele and Karr, 1997). Bekele and Karr (1997) presented the only detailed account of *Fusarium* spp. in Ethiopia based on a 1987-1988 diagnostic survey. They reported more than 19 *Fusarium* spp. in association with head blight and other fungal diseases of stored wheat (*Triticum aestivum* L.) seeds. Adequate information on the importance of *Fusarium* spp. from the horn of Africa in general, and from Ethiopia in particular, is therefore lacking.

Morphological identification of *Fusarium* spp. involves examination of cultural characteristics and microscopic features such as presence or absence of micro- and macroconidia, and chlamydospores; and the shape of the chlamydospores, conidia and the conidiogenous cells bearing them (Nelson *et al.*, 1983). However, these features may vary with cultural conditions (Waite and Stover, 1960)

making routine identifications difficult. The presence of different taxonomic systems further complicates morphology-based diagnoses (Nelson, 1991). To circumvent these problems, vegetative compatibility (Puhalla, 1985; Bosland and Williams, 1987) and an array of molecular techniques have been used. For example, isozyme analyses (Huss *et al.*, 1996; Yli-Mattila *et al.*, 1996; Abd-Elaah, 1998; Boshoff *et al.*, 1999; Láday *et al.*, 2000), electrophoretic patterns of soluble proteins (Glynn and Reid, 1969; Partridge *et al.*, 1984), electrophoretic karyotypes (Fekete *et al.*, 1993; Migheli *et al.*, 1993; Xu *et al.*, 1995), randomly amplified polymorphic DNA (Yli-Mattila *et al.*, 1996), restriction fragment length polymorphism (Manicom *et al.*, 1987; 1993; Donaldson *et al.*, 1995; Edel *et al.*, 1996; Bagley *et al.*, 1997; Baayen *et al.*, 2000b), amplified fragment length polymorphism (Abdel-Satar *et al.*, 2003), and DNA sequence comparisons (O'Donnell, 1992; 1997; Baayen *et al.*, 2001) have been employed to varying degrees in the study of *Fusarium* spp.

The objective of this study was to add to the available information on *Fusarium* spp. in Ethiopia by determining the species commonly isolated from agricultural soil samples and plant tissues. For this purpose both morphology- and DNA-based procedures were used. The molecular characterization of Ethiopian isolates was accomplished using DNA sequence information from two protein-coding genes (translation Elongation Factor 1α [EF-1α] and β-tubulin). Morphological characterization of the isolates was mainly based on the classification scheme of Nelson *et al.* (1983).

MATERIALS AND METHODS

Sample collection

Soil samples were collected in September 2000 at 30-50 km intervals from farms along the five major routes out of Addis Ababa (Fig. 1). Four composite samples were collected from an area of 4-6 m^2 and a depth of 3-15 cm along the diagonals of each farm visited. Soil samples were kept at 4°C for 2-4 weeks until isolation of *Fusarium* spp. Wheat and barley (*Hordeum vulgare* L.) samples were collected in August 2001, from 23 farms at 10-15 km intervals along the major roads in the Shoa region (Fig. 1). From each farm, samples were collected at five spots along the diagonals of the field, where 10-15 plants that were close to harvesting were sampled from an area of about 4 m^2 at each spot. Stem samples were collected from plants cut close to the ground. Banana (*Musa acuminata* Colla) root samples were also collected in August 2001 from 100 Cavendish plants grown on a commercial farm at Arbaminch, Southern Ethiopia (Fig. 1). Sampled banana plants were at least 10 m apart from each other. Five to eight pieces of root, each at least 5 cm in length, were taken from the selected banana plants at 5-10 cm depth from the surface of the ground.

Isolation of* Fusarium *strains

Fusarium spp. were isolated from the soil samples following a modification of the dilution plate method (Nelson *et al.*, 1983). Air-dried soil samples (30-50 g) were first suspended in 100 ml of distilled water, and then diluted 100-fold. One ml of each diluted suspension was plated on a *Fusarium*-selective pentachloronitrobenzene (PCNB) medium (Nash and Snyder, 1962). Inoculated plates were incubated upside down at 25°C for 3-5 days under ordinary white fluorescent light. Fungal colonies were cut out and transferred to MEA medium (2% [w/v] malt extract, 1.5% [w/v] agar) and incubated for 10-15 days at 25°C in the dark. Spores were gently washed from the surface of the cultures with 3-5 ml of 15% (v/v) glycerol (sterile aqueous solution) and stored as mixed spore suspensions at –80°C for later use.

Plant tissues (wheat stems, barley stems and banana roots) were thoroughly washed under running tap-water, cut into 2-3 cm pieces, and surface sterilized by soaking them in NaOCl (1.5% v/v) for two minutes. The plant tissues were rinsed in three changes of distilled water to remove the NaOCl, and dried by passing them a few times through the flame of a spirit lamp. Three or four pieces were then placed on PCNB plates, and *Fusarium* spp. isolated from them as described above.

Single spore cultures were prepared from mixed spore suspensions that were stored at –80ºC. A drop of spore suspension was diluted with 1 ml of sterile distilled water, and spread on a sterile water agar medium (WA, 1.5% w/v). After incubation at 25°C for 16-24 hours under fluorescent light, single germinating spores were identified using stereomicroscope and aseptically transferred to MEA plates. The plates were incubated in the dark at 25°C for 10-15 days, after which, spore suspensions were prepared and stored at –80ºC as described above. All isolates were deposited in the *Fusarium* Culture Collection (FCC) at the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

Morphology-based identification

For morphological identification, single spore isolates were grown for 10-15 days on Potato Dextrose Agar (PDA) medium (Nelson *et al.*, 1983), and on Carnation Leaf Agar (CLA) medium prepared following a modification of the method described by Fisher *et al.* (1982). Young leaves from carnations (*Dianthus carophyllus* L.) were cut into small pieces of approximately 5 mm^2, placed in glass Petri dishes and autoclaved for 20 min at 120 lb. CLA was prepared by aseptically placing two or three sterile leaf pieces onto Petri dishes and floating them in sterile WA. As described by Nelson *et al*. (1983), gross cultural characteristics of each isolate were determined

from 10-15-day old PDA cultures, whereas microscopic features of conidia, conidiophores and chlamydospores were determined based on 10-15-day old CLA cultures.

DNA-based identification

DNA was extracted from the isolates using CTAB (N-cetyl-N,N,N-trimethyl-ammonium bromide) (Murray and Thompson, 1980). For this purpose mycelium was scraped from the surface of cultures on MEA, and transferred to Eppendorf tubes containing sterile sand and 700 µL extraction buffer (5% [w/v] CTAB, 1.4 M NaCl, 0.2% [v/v] 2-mercaptoethanol, 20 mM ethylene diamine tetraacetate, 10 mM Tris-HCl [pH 8.0], and 1% [w/v] polyvinylpyrrolidone). The mixture was homogenized; incubated for 1 hr at 60°C, and centrifuged at 9,300 *g* for 10 min. DNA was extracted from the aqueous phase by repeated phenol-chloroform (1:1) extractions followed by a final chloroform extraction to remove residual phenol. The DNA was then precipitated with 0.1 volume of 3 M sodium acetate (pH 5.4) and 0.6 volume of 2-propanol, and incubated at –20°C overnight. Precipitated DNA was pelleted by centrifugation at 15,700 *g* for 30 min at 4°C, washed with 70% ethanol, air-dried, and dissolved in deionised water.

DNA was isolated from one to four representatives of each of the species characterized using morphology. However, DNA was extracted from all of the isolates morphologically identified as *F. oxysporum* and *F. solani*, as these were the most commonly isolated species. DNA was also extracted from 18 *formae speciales* of *F. oxysporum* that were obtained from the Centraalbureau voor Schimmelcultures (CBS) in the Netherlands (Table 1).

The isolated DNAs were then used as templates to PCR-amplify ~ 656 base pairs (bp) and ~ 290 bp of the genes encoding EF-1α and β-tubulin, respectively (Fig. 2). This was accomplished with primers EF1 and EF2 (O'Donnell *et al.*, 1998b) for EF-1α, and 2A and 2B (Glass and Donaldson, 1995) for β-tubulin. PCR mixtures contained reaction buffer (10 mM Tris-HCl [pH 8.3], 2.5 mM $MgCl_2$, 50 mM KCl), 2.5 µM of each dNTP, 0.20 µM of each primer, 0.05 U/µL *Taq* Polymerase (Roche, USA), and approximately 4 ng/µL template DNA. The PCR cycling conditions included an initial denaturation at 94°C for 4 min; 35 cycles of denaturation at 94°C for 30 sec, annealing for 30 sec at 56°C (for EF1 and EF2) or 68°C (for 2A and 2B), and extension at 72°C for 30 sec; and a final extension at 72ºC for 10 min. PCR products were purified using the QIAquick PCR purification kit (QIAGEN, GmbH, Germany), and sequenced in both directions using the same primers as for the respective PCRs. Sequencing was carried out with the BigDye terminator sequencing kit (Version 3.2, Applied Biosystems, USA) and an ABI PRISM™ 3100 DNA

sequencer (Applied Biosystems). All PCRs and sequencing reactions were performed on a GeneAmp PCR System 9700 (Applied Biosystems).

Gene sequences were assembled with Sequence Navigator (Version 1.0.1, Applied Biosystems). The β-tubulin and EF-1α nucleotide sequences for each isolate were then compared to those in the public domain databases (National Centre for Biotechnology Information at www.ncbi.nih.gov, GenBank; and the *Fusarium* database at http://fusarium.cbio.psu.edu) using the Basic Local Alignment Search Tool for Nucleotide sequences (BLASTN, Altschul *et al.*, 1997). Sequence navigator was also used to predict the amino acid sequences encoded by the nucleotide sequences. The presence of introns and their positions in these nucleotide sequences were determined by comparing the predicted amino acid sequences with relevant amino acid sequences in the GenBank.

Sequences were aligned using ClustalX (Version 1.8, Thompson *et al.*, 1997), after which the alignments were corrected manually where needed. These alignments included all of the newly generated sequences as well as those for 33 representatives of known *Fusarium* spp. that were downloaded from GenBank (Table 1). However, no β-tubulin sequences were available in the database for *F. equiseti* and *F. solani*, and EF-1α sequences for *F. avenaceum* were inordinately short to include in this study. Additionally, EF-1α and β-tubulin sequences associated with individual strains could not be found for most of the representative species in GenBank (Table 1). In some cases, therefore, EF-1α and β-tubulin sequences from different strains representing the same species were used in the combined sequence dataset. To assess how this might affect the results, parsimony analyses were done both with and without the GenBank sequences.

Phylogenetic relationships were estimated with PAUP (Phylogenetic Analysis Using Parsimony, Version 4.0b 10, Swofford, 2002), by heuristic searches using 1,000 random addition sequences and tree bisection-reconstruction, with the 'branch swapping' option set on 'best trees only'. All characters were weighted equally and alignment gaps were treated as missing data. *Cylindrocladium pacificum* (strain IMI35428) was used as the outgroup taxon. Bootstrap analyses (Hillis and Bull, 1993) were performed using 1,000 replications. PAUP was also used to test combinability of the EF-1α and β-tubulin datasets using the partition homogeneity test by heuristic searches employing 500 repartitions.

Statistical analyses

Association between farm types (by crop) and the number and types of *Fusarium* species recovered from soil samples collected from these farms was determined using the Chi-square test.

RESULTS

Fusarium *isolates*

Three hundred and twenty soil samples from 80 agricultural fields; 100 root samples from 100 banana plants; and 115 stem samples from wheat and barley plants from 23 farms were collected during the survey. A total of 250 *Fusarium* strains were isolated from these samples. Among these strains, 183 were isolated from soil, 41 from banana roots, and 26 from wheat and barley stems.

Based on morphology and DNA sequence information, a total of nine *Fusarium* spp. were identified. These were *F. oxysporum, F. redolens*, *F. solani, F. acutatum, F. equiseti, F. dlamini, F. avenaceum* and *F. lactis*. Strains that potentially represent a novel un-described species were also identified. This species was isolated from maize (*Zea mays* L.), barley and 'tef' (*Eragrostis tef* [Zuccagni] Trotter) farms in the Shoa region (Fig. 1), and is represented by strain FCC4649. *F. oxysporum* and *F. solani* accounted for more than 42% of the 183 soil isolates (Table 3). There was an association between the type of farm and the number of *Fusarium* spp. obtained from it (Table 4). Thus, the greatest number of soil isolates, particularly of *F. oxysporum*, was obtained from 'tef' farms followed by barley farms, whereas the greatest number of *F. solani* from the soil, was obtained from barley farms followed by 'tef' farms. Among the 26 *Fusarium* strains that were isolated from wheat and barley stem samples, there were nine *F. avenaceum*, eight *Fusarium* sp. (represented by FCC4649), three *F. oxysporum*, and six *F. solani* isolates. *F. oxysporum* (12 isolates), *F. solani* (14 isolates), *F. redolens* (9 isolates) and *F. dlamini* (6 isolates) constituted the 41 isolates obtained from banana root samples.

Although all of the 47 *F. oxysporum* and the 64 *F. solani* isolates obtained from Ethiopia were sequenced for both genes, only isolates representing different haplotypes based on the two genes are shown to facilitate readability of the trees. However, all of the sequences generated in this study have been deposited in the GenBank under accession numbers DQ220021 - DQ220082 and DQ220207 - DQ220247 (for β-tubulin), and DQ220083 – DQ220144 and DQ220248 – DQ220288 (for EF-1α).

The partition homogeneity test showed that the EF-1α and β-tubulin data represent homogenous partitions ($p = 0.018$). Analyses of the combined EF-1α and β-tubulin datasets generated an overall *Fusarium* phylogenetic tree that separated the taxa into two major groups (Fig. 3). One of these groups exclusively harboured isolates of the *F. solani-Haematonectria haematococca* complex (100% bootstrap support [BP]). All of the isolates in this group were obtained from Ethiopia and

included no known representatives in the form of sequences obtained from GenBank, as β-tubulin sequences were not available from previous studies for the *F. solani-H. haematococca* complex. The other group (89% BP) consisted of the remaining 41 *Fusarium* spp., which further separated into four clades (Clades 1 - 4). Clade 1 (86% BP) was represented by two sub-clades harbouring isolates from the *F. redolens-F. hostae* complex (100% BP) and the *F. oxysporum* complex (97% BP), as well as 25 other *Fusarium* spp. most of which represented the *Gibberella fujikuroi* complex. Clade 2 (95% BP) harboured *F. avenaceum*, *F. equiseti*, and *F. flocciferum*. Clade 3 (100% BP) exclusively consisted of *F. lateritium*. Clade 4 (99% BP) included the Ethiopian isolate FCC4649, as well as *F. venenatum, F. graminearum, F. pseudograminearum, F. cortaderiae, F. brasilicum, F. cerealis* and *F. lunulosporum*.

Trees inferred from the EF-1α data set (Fig. 4) had the same topology as those inferred from the combined dataset, except that the EF-1α trees failed to resolve the closely related *F. avenaceum* and *F. equiseti* isolates. This suggests that the treatment of sequences from different strains representing the same species, as if they were from the same strain, did not affect the results. However, the BP values for some of the nodes were lower for the EF-1α than the combined data (Table 5). Overall, the β-tubulin data set provided relatively little resolution (Fig. 5, Table 5). It also failed to resolve Clade 2 species, and instead placed *F. equiseti* and *F. avenaceum* with *F. dlamini* and the unresolved species of Clade 1, respectively.

The *F. oxysporum* and *F. solani* complexes had several moderately to well-supported (>70% BP) subgroups (Fig. 3). In the combined dataset, four of the *F. solani* clusters received BP values of more than 90%. Three of the *F. oxysporum* clusters had 90% or higher BP support on the tree inferred from the combined dataset. Among the *F. oxysporum* isolates included in this study, most of the Ethiopian isolates grouped together (85% BP, Fig. 3). Among the 47 Ethiopian *F. oxysporum* isolates, there were nine, 11 and 14 haplotypes based on the β-tubulin, the EF-1α and the combined sequence information, respectively. Similarly, among the 64 *F. solani* isolates, these haplotypes were seven, 15 and 17, in that order. The two *F. avenaceum* isolates included in the study had identical sequences at both loci, whereas the two *F. dlamini* isolates had different haplotypes at both loci. Isolates of *F. redolens* and *F. equiseti* had only one β-tubulin haplotype and two haplotypes each based on the EF-1α. The two *F. acutatum* isolates had one haplotype based on EF-1α and two haplotypes based on each of the β-tubulin and the combined sequence information. In both *F. redolens* and *F. equiseti*, only a single isolate had a different EF-1α haplotype from the rest of the isolates in the respective group. Only one isolate was included in the study from each of the *F.*

lactis and the potentially new *Fusarium* sp., and hence there were only single haplotypes for these species based on either the individual or combined sequence information.

Within the β-tubulin alignment, the two introns (TI-1 and TI-2) accounted for about 56% of the total variable nucleotide sites, with 54% of these being restricted to the first intron (TI-1, Fig. 2). All variations in the exons represented silent or synonymous substitutions and were exclusively restricted to third codon positions. Within the EF-1α alignment, more than 94% of variable sites were restricted to the three introns, of which EI-2 (Fig. 2) harbored more than 70%, and EI-1 harbored more than 17% of the total variation. The EF-1α exons accounted only for about 5% of the total variability and were restricted to third codon positions. O'Donnell *et al.* (1998b) also reported a similar level of variability within EF-1α exons, which they attributed to two synonymous substitutions in the middle exons (Fig. 2).

DISCUSSION

In this survey, nine *Fusarium* spp. were encountered, some of which have previously been implicated as causal agents of various types of crop diseases. Morphological identification of some of these species was difficult. At first for example, the *F. redolens* isolates were mistaken for *F. oxysporum*, and it was only after DNA sequence comparisons that these isolates were correctly identified as *F. redolens*. In another case, strains could not be identified based on morphology, and sequence analyses indicated that they might represent a new *Fusarium* sp. DNA sequence similarity search (BLASTN) as a means of diagnosing species was not always unambiguous since, in some cases, sequence information from the β-tubulin gene found the highest similarity in different species than those obtained using EF-1α gene sequences. Phylogenetic analyses, however, resolved all such ambiguities and enabled accurate identification of species.

Fusarium spp. are commonly found in agricultural soils (Burgess, 1981). Accordingly, most of the soil samples collected in this study had *Fusarium* spp. in them. Surprisingly, however, some samples did not harbour *Fusarium* isolates (results not shown). This may be because a number of these samples came from areas that had not received precipitation for several years. Soil samples collected from such areas (mostly from the South Eastern parts of Ethiopia) were very hot, dry and dusty at the time of collection. Plant residues were also not evident in the soils, as drought had prevailed for a protracted period of time. This climatic condition might have affected the *Fusarium* population in these areas. In some cases, it was not possible to recover *Fusarium* isolates from the

mixed spore suspensions that were kept at –80°C in 15% glycerol, suggesting that this may not be the best method for the preservation of some isolates. Therefore, it is difficult to discount the possibilities that this study might have underestimated the diversity of the *Fusarium* spp., nor that the sampling procedures might have skewed the species representation in the various soil and tissue samples.

The association observed between crop types and the number and type of *Fusarium* spp. isolated from the farms suggests that 'tef' and barley residues may be suitable for the off-season survival of *Fusarium* spp., particularly for *F. oxysporum* and *F. solani*. However, this apparent association may not be real because most subsistence farmers in Ethiopia practice crop rotation fairly regularly. Therefore, the type of crops used in a rotation may affect the spectrum and density of *Fusarium* spp. that may be found at a particular time. Consequently, the high number of isolates recovered from 'tef' and barley farms might not be because these crops are susceptible to colonisation by *Fusarium* spp., since previous crops might have influenced the population of *Fusarium* spp. Another factor that could influence the occurrence of *Fusarium* spp. is the fact that farmers employ different types of fertilizers including manure and compost, which also affect the population of *Fusarium* spp. depending on their biotic and abiotic contents (Raviv *et al.*, 1998). If the type of fertilizer used differs among farms, then the observed association might rather be due to the type of fertilizer used than the type of crop planted. Farmers also commonly plant small neighbouring plots of land with different crops because of the small size (< 1 ha) of their total land holding. Consequently, movement of *Fusarium* spp. between neighbouring farms together with soil and farm tools is likely to be common during the course of crop production, and this might affect the populations of *Fusarium* spp. in these farms.

F. avenaceum, *F. oxysporum* and *F. solani* were commonly isolated from wheat and barley stem samples. Strains representing a potentially new *Fusarium* sp. were also isolated from barley soils and plants. These species were not tested for pathogenicity on wheat or barley. However, previous studies have shown that *F. avenaceum*, *F. oxysporum* and *F. solani* were among those species isolated from wheat seeds collected from various sources, and implicated as causal agents of head blight and other fungal diseases of wheat (Bekele and Karr, 1997). As such, these species may be considered important to wheat cultivation in Ethiopia.

Panama wilt disease is caused by *F. oxysporum* f.sp. *cubense* (Stover, 1981). The fungus attacks the vascular tissues of banana plants, restricting the transportation of water, thereby resulting in the wilting and, eventually, death of the plants (Stover, 1981). The banana root samples used in this

study were taken from apparently healthy Cavendish banana plantations. The *F. oxysporum* isolates from these samples have not been tested for pathogenicity. But, it is likely that *F. oxysporum* plays a role in the wilt diseases observed by some growers in the area. To the best of my knowledge, however, there has been no previous report on Panama wilt disease in Ethiopia. Closer examination of the *Fusarium* spp. associated with these plants, particularly virulence towards the host, and incorporation of this knowledge into the existing disease management schemes would be very important.

DNA-based studies have provided not only an alternative, but also a more reliable tool for the identification of morphologically closely related *Fusarium* spp. (Baayen *et al.*, 2001). One such approach is a simple sequence similarity search (*e.g.* BLASTN) to public domain sequence databases. However, the success of this approach is heavily dependent on the sequence information available in these databases. This is well demonstrated by the β-tubulin sequences that were less useful than the EF-1α sequences for diagnosing species, due to limited *Fusarium* β-tubulin entries in GenBank and their absence in the *Fusarium* database. For example, the highest similarity obtained using the β-tubulin sequence information for the *F. solani* isolate (FCC3815) was with those of *Acremonium chrysogenum* and/or *Phaeoacremonium aleophilum*. This was due to the absence of β-tubulin sequences of *F. solani* strains in GenBank, and because the best matches happened to be with these species. *F. solani* is reported as more closely related to *Acremonium* spp. than some *Fusarium* spp. (Hennequin *et al.*, 1999). Consequently, the fact that the *F. solani* β-tubulin sequences from this study received the highest similarity with these species was to be expected in the absence of β-tubulin sequence information for *F. solani* isolates in GenBank. However, the EF-1α sequences of all the *F. solani* isolates in this study were most similar to those of known isolates of *F. solani* or *Haematonectria haematococca* (the sexual form of *F. solani*), which is in line with morphological identifications.

Interpretation of BLASTN results may also be complicated by the fact that a gene sequence for an isolate may be similar to sequences from isolates of several other species. For example, the range of sequence variation in the β-tubulin gene region sequenced in this study was only 1-2% (99-98% similarity) among the *F. oxysporum, F. inflexum, F. subglutinans* and *F. commune* strains, which had the highest sequence similarity to the Ethiopian *F. oxysporum* isolates (*e.g.* isolates 3618, 3753; Table 2). Furthermore, BLASTN-based diagnoses with one gene may differ from those of another. For example, the β-tubulin sequence of the Ethiopian *F. lactis* isolate (FCC325), was most similar to those of *F. nygamai* (GenBank Accession U34426), while its EF-1α sequence was most similar

to those of *F. lactis* (GenBank Accession AF160309). Because of these ambiguities associated with the sequence similarity-based species diagnoses, phylogenetic analyses were used to identify the isolates obtained from Ethiopian soils and plant tissues.

Phylogenetic analyses solved all of the ambiguities associated with BLASTN analyses. For example the Ethiopian *F. lactis* isolate (FCC4637) was diagnosed as *F. nygamai* based on β-tubulin BLASTN results, and as *F. lactis* based on EF-1α BLASTN results (see above). However, phylogenetic analyses grouped this isolate (FCC4637) with the *F. lactis* sequence obtained from GenBank, confirming that FCC4637 represents *F. lactis*. The Ethiopian *F. redolens* isolates were identified as *F. oxysporum* based on morphology. Similarity search using EF-1α and β-tubulin sequences from these isolates had the best matches with either *F. redolens* or *F. oxysporum*. Phylogenetic analyses, however, unambiguously identified these isolates as *F. redolens*. For the remaining Ethiopian isolates, where results of morphological identification correlated with the similarity search results, phylogenetic analyses results also confirmed these identifications.

Phylogenetic studies in *Fusarium* have mostly been limited to isolates of one or a few closely related species. For example, some studies targeted phylogenetic relations among strains representing worldwide collections of particular species (Bentley *et al.*, 1995; O'Donnell *et al.*, 2000b; Jiménez-Gasco *et al.*, 2002). Others have attempted to elaborate phylogenetic relations among morphologically closely related species (Guadet *et al.*, 1989; O'Donnell *et al.*, 1998a). Still others have aimed at revealing evolutionary relations among different *Fusarium* species attacking a family of plants (Bosland and Williams, 1987; Gordon *et al.*, 1989; Manicom and Baayen, 1993; O'Donnell *et al.*, 1999; Pasquali *et al.*, 2003). Included in this study were representatives of all *Fusarium* spp. for which there was EF-1α and β-tubulin sequence information in GenBank. As such, and to the best of my knowledge, this is the first attempt to elucidate the phylogenetic relationships among such a large number of *Fusarium* spp. Aligning the sequences from these diverse species was challenging because the sequences involved large variations. Selecting an appropriate outgroup was also problematic. For example, some *Fusarium* spp. are reported to be more closely related to some species of *Cylindrocarpon* and *Acremonium* than to other *Fusarium* spp. (Hennequin *et al.*, 1999). This indicated that the genera *Cylindrocarpon* and *Acremonium*, which are closely related to *Fusarium,* would not make suitable outgroups. The use of more distant relatives like *Neurospora* would increase the chance of phylogenetic artefacts such as long-branch-attraction (Lyons-Weiler and Hoelzer, 1997). Eventually *Cylindrocladium pacificum* was chosen as it was a member of a well-defined monophyletic group that has not been reported to include *Fusarium* spp.

Parsimony analyses of the combined sequence information separated the *Fusarium* spp. into two main groups. One of these groups consisted exclusively of isolates from the *F. solani-H. haematococca* species complex. A similar evolutionary separation between *F. solani* (section *Martiella*) and isolates from the *Fusarium* sections *Elegans, Liseola, Discolor* and *Spicarioides* has been reported using sequence information from the large subunit rRNA gene (Guadet *et al.*, 1989; Edel *et al.*, 1996). Some *F. solani* isolates have also been reported to have a closer 28S rDNA sequence similarity (percentage homology) with homologous sequences from isolates of *Cylindrocarpon tonkinense* than other *F. solani* isolates (Hennequin *et al.*, 1999). However, only representatives for *Fusarium* were included here; thus the monophyly of this genus and whether the *F. solani-H. haematococca* species complex shares a more recent common ancestor with species from other related genera such as *Acremonium* and *Cylindrocarpon* could not be tested in this study.

The second major group emerging from the DNA sequence comparisons consisted of four well-supported clades (Clades 1 - 4). Although the exact relationships among these clades are unresolved, the data supported the relationship within the clades relatively well. For example, the ancestor of Clade 1 appeared to have diverged to form the *F. redolens-F. hostae* complex and the cohesive, but largely unresolved group, that mostly included *Fusarium* spp. from the *G. fujikuroi* complex and *F. nisikadoi*. This cohesive group also included the *F. oxysporum* complex, which together with *F. inflexum* and *F. foetens*, formed a monophyletic group. Although both *F. redolens* and *F. oxysporum* are grouped within the section *Elegans*, they lack a sister group relationship (O'Donnell *et al.*, 1998a; Gams *et al.*, 1999; Baayen *et al.*, 2000a; 2001). Consistent with the results of this study, a closer relationship between *F. oxysporum* and *F. nisikadoi* (*F. nisikadoi-F. miscanthi* clade) has also been reported elsewhere (Gams *et al.*, 1999). Although sections in *Fusarium* are based on morphological features such as cultural characteristics and microscopic features of the macro- and microconidia (Nelson *et al.*, 1983), these traits are often not synapomorphic. Consequently, as has also been suggested by other researchers (Waalwijk *et al.*, 1996), *F. redolens* should be placed in a different section than *Elegans* for this section to be phylogenetically meaningful.

Comparison of the various diagnostic approaches employed here revealed that the EF-1α BLASTN results corresponded well with those of the combined EF-1α + β-tubulin phylogeny. This suggests that rapid identification of most isolates may be accomplished with BLASTN of EF-1α gene sequence. The EF-1α sequence provided a better resolution mainly because of the larger size and number of introns it contains. The EF-1α gene sequence also had more parsimony informative sites than the β-tubulin gene sequence used. Variable sites in exons accounted only for a small fraction

of the total variation in both gene fragments. Moreover, nucleotide variations in exons did not result in different putative amino acid residues. This is probably because of the functional constraints in both gene products, which play a role in protein synthesis and the cytoskeletal system, respectively.

Most of the *Fusarium* spp. encountered were initially identified using morphology, and these identifications mostly corresponded well with the identifications based on DNA sequence data. For some isolates, however, the morphological identification was later rejected, as it did not correspond with BLASTN results and the sequence-based phylogeny. An excellent example of this situation is the Ethiopian *F. redolens* isolates, which were initially identified as *F. oxysporum* based on morphology. *F. redolens* has been considered until recently to be conspecific with *F. oxysporum* or a variety of this species (Snyder and Hansen, 1940; Gordon, 1952; Booth, 1971; 1975; Nelson *et al.*, 1983). This shows the difficulty associated with the morphological differentiation between the two species, which Baayen and Gams (1988) attributed to the presence of intermediate forms.

Isolate FCC4649 could not be accurately identified based on morphology. The BLASTN analyses of EF-1α gene sequence for this isolate revealed that the highest similarity was with that of *F. equiseti*, whereas the β-tubulin sequences of FCC4649 found the highest similarity with that of *F. pseudograminearum.* However, the phylogenetic analyses showed that this taxon represents a unique lineage separate from both *F. equiseti* and *F. pseudograminearum*. In the tree inferred from the combined sequence information, both of these taxa grouped within Clade 4, where isolate FCC4649 formed the most basal group. Consequently, it may represent a hitherto un-described and novel species of *Fusarium*. Strain FCC4649 was mostly isolated from barley soils and stems in the Shoa region in Ethiopia. As such it may represent a species of significance to barley in this region.

F. solani is a complex species composed of 10 *formae speciales* (Matuo and Snyder, 1973) and seven biological species, which could be differentiated using DNA sequence information (Crowhurst *et al.*, 1991; Suga *et al.*, 2000). A separate study (Chapter 3 of this thesis) including representative sequences of the *F. solani-H. haematococca* complex obtained from GenBank has indicated that the Ethiopian *F. solani* isolates used in this study form separate lineages within Clade 3 of the *F. solani-H. haematococca* complex designated by O'Donnell *et al.* (2000a). Morphological identification of this species is relatively easy, although it may be confused with *F. oxysporum*. However, the relatively longer monophialides of *F. solani sensu lato* are quite different from those of *F. oxysporum*.

The *F. oxysporum* complex consists of more than 120 *formae speciales* and races (Armstrong and Armstrong, 1981), and non-pathogenic members that make up a significant component of its genetic diversity. It is resolved into at least three well-supported clades based on DNA sequence information (O'Donnell *et al.*, 1998b; Baayen *et al.*, 2000a). The BLASTN results using *F. oxysporum* sequences had *F. inflexum* among the highest matches. Also, the *F. inflexum* strain included in the phylogenetic analyses grouped within the *F. oxysporum* complex. I have not found reports on the phylogeny of *F. inflexum*. However, the phylogenetic analyses in this study clearly indicated that *F. inflexum* is a member of the *F. oxysporum* complex.

This study adds to the very incomplete information available on *Fusarium* spp. in Ethiopian agriculture by providing an overview of the diversity of species that are present in agricultural soils. It sheds some light on the *Fusarium* species that are found in wheat and barley fields in the Shoa region, and the banana plantations at Arbaminch. It also highlights the phylogenetic relationships among the different *Fusarium* spp. studied. I believe it will serve as a foundation for the further study of *Fusarium* species, particularly from Ethiopia.

Table. 1. *Fusarium* strains included in the study, and accession numbers of EF-1α and β-tubulin sequences downloaded from GenBank.

Isolate	Strain No [a, b]	GenBank Acc. No. (Gene) [c]
F. acutatum	-	AF160276 (EF), U34486 (BT)
F. anthophilum	KSU05007 (BT)	AY222292 (BT), AF160292 (EF)
F. bactridioides	-	AF160290 (EF), U34489 (BT)
F. begoniae	NRRL31851 (EF, BT)	AY329045 (BT), AY329036 (EF)
F. brasilicum	NRRL31281(EF, BT)	AY452964 (EF) AY452956 (BT)
F. brevicatenulatum	-	AF160265 (EF), U61623 (BT)
F. bulbicola	KSU03814 (BT)	AY222287 (BT), AF160294 (EF)
F. cerealis	NRRL13393 (EF)	AF006361 (BT), AF212467 (EF)
F. circinatum	NRRL26432 (EF), MRC7870 (BT)	AF366555 (BT), AF333929 (EF)
F. concentricum	NRRL2994 (EF, BT)	AF333951 (EF), AF333935 (BT)
F. cortaderiae	NRRL29306 (EF, BT)	AY225898 (BT), AY225886 (EF)
F. denticulatum	-	AF160269 (EF), U61628 (BT)
F. dlamini	-	AF160277 (EF), U34485 (BT)
F. flocciferum	VI02440 (EF)	AJ543574 (EF), U85570 (BT)
F. foetens	NRRL31947 (EF, BT)	AY320125 (BT), AY320107 (EF)
F. fractiflexum	NRRL28854 (EF, BT)	AF333932 (EF), AF333948 (BT)
F. globosum	FRC-M8014 (EF)	AY337441 (EF), U61635 (BT)
F. graminearum	NRRL29169 (EF), NRRL26157 (BT)	AF107857 (BT), AF212461 (EF)
F. guttiformae	NRRL22945 (BT)	AY222287 (BT), AF160297 (EF)
F. hostae	NRRL29889 (EF, BT)	AY329042 (BT), AY329034 (EF)
F. inflexum	O-1244 (BT)	AF331804 (BT), AF331814 (EF)
F. lactis	-	AF160272 (EF), U61551 (BT)
F. lateritium Clade 1	FRCL376 (EF, BT)	AY707170 (EF), AY707152 (BT)
F. lateritium Clade 2a	FRCL200 (EF, BT)	AY707168 (EF), AY707150 (BT)
F. lateritium Clade 2b	FRCL120 (EF, BT)	AY707167 (EF), AY707149 (BT)
F. lateritium Clade 3	FRCL112 (EF, BT)	AY707166 (EF), AY707148 (BT)
F. lunulosporum	VI02442 (EF)	AJ543601 (EF), U85571 (BT)
F. napiforme	-	AF160266 (EF), U34428 (BT)
F. nisikadoi	NRRL25183 (EF)	AF324330 (EF), U61633 (BT)
F. o. chrysanthemi	129.81 (CBS), 3460 (FCC)	This study
F. o. conglutinans	186.53 (CBS), 3171 (FCC)	"
F. o. cucurbitacearum	680.89 (CBS), 3461 (FCC)	"
F. o. elaedis	783.83 (CBS). 3184 (FCC)	"
F. o. gladioli	137.97 (CBS), 3173 (FCC)	"
F. o. lini	259.51 (CBS), 3174 (FCC)	"
F. o. lycopersici	413.90 (CBS), 3175 (FCC)	"
F. o. nicotianae	179.32 (CBS), 3186 (FCC)	"

Isolate	Strain No [a, b]	GenBank Acc. No. (Gene) [c]
F. o. niveum	419.90 (CBS), 3177 (FCC)	"
F. o. passiflorae	744.79 (CBS), 3187 (FCC)	"
F. o. perniciosum	794.70 (CBS), 3188 (FCC)	"
F. o. phaseoli	935.73 (CBS), 3178 (FCC)	"
F. o. pisi	127.73 (CBS), 3179 (FCC)	"
F. o. radicis-lycopersici	101587 (CBS), 3180 (FCC)	"
F. o. raphanai	488.76 (CBS), 3181 (FCC)	"
F. o. redolens	489.97 (CBS), 3459 (FCC)	"
F. o. tulipae	195.65 (CBS), 3176 (FCC)	"
F. o. vasinfectum	411.90 (CBS). 3183 (FCC)	"
F. phyllophilum	-	AF160274 (EF), U34487 (BT)
F. proliferatum	KSU12913 (EF, BT)	AY660014 (EF), AY660013 (BT)
F. pseudoanthophilum	-	AF160264 (EF), U61631 (BT)
F. pseudocircinatum	-	AF160271 (EF), U34482 (BT)
F. pseudograminearum	NRRL28338 (EF, BT)	AF212471 (EF), AF107882 (BT)
F. pseudonygamai	NRRL13592 (BT)	U34476 (BT), AF160263 (EF)
F. ramigenum	-	AF160267 (EF), U61554 (BT)
F. redolens	O681(BT, EF)	AF331806 (BT), AF331816 (EF)
F. succisae	KSU03832 (BT)	AF158344 (EF), AY222289 (BT)
F. udum	-	AF160275 (EF), U34488 (BT)
F. venenatum	VI01179 (EF)	U85577 (BT), AJ543635 (EF)

[a] Strain numbers for isolates used. CBS = Centraalbureau voor Schimmelcultures, Utrecth, Netherlands. FCC = *Fusarium* Culture Collection, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

[b] EF and BT, indicate the strain(s) from which the EF-1α and β-tubulin sequences, respectively, were obtained.

[c] GenBank accession number for EF-1α (EF) and β-tubulin (BT) sequences from the respective strain in the second column.

Table 2. Diagnoses of the Ethiopian *Fusarium* isolates using morphology, BLASTN and phylogenetic analyses of EF-1α and the β-tubulin gene sequences.

Isolate	Species ID[a]	EF-1α-based BLASTN result (Fusarium database)				EF-1α-based BLASTN result (GenBank)				β-tubulin-based BLASTN result (GenBank)			
		Top 100 hits[b]	% Sim.[c]	Bit score[d]	E-value	Top 100 hits[b]	% Sim.[c]	Bit score[d]	E-value	Top 100 hits[b]	% Sim.[c]	Bit score[d]	E-value
4639, FP141	*F. dlamini* (M, P)	*F. dlamini* (2)	99	1225	0	*F. dlamini* (1)	97	1178	0	*F. dlamini* (1)	98	551	$3e^{-154}$
		G. fujikuroi (1)	96	1061	0	*F. foetens* (6)	94-95	942	0	*F. oxysporum* (88)	98-97	551-504	$3e^{-145}$–$7e^{-140}$
		F. oxysporum (41)	96-94	983-946	0	*F. fractiflexum* (2)	94	936	0	*F. subglutinans* (11)	97	498	$4e^{-138}$
		F. commune (3)	95	973-955	0	*F. acutatum* (1)	94	932	0				
		F. foetens (18)	95	965	0	*F. oxysporum* (3)	94	930	0				
4638	*F. acutatum* (M, P)	*F. acutatum* (2)	99	1239	0	*F. acutatum* (1)	95	1205	0	*F. acutatum* (1)	99	535	$2e^{-149}$
		G. xylarioides (10)	96-95	993-912	0	*F. udum* (1)	96	965	0	*G. xylarioides* (18)	97-96	504-478	$7e^{-140}$- $4e^{-132}$
		F. udum (2)	96	991	0	*G. xylarioides* (22)	96	965-521	$0\text{-}1e^{-145}$	*F. phyllophilum* (1)	97	504	$7e^{-140}$
		F. phyllophilum (2)	96	955	0	*F. nygamai* (1)	95	944	0	*F. oxysporum* (69)	96	490-472	$1e^{-135}$-$3e^{-130}$
		F. nygamai (1)	94	948	0	*F. phylophilum* (1)	94	938	0	*F. denticulatum* (1)	96	480	$1e^{-132}$
2929, 3697, 3736, 2929, 3677, 3750, 3760, 3803	*F. oxysporum* (M, P)	*F. oxysporum* (100)	99-97	1251-1120	0	*F. oxysporum* (99)	100	1209-1140	0	*F. oxysporum* (88)	99-98	569-541	$1e^{-159}$- $3e^{151}$
						F. inflexum (1)	99	1201	0	*F. inflexum* (1)	99	551	$3e^{-154}$
										F. subglutinans (11)	98	537	$5e^{-150}$
3753, 3786, 3812,	*F. oxysporum* (M, P)	*F. oxysporum* (100)	100-97	1267-1124	0	*F. oxysporum* (99)	100	1209-1140	0	*F. oxysporum* (88)	99-98	569-541	$1e^{-159}$- $3e^{151}$
						F. inflexum (1)	99	1201	0	*F. inflexum* (1)	99	551	$3e^{-154}$
										F. subglutinans (11)	98	537	$5e^{-150}$
3618, 3641, 3725, 3807	*F. oxysporum* (M, P)	*F. oxysporum* (100)	99-97	1241-1124	0	*F. oxysporum* (100)	100	1269-1215	0	*F. oxysporum* (87)	99	549	$1e^{-159}$-$1e^{-153}$
										F. subglutinans (12)	98	545	$2e^{-152}$
										F. commune (1)	98	543	$8e^{-152}$
2930	*F. solani* (M, P)	*N. ipomoeae* (1)	97	1110	0	*N. ipomoeae* (1)	97	1146	0	*P. aleophilum* (1)	95	236	$3e^{-159}$
		F. solani (93)	95-90	1065-676	0	*F. solani* (16)	97	892-295	0	*F. oxysporum* (59)	95-94	228-214	$6e^{-57}$-$4e^{-52}$
		N. africana (2)	95	1086	0	*N. vasinfecta* (6)	96	692-646	0	*F. foetens* (23)	95	224	$1e^{-55}$
		N. vasinfecta (2)	95	1063	0	*N. plagianthi* (1)	96	688	0	*F. subglutinans* (17)	94	220-212	$2e^{-54}$-$4e^{52}$
		N. ornamentata (2)	94	995	0	*N. africana* (1)	96	684	0	*F. proliferatum* (2)	94	220	$2e^{-54}$
3613, 2934, 3809	*F. solani* (M, P)	*N. ipomoeae* (1)	97	1136	0	*N. ipomoeae* (1)	97	1162	0	*P. aleophilum* (1)	94	228	$1e^{-135}$
		F. solani (95)	95-90	1084-458	$0\text{-}e^{-130}$	*F. solani* (21)	97-94	906-424	$0\text{-}8e^{-157}$	*A. chrysogenum* (1)	94	226	$2e^{-56}$
		N. vasinfecta (2)	95	1076	0	*N. plagianthi* (1)	96	704	0	*N. tetrasperma* (1)	93	218	$6e^{-54}$
		N. africana (2)	95	1059	0	*N. vasinfecta* (6)	96	684-654	0	*G. endodonta* (1)	93	218	$6e^{-54}$
										N. calospora (1)	93	218	$6e^{-54}$
3734, 3623, 3686, 2926, 3625, 3670	*F. solani* (M, P)	*N. africana* (2)	97	1146	0	*F. solani* (38)	99-96	1172-406	$0\text{-}3e^{-110}$	*P. aleophilum* (1)	95	236	$3e^{-159}$
		F. solani (94)	97-92	1132-827	0	*N. vasinfecta* (8)	97	1164-1110	0	*F. oxysporum* (59)	95-94	228-214	$6e^{-57}$-$4e^{-52}$
		N. vasinfecta (2)	96	1122	0	*N. borneensis* (1)	95	1144-985	0	*F. foetens* (23)	95	224	$1e^{-55}$
		N. ornamentata (2)	96	1074	0					*F. subglutinans* (17)	94	220-212	$2e^{-54}$-$4e^{-52}$
										F. proliferatum (2)	94	220	$2e^{-54}$
4635, 3672	*F. solani* (M, P)	*N. ipomoeae* (1)	97	1132	0	*N. ipomoeae* (1)	97	1160	0	*A. chrysogenum* (1)	95	242	$4e^{-61}$
		F. solani (87)	95-91	1080-454	$0\text{-}e^{-129}$	*F. solani* (21)	97-95	904-391	$0\text{-}6e^{-161}$	*M. camptospora* (1)	94	230	$2e^{-57}$
		N. africana (2)	95	1076	0	*N. plagianthi* (1)	96	710	0	*P. aleophilum* (1)	94	228	62^{-57}
		N. vasinfecta (2)	95	1053	0	*N. vasinfecta* (6)	96	682-658	0	*C. sorghicola* (4)	94	228	$6e^{-57}$
		N. ornamentata (2)	93	985	0					*N. radicicola* (25)	94-93	226-218	$2e^{-56}$-$6e^{-54}$

Isolate	Species ID[a]	EF-1α-based BLASTN result (Fusarium database)				EF-1α-based BLASTN result (GenBank)				β-tubulin-based BLASTN result (GenBank)			
		Top 100 hits[b]	% Sim.[c]	Bit score[d]	E-value	Top 100 hits[b]	% Sim.[c]	Bit score[d]	E-value	Top 100 hits[b]	% Sim.[c]	Bit score[d]	E-value
3735, 3695,	*F. solani* (M, P)	*N. ipomoeae* (1)	97	1140	0	*N. ipomoeae* (1)	97	1168	0	*P. aleophilum* (1)	94	228	$1e^{-135}$
3723, 3816		*F. solani* (38)	95-93	1104-404	$0\text{-}e^{-114}$	*F. solani* (20)	97-96	912-391	$0\text{-}2e^{-105}$	*A. chrysogenum* (1)	94	226	$2e^{-56}$
		N. vasinfecta (2)	95	1102	0	*N. plagianthi* (1)	96	718	0	*N. tetrasperma* (1)	93	218	$6e^{-54}$
		N. africana (2)	95	1076	0	*N. vasinfecta* (6)	96	690-658	0	*G. endodonta* (1)	93	218	$6e^{-54}$
		N. ornamentata (2)	94	1013	0					*N. calospora* (1)	93	218	$6e^{-54}$
3815	*F. solani* (M, P)	*F. solani* (85)	98-90	1259-460	$0\text{-}e^{-131}$	*H. haematococca* (31)	99-94	1259-345	$0\text{-}9e^{-92}$	*P. aleophilum* (1)	95	236	$3e^{-59}$
		N. vasinfecta (2)	95	1031	0	*N. vasinfecta* (7)	95	1055-979	0	*A. chrysoginum* (1)	95	234	$1e^{-58}$
		N. africana (2)	94	1023	0	*N. borneensis* (1)	94	1051	0	*F. oxysporum* (46)	94-93	228-214	$6e^{-57}\text{-}1e^{-52}$
		N. ornamentata (2)	94	944	0	*N. africana* (1)	94	1037	0	*F. foetens* (18)	94	224	$1e^{-55}$
		N. ipomoeae (2)	92	884	0					*M. camptospora* (1)	93	222	$4e^{-55}$
4640, 4644	*F. oxysporum* (M)	*F. redolens* (31)	100-99	1298-1251	0	*F. redolens* (36)	100	1298-1084	0	*F. oxysporum* (89)	100-96	575-486	$2e^{-161}\text{-}2e^{-134}$
	F. redolens (P)	*F. hostae* (15)	97	1154-1090	0	*F. oxysporum* (37)	99	1237-410	$0\text{-}2e^{-111}$	*F. redolens* (4)	100	549-529	$1e^{-153}\text{-}1e^{147}$
		F. polyphialidicaum (2)	89	632	0	*F. hostae* (14)	97	1160-1096	0	*F. hostae* (7)	100	549	$1e^{-153}$
		F. commune (6)	92	448-478	e^{-127}	*F. concentricaum* (1)	96	418	$8e^{-114}$				
		F. oxysporum (40)	92-91	408-333	$e^{-127}\text{-}1e^{-92}$								
4647, 4645	*F. avenaceum* (M, P)	*F. avenaceum* (4)	99-97	1237-311	$4e^{-86}$	*F. avenaceum* (26)	99	1247-739	0	*F. dlamini* (1)	100	569-541	$1e^{-159}\text{-}3e^{151}$
		F. acuminata (1)	97	1128	0	*F. arthrosporioides* (25)	99	1239-1215	0	*F. oxysporum* (88)	97-96	551	$3e^{-154}$
		F. negundis (1)	95	1049	0	*F. tricinctum* (11)	96	1070-1049	0	*F. subglutinans* (11)	96	537	$5e^{-150}$
		F. reticulatum (1)	95	1049	0	*F. flocciferum* (3)	95	1045-975	0				
		F. tricinctum (1)	95	1031	0	*F. torulosum* (15)	95	1035-1003	0				
4637	*F. lactis* (M, P)	*G. fujikuroi* (1)	98	1178	0	*F. lactis* (1)	98	1116	0	*F. nygamai* (1)	100	551	$3e^{-154}$
		F. lactis (2)	98	1170	0	*F. nygamai* (1)	96	1047	0	*F. denticulatum* (1)	99	535	$2e^{-149}$
		F. nygamai (1)	96	1061	0	*F. napiformae* (1)	96	1037	0	*F. brevicatenulatum* (1)	99	535	$2e^{-149}$
		F. napiformae (1)	96	1051	0	*F. ramigenum* (1)	96	1007	0	*F. pseudonygamai* (1)	98	527	$5e^{-147}$
		F. brevicatenulatum (1)	95	1037	0	*F. brevicatenulatum* (1)	95	1003	0	*G. moniliformis* (1)	98	523	$8e^{-146}$
4649	*Fusarium* sp. (M, P)	*F. equiseti* (4)	98-94	1144-965	0	*F. equiseti* (15)	98-93	1148-858	0	*G. pulicaris* (3)	93	420-394	$8e^{-115}\text{-}5e^{-107}$
		F. scripi (2)	94	856	0	*F. pseudograminearum* (4)	93	365-325	$1e^{-97}\text{-}9e^{-86}$	*F. pseudograminearum* (18)	93-91	404-353	$5e^{-110}\text{-}2e^{-94}$
		F. pallidoroseum (2)	91	755	0	*G. moniliformis* (9)	97	335-327	$9e^{-89}\text{-}2e^{-86}$	*G. zeae* (40)	92	396-361	$1e^{-107}\text{-}7e^{-97}$
		F. camptoceras (1)	91	696	0	*F. kyushuense* (1)	97	335	$9e^{-89}$	*F. cerealis* (7)	92	396-355	$1e^{-107}\text{-}4e^{-95}$
		F. musarum (1)	91	341	$4e^{-95}$	*F. venenatum* (5)	97	333	$3e^{-88}$	*F. venenatum* (11)	92	394	$5e^{-107}$

[a] Species identified based on morphology (M) (Nelson *et al.*, 1983) or phylogeny (P).
[b] The first five species in a list of the top 100 matches obtained using BLASTN analyses (numbers in parentheses indicate the number of hits for the particular species).
[c] The percentage similarity in nucleotide sites between the query sequence and that of the corresponding strain.
[d] The bit score (S') between two sequences is given as (λS-lnK)/ln2, where K and λ are the normalized scales for the search space size and the scoring system, respectively (Altschul *et al.*, 1990).
[e] The e-value indicates the expected number of high-scoring segment pairs (HSPs) with a bit score of at least S'. The e-value corresponding to a given bit score is calculated as: $e = mn\ s^{-S'}$, where m and n are the lengths of the two sequences being compared (Altschul *et al.*, 1990).

Note that *A. chrysogenum* belongs to *Aleophilum*; *C. sorghicola* belongs to *Claviceps*; *G. fujikuroi, G. moniliformis* and *G. zeae* belong to *Gibberella; G. endodonata* belongs to *Gelasinospora*; *M. camptospora* belongs to *Mariannae*; *N. ipomoeae* and *N. plagianthi* belong to *Nectria*; *N. africana*, *N. ornamentata* and *N. vasinfecta* belong to *Neocosmospora*; *N. radicola* belongs to *Neonectria*; *N. terasperma* and *N. calospora* belong to *Neurospora; P. aleophilum* belongs to *Pheaoacremonium*; and the rest of the species belong to *Fusarium.*

Table 3. *Fusarium* spp. isolated from agricultural soil samples categorized according to the previous crop on the farm from which the soil samples were collected.

Type of farm	**No. of farms**	***Fusarium* spp. isolated [a]**	**Total No. of isolates**
'Tef'	25	*F. oxysporum* (9), *F. solani* (10), *F. dlamini* (10), *F. redolens* (5), *Fusarium* sp. (5), *F. avenaceum* (6) , *F. lactis* (6), *F. equiseti* (4)	55
Barley	12	*F. oxysporum* (4), *F. redolens* (7), *F. solani* (15), *F. dlamini* (10), *Fusarium* sp. (4), *F. equiseti* (2)	42
Maize	16	*F. oxysporum* (5), *F. acutatum* (6), *F. solani* (7), *Fusarium* sp. (7), *F. equiseti* (2)	27
Sorghum (*Sorghum bicolor* [L.] Moench)	13	*F. oxysporum* (4), *F. avenaceum* (5), *F. solani* (4), *F. redolens* (9), *F. acutatum* (4)	26
Wheat	8	*F. oxysporum* (8), *F. solani* (2), *F. redolens* (2), *F. acutatum* (6)	18
Pulses	6	*F. oxysporum* (2), *F. solani* (6), *F. redolens* (7)	15

[a] Numbers in parentheses show number of isolates.

Table 4. Chi-square test of association between *Fusarium* spp. isolated from soil samples and type of farm (by crop) from which the soil samples were collected.

	Percentage contribution of farm type to the total number of soil isolates		
Type of farm	*Fusarium* spp.	*F. oxysporum*	*F. solani*
'Tef'	30.05	28.13	22.73
Maize	14.75	15.62	15.90
Sorghum	14.21	12.50	9.09
Barley	22.95	12.50	34.09
Wheat	9.84	25.00	4.55
Pulses	8.20	6.25	13.64
X^2 value	0.000	0.003	0.007

Table 5. Sequence alignment phylogenetic tree characteristics based on the individual and combined EF-1α and β-tubulin partitions.

Partition	Aligned positions [a]	Constant positions [b]	Pars. info. sites [c]	MPT [d]	Tree scores [e]	BS ≥ 50% [f]
EF-1α	679	271 39.9%	331 48.75%	1276	L = 1308, CI = 0.531, RI = 0.885, RC = 0.469	60
β-tubulin	295	174 58.9%	74 25.1%	295	L = 298, CI = 0.597, RI = 0.901, RC = 0.538	32
Combined	974	445 45.7%	405 41.6%	1528	L = 1633, CI = 0.534, RI = 0.884, RC = 0.472	65

[a] Number of aligned sites including gaps.

[b] Number and percentage of constant sites.

[c] Number and percentage of parsimony informative sites.

[d] Number of most parsimonious trees.

[e] Tree scores (L = tree length, CI = consistency index, RI = retention index, RC = rescaled consistency index).

[f] Number of nodes with bootstrap values of 50% or higher.

[h] Number of haplotypes (types of identical sequences).

Fig. 1. Routes followed during soil sample collection, and locations of the wheat and barley (Shoa, encircled), and banana (Arbaminch) farms visited.

Fig. 2. Primer maps for the β-tubulin (A) and EF-1α (B) gene sequences used in this study. Arrows indicate binding sites of the primers 2A and 2B (for β-tubulin) and EF1 and EF2 (for EF-1α). These maps are based on the β-tubulin and EF-1α gene sequences for *F. oxysporum* and correspond to amino acid (aa) positions 1-86 of its EF-1α sequence (AY450432.1) and aa positions 16-82 of its β-tubulin sequence (AF008537). Introns (open boxes) are indicated with TI (for β-tubulin) and EI (for EF-1α), whereas exons are indicated with shaded boxes. Numbers inside the boxes indicate the length of exons (aa) and introns (number of nucleotides and gaps in the aligned sequences).

Fig. 3. One of 1528 most parsimonious trees inferred from the combined EF-1α and β-tubulin partitions using 42 *Fusarium* spp. Bootstrap values of 70% and higher are indicated. Ethiopian isolates are indicated by 'Eth', whereas those obtained from the CBS are indicated by 'CBS'. The remaining are strains for which sequence information was obtained from GenBank. *Cylindrocladium pacificum* was used as an outgroup taxon.

Fig. 4. One of 1270 most parsimonious trees inferred from the EF-1α partition using 42 *Fusarium* spp. Bootstrap values of 70% and higher are indicated. Ethiopian isolates are indicated by 'Eth', whereas those obtained from the CBS are indicated by 'CBS'. The remaining are strains for which sequence information was obtained from GenBank. *Cylindrocladium pacificum* was used as an outgroup taxon.

Fig. 5. One of 295 most parsimonious trees inferred from the b-tubulin partition using 42 *Fusarium* spp. Bootstrap values of 70% and higher are indicated. Ethiopian isolates are indicated by 'Eth', whereas those obtained from the CBS are indicated by 'CBS'. The remaining are strains for which sequence information was obtained from GenBank. *Cylindrocladium pacificum* was used as an outgroup taxon.

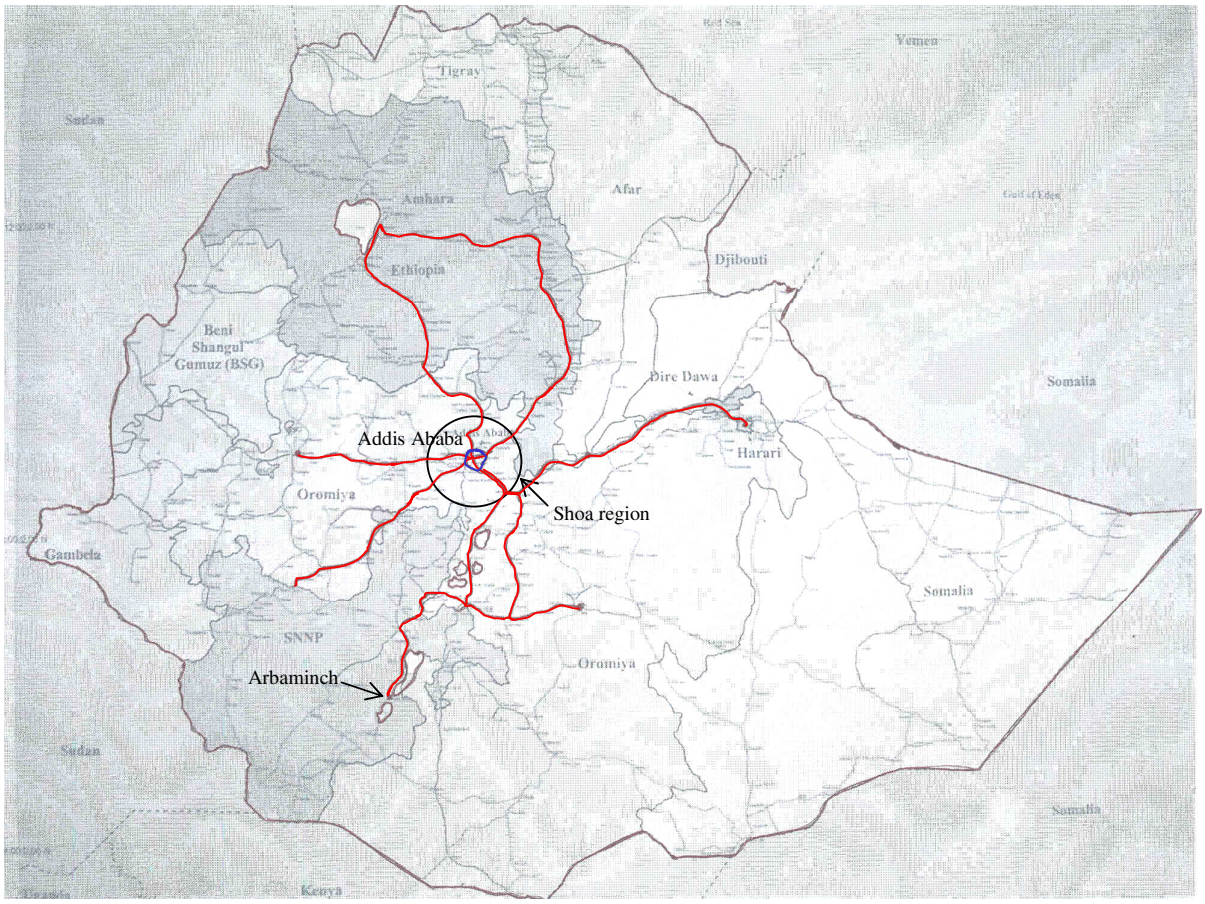

Red Sea
Yemen
Tigray
Sudan
Afar
Amhara
Djibouti
Ethiopia
Beni
Shangul
Gumuz (BSG)
Dire Dawa
Somalia
Addis Ababa
Harari
Oromiya
Shoa region
Gambela
Somalia
SNNP
Oromiya
Arbaminch
Sudan
Somalia
Kenya

Fig. 1.

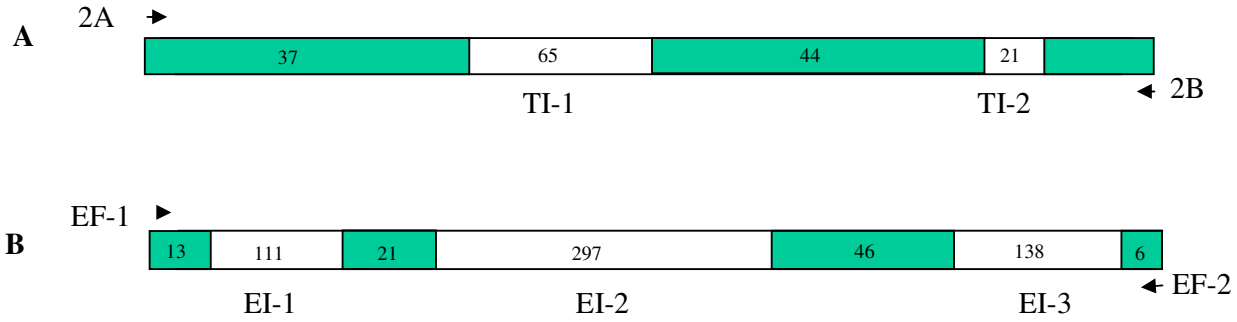
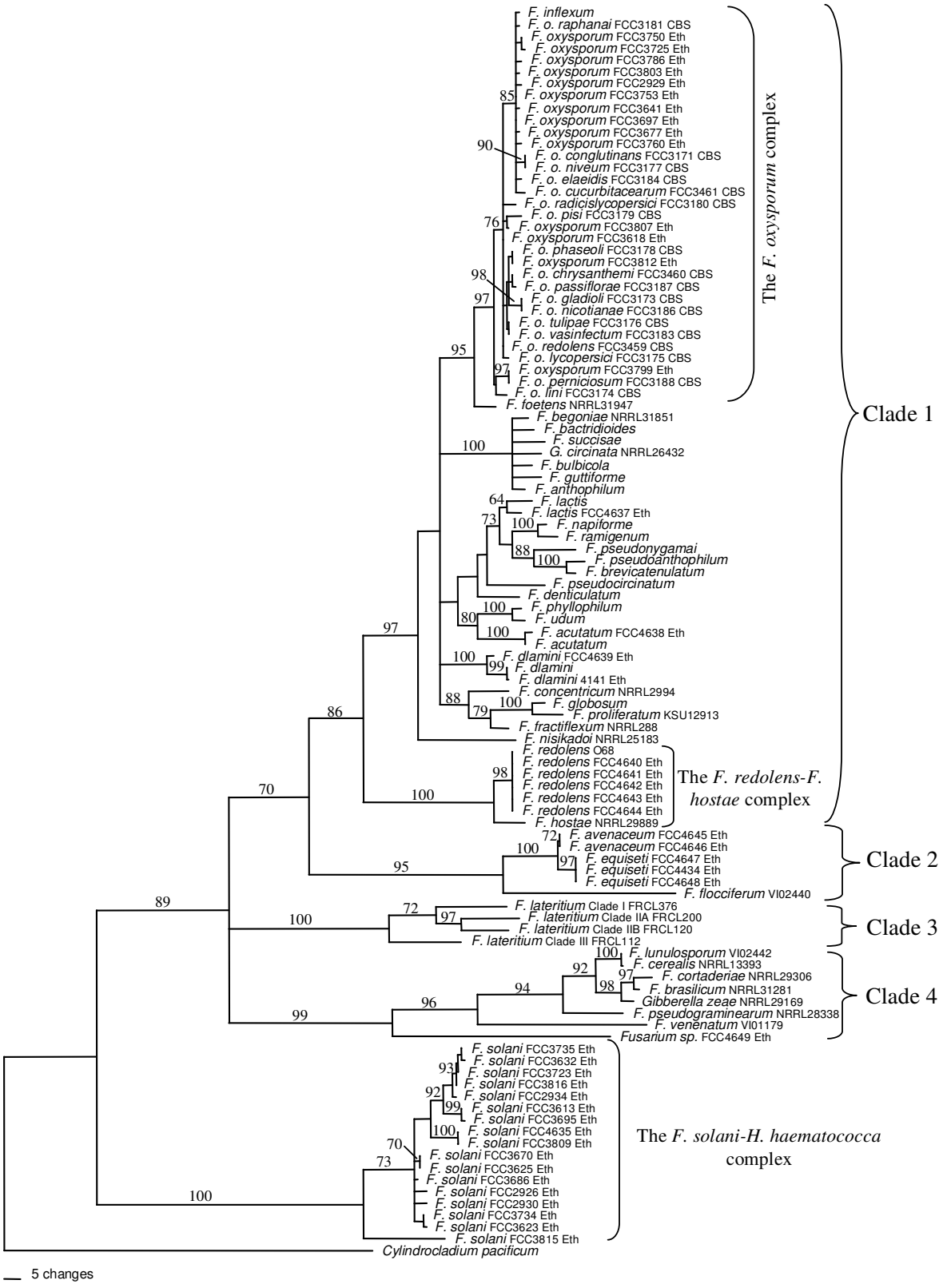

A
2A
37
65
44
21
2B
TI-1
TI-2
B
EF-1
13
111
21
297
46
138
6
EF-2
EI-1
EI-2
EI-3

Fig. 2.


F. inflexum
F. o. raphanai FCC3181 CBS
F. oxysporum FCC3750 Eth
F. oxysporum FCC3725 Eth
F. oxysporum FCC3786 Eth
F. oxysporum FCC3803 Eth
F. oxysporum FCC2929 Eth
F. oxysporum FCC3753 Eth
F. oxysporum FCC3641 Eth
F. oxysporum FCC3697 Eth
F. oxysporum FCC3677 Eth
F. oxysporum FCC3760 Eth
F. o. conglutinans FCC3171 CBS
F. o. niveum FCC3177 CBS
F. o. elaeidis FCC3184 CBS
F. o. cucurbitacearum FCC3461 CBS
F. o. radicislycopersici FCC3180 CBS
F. o. pisi FCC3179 CBS
F. oxysporum FCC3807 Eth
F. oxysporum FCC3618 Eth
F. o. phaseoli FCC3178 CBS
F. oxysporum FCC3812 Eth
F. o. chrysanthemi FCC3460 CBS
F. o. passiflorae FCC3187 CBS
F. o. gladioli FCC3173 CBS
F. o. nicotianae FCC3186 CBS
F. o. tulipae FCC3176 CBS
F. o. vasinfectum FCC3183 CBS
F. o. redolens FCC3459 CBS
F. o. lycopersici FCC3175 CBS
F. oxysporum FCC3799 Eth
F. o. perniciosum FCC3188 CBS
F. o. lini FCC3174 CBS
F. foetens NRRL31947
The F. oxysporum complex
F. begoniae NRRL31851
F. bactridioides
F. succisae
G. circinata NRRL26432
F. bulbicola
F. guttiforme
F. anthophilum
F. lactis
F. lactis FCC4637 Eth
F. napiforme
F. ramigenum
F. pseudonygamai
F. pseudoanthophilum
F. brevicatenulatum
F. pseudocircinatum
F. denticulatum
F. phyllophilum
F. udum
F. acutatum FCC4638 Eth
F. acutatum
F. dlamini FCC4639 Eth
F. dlamini
F. dlamini 4141 Eth
F. concentricum NRRL2994
F. globosum
F. proliferatum KSU12913
F. fractiflexum NRRL288
F. nisikadoi NRRL25183
F. redolens O68
F. redolens FCC4640 Eth
F. redolens FCC4641 Eth
F. redolens FCC4642 Eth
F. redolens FCC4643 Eth
F. redolens FCC4644 Eth
F. hostae NRRL29889
The F. redolens-F. hostae complex
Clade 1
F. avenaceum FCC4645 Eth
F. avenaceum FCC4646 Eth
F. equiseti FCC4647 Eth
F. equiseti FCC4434 Eth
F. equiseti FCC4648 Eth
F. flocciferum VI02440
Clade 2
F. lateritium Clade I FRCL376
F. lateritium Clade IIA FRCL200
F. lateritium Clade IIB FRCL120
F. lateritium Clade III FRCL112
Clade 3
F. lunulosporum VI02442
F. cerealis NRRL13393
F. cortaderiae NRRL29306
F. brasilicum NRRL31281
Gibberella zeae NRRL29169
F. pseudograminearum NRRL28338
F. venenatum VI01179
Fusarium sp. FCC4649 Eth
Clade 4
F. solani FCC3735 Eth
F. solani FCC3632 Eth
F. solani FCC3723 Eth
F. solani FCC3816 Eth
F. solani FCC2934 Eth
F. solani FCC3613 Eth
F. solani FCC3695 Eth
F. solani FCC4635 Eth
F. solani FCC3809 Eth
F. solani FCC3670 Eth
F. solani FCC3625 Eth
F. solani FCC3686 Eth
F. solani FCC2926 Eth
F. solani FCC2930 Eth
F. solani FCC3734 Eth
F. solani FCC3623 Eth
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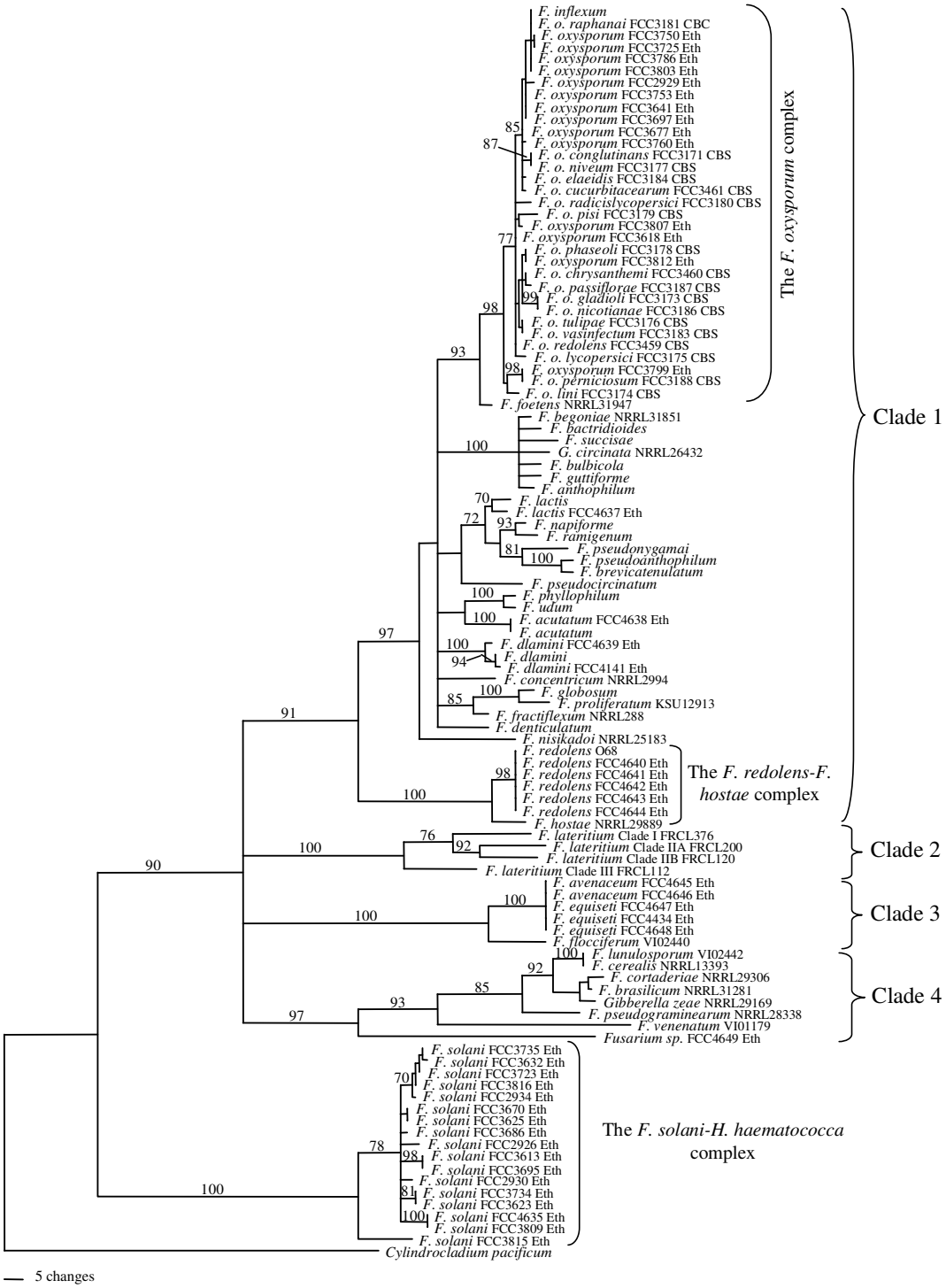
The F. solani-H. haematococca complex
Cylindrocladium pacificum
5 changes

Fig. 3.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F. oxysporum FCC3786 Eth
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F. oxysporum FCC2929 Eth
F. oxysporum FCC3753 Eth
F. oxysporum FCC3641 Eth
F. oxysporum FCC3697 Eth
F. oxysporum FCC3677 Eth
F. oxysporum FCC3760 Eth
F. o. conglutinans FCC3171 CBS
F. o. niveum FCC3177 CBS
F. o. elaeidis FCC3184 CBS
F. o. cucurbitacearum FCC3461 CBS
F. o. radicislycopersici FCC3180 CBS
F. o. pisi FCC3179 CBS
F. oxysporum FCC3807 Eth
F. oxysporum FCC3618 Eth
F. o. phaseoli FCC3178 CBS
F. oxysporum FCC3812 Eth
F. o. chrysanthemi FCC3460 CBS
F. o. passiflorae FCC3187 CBS
F. o. gladioli FCC3173 CBS
F. o. nicotianae FCC3186 CBS
F. o. tulipae FCC3176 CBS
F. o. vasinfectum FCC3183 CBS
F. o. redolens FCC3459 CBS
F. o. lycopersici FCC3175 CBS
F. oxysporum FCC3799 Eth
F. o. perniciosum FCC3188 CBS
F. o. lini FCC3174 CBS
F. foetens NRRL31947
The F. oxysporum complex
F. begoniae NRRL31851
F. bactridioides
F. succisae
G. circinata NRRL26432
F. bulbicola
F. guttiforme
F. anthophilum
F. lactis
F. lactis FCC4637 Eth
F. napiforme
F. ramigenum
F. pseudonygamai
F. pseudoanthophilum
F. brevicatenulatum
F. pseudocircinatum
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F. denticulatum
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F. hostae NRRL29889
The F. redolens-F. hostae complex
Clade 1
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F. lateritium Clade IIB FRCL120
F. lateritium Clade III FRCL112
Clade 2
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F. flocciferum VI02440
Clade 3
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Gibberella zeae NRRL29169
F. pseudograminearum NRRL28338
F. venenatum VI01179
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Clade 4
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F. solani FCC3686 Eth
F. solani FCC2926 Eth
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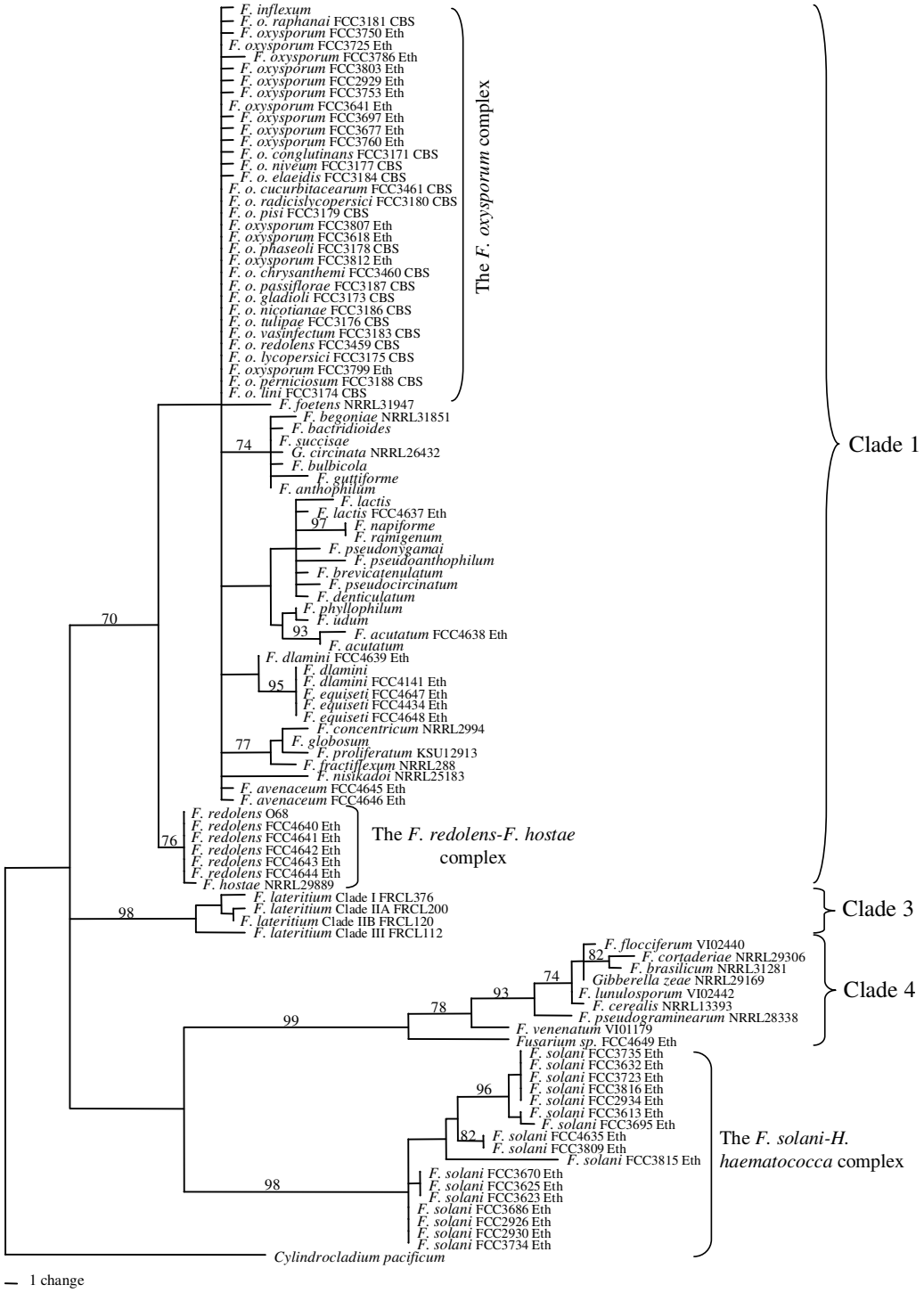
F. solani FCC3623 Eth
F. solani FCC4635 Eth
F. solani FCC3809 Eth
F. solani FCC3815 Eth
The F. solani-H. haematococca complex
Cylindrocladium pacificum
5 changes

Fig. 4.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F. oxysporum FCC2929 Eth
F. oxysporum FCC3753 Eth
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F. o. conglutinans FCC3171 CBS
F. o. niveum FCC3177 CBS
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F. oxysporum FCC3812 Eth
F. o. chrysanthemi FCC3460 CBS
F. o. passiflorae FCC3187 CBS
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F. o. nicotianae FCC3186 CBS
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F. o. vasinfectum FCC3183 CBS
F. o. redolens FCC3459 CBS
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F. oxysporum FCC3799 Eth
F. o. perniciosum FCC3188 CBS
F. o. lini FCC3174 CBS
The F. oxysporum complex
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F. begoniae NRRL31851
F. bactridioides
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F. solani FCC3734 Eth
The F. solani-H. haematococca complex
Cylindrocladium pacificum
1 change
Clade 1
Clade 3
Clade 4
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Fig. 5

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CHAPTER 3

Characterization of *Fusarium solani* isolates from Ethiopia using Amplified Fragment Length Polymorphism (AFLP) and DNA sequence information

ABSTRACT

Fusarium solani is an important fungal pathogen that causes diseases on at least 87 genera and many hundreds of species of plants. This fungus represents one of the two *Fusarium* species most commonly isolated from agricultural soils and plant tissues in Ethiopia. Forty-three Ethiopian *F. solani* isolates were studied using Amplified Fragment Length Polymorphism (AFLP) and nucleotide sequences of the translation Elongation Factor 1α (EF-1α) and β-tubulin genes. Phylogenetic analyses of the EF-1α sequence data aggregated all the Ethiopian isolates in a strongly supported group. This group correlated with Clade 3 of the *F. solani-Haematonectria haematococca* species complex that has been characterized previously. Within this clade, the Ethiopian *F. solani* isolates separated into six well-supported lineages, corresponding with the six lineages that emerged from the AFLP analyses. AFLPs further separated the six lineages into two clusters. The presence of three β-tubulin nucleotide sites that were fixed differently between the two clusters also supported the separation of these clusters. However, the genetic differentiation between the two clusters was small. Taken together, these data suggest that the examined Ethiopian *F. solani* isolates represent two different entities that are either in the process of separating into two different species or groups that have recently converged into a single species.

INTRODUCTION

Fusarium solani (Mart.) Appel & Wollenweber. emend. Snyd. & Hans. (teleomorph *Haematonectria haematococca* (Berk. & Broome) Samuels & Nirenberg) is a widely distributed soil-borne fungus that is pathogenic to at least 111 plant species spanning 87 genera (Kolattukudy and Gamble, 1995). Amongst the many diseases caused by this fungus, Sudden Death Syndrome (SDS) of soybean (*Glycine max*) is one of the most economically important (Achenbach *et al.*, 1996; 1997; Roy, 1997; Anderson and Tenuta, 1998; Aoki *et al.*, 2003). This disease has resulted in as much as 90% yield loss in some areas of the ten top soybean-growing countries of the world (Wrather *et al.*, 1994). *F. solani* also causes wilt and rot diseases in a wide variety of crops (Campbell and Collins, 1987; Hawthorne *et al.*, 1992; Li *et al.*, 1995). Moreover, the fungus has medical and/or veterinary significance since some isolates have been implicated in infections of the nails (Baran *et al.*, 1997) and the skin, especially in immuno-compromised individuals (Leu *et al.*, 1995; Girardi *et al.*, 1999; Gupta *et al.*, 2000). Furthermore, some *F. solani* isolates have been shown to be pathogenic to some crustaceans (Van Etten and Kistler, 1988).

F. solani resides in the section *Martiella* (Wollenweber and Reinking, 1935), which may also include other *Fusarium* spp. depending on the taxonomic system used. For example, Booth (1971) recognized four species in this section, whereas Wollenweber and Reinking (1935), and Gerlach and Nirenberg (1982) recognized five and six species, respectively. However, Nelson *et al.* (1983) and Snyder and Hansen (1941) recognized only *F. solani* in this section. Routine identification of this species is, therefore, complicated and is typically accomplished using morphology, host-specificity, sexual compatibility, DNA sequence analyses or combinations of these characters.

Morphologically, *F. solani* is identified by the shape of its macroconidia, chlamydospores and the presence of long phialides. However, the shape of the macroconidia can vary. For example, macroconidia of isolates from temperate regions are usually 'sausage-shaped' (wide, slightly curved and with rounded apical cells), whereas macroconidia of isolates from the tropics are narrower and longer with more distinct basal cells (Burgess *et al.*, 1994). Macroconidial morphology also varies with culture conditions (Matuo and Snyder, 1973). The most important limitations of morphological identification of *F. solani*, however, lie in the inability to distinguish among the different biological species, and the presence of genetically diverse mitotic strains sharing common morphological features.

Based on host-specificity, *F. solani* has been divided into 10 *formae speciales* (Matuo and Snyder, 1973), of which *forma specialis cucurbitae* contains two distinct races (Toussoun and Snyder, 1961). *Formae speciales* and races are determined based on pathogenicity to specific hosts. However, pathogenicity tests are cumbersome, time consuming and often inconclusive because they are affected by factors such as the environment and genetic makeup of the host species (Kraft and Haglund, 1978; Armstrong and Armstrong, 1981; Hart and Endo, 1981; Correll, 1991). Moreover, virulence of a strain can be lost, resulting in lack of repeatability of experiments (Windels, 1991).

The two races of *F. solani* f.sp. *cucurbitae*, and five of the remaining nine *formae speciales* are heterothallic having *N. haematococca* Berk. et Br. as the teleomorph. All seven of the teleomorph states are reproductively isolated and each represents a distinct biological species (designated as mating population [MP] I – VI, Matuo and Snyder, 1973; Van Etten and Kistler, 1988). Isolates are grouped into these MPs using mating tests. However, like pathogenicity tests, mating tests are also unwieldy and time consuming (Kraft and Haglund, 1978; Armstrong and Armstrong, 1981; Hart and Endo, 1981; Correll, 1991), and only positive mating tests provide guaranteed identification (Matuo and Snyder, 1973).

To circumvent the shortcomings associated with classifications based on morphology, pathogenicity, and mating tests, various molecular tools have been employed to characterize *F. solani* isolates. Of these methods, analysis of DNA sequences appears to be the most informative. For example, based on sequence data for the 28S ribosomal DNA (rDNA), the Internal Transcribed Spacer (ITS) region, and translation Elongation Factor 1α (EF-1α), O'Donnell (2000) recognized more than 26 "phylogenetic species" in the *F. solani-H. haematococca* species complex. Other DNA-based techniques that have been used for classifying isolates of *F. solani* include, Random Amplified Polymorphic DNA (RAPD) (Crowhurst *et al.*, 1991), Restriction Fragment Length Polymorphism (RFLP) of PCR amplified products (PCR-RFLP) (Suga *et al.*, 2000), and karyotyping (Nazareth and Bruschi, 1994; Taga *et al.*, 1998; Suga *et al.*, 2002). Amplified Fragment Length Polymorphism (AFLP, Vos *et al.*, 1995), has been used in the taxonomy and phylogeny of various *Fusarium* spp. (Majer *et al.*, 1996; Baayen *et al.*, 2000; Kiprop *et al.*, 2002; Abdel-Satar *et al.*, 2003). AFLP offers two major advantages over the other DNA-based tools most often used in taxonomic and phylogenetic studies of *F. solani*. Firstly, it samples widely in the genome rather than considering specific regions as is the case with nucleotide sequence and PCR-RFLP analyses (Majer *et al.*, 1996; Baayen *et al.*, 2000). Secondly, AFLP is highly reproducible, which is unlike RAPD analyses (Vos *et al.*, 1995). Despite these advantages, AFLP has not been previously used in the *F. solani* complex.

The occurrence of *Fusarium* spp. in general and *F. solani* in particular, has not been well documented in Ethiopian agriculture. The only detailed account of *Fusarium* spp. was made by Bekele and Karr (1997), where more than 19 different species including *F. solani* were listed associated with head blight and various other diseases of stored seeds of wheat. However, a recent study (Chapter 2 of this thesis) has shown that *F. solani* is one of the two *Fusarium* spp. most commonly occurring in agricultural soils and plant tissues in Ethiopia. The objective of this study was, therefore, to assess phylogenetic relationships and genetic diversity of *F. solani* isolates obtained from agricultural soils and plant tissues from Ethiopia using AFLP and nucleotide sequence analyses.

MATERIALS AND METHODS

Isolates

The 43 isolates of *F. solani* included in this study (Table 1) were obtained from agricultural soils and plant tissues collected from Ethiopia as part of a previous study (Chapter 2 of this thesis). *F. oxysporum* f.sp. *dianthi* (CBS No. 491.97) obtained from the Centraalbureau voor Schimmelcultures (CBS), Utrecht, Netherlands, was included as an outgroup taxon in sequence analyses. All isolates used in this study are maintained in the *Fusarium* Culture Collection (FCC) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

DNA extraction and sequencing

DNA was extracted from isolates using the CTAB (N-cetyl-N,N,N-trimethyl-ammonium bromide) method (Murray and Thompson, 1980). For this purpose, isolates were grown on malt extract agar medium (2% [w/v] malt extract and 1.5% [w/v] agar) in Petri dishes for 7-10 days at 25°C. Mycelium was then scraped from the surface of cultures and transferred to Eppendorf tubes containing sterile sand and 700 µL extraction buffer. The extraction buffer consisted of 5% (w/v) CTAB, 1.4 M NaCl, 0.2% (v/v) 2-mercaptoethanol, 20 mM EDTA (ethylene diamine tetraacetate, pH 8.0), 10 mM Tris-HCl (pH 8.0), and 1% (w/v) PVP (polyvinylpyrrolidone). The mixture was ground; incubated for 1 hr at 60°C, and centrifuged at 9,300 *g* for 10 min. DNA was extracted from the aqueous phase by repeated phenol-chloroform (1:1) extractions followed by a final chloroform extraction to remove residual phenol. The DNA was then precipitated with 0.1 volume of 3 M sodium acetate (pH 5.4) and 0.6 volume of 2-propanol, and incubated at –20°C overnight.

Precipitated DNA was pelleted by centrifugation at 15,700 *g* for 30 min at 4°C, washed with 70% ethanol, air-dried, and dissolved in deionised water.

EF-1α (~ 656 base pair [bp]) and β-tubulin (~ 290 bp) gene fragments were PCR-amplified using primer pairs EF1 and EF2 (O'Donnell *et al.*, 1998), and 2A and 2B (Glass and Donaldson, 1995), respectively. PCR mixtures contained reaction buffer (10 mM Tris-HCl [pH 8.3], 2.5 mM $MgCl_2$, 50 mM KCl), 2.5 µM of each dNTP, 0.20 µM of each primer, 0.05 U/µL *Taq* Polymerase (Roche, USA), and approximately 4 ng/µL template DNA. The PCR cycling conditions consisted of an initial denaturation at 94°C for 4 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing for 30 sec at 56°C (for EF1 and EF2) or 68°C (for 2A and 2B), and extension at 72°C for 30 sec. PCRs were terminated after a final extension at 72°C for 10 min. PCR products were purified using the QIAquick PCR purification kit (QIAGEN, GmbH, Germany), and sequenced in both directions using the same primers as for the respective PCRs. For this purpose, the BigDye terminator sequencing kit (Version 3.2, Applied Biosystems, USA) and an ABI PRISM™ 3100 DNA sequencer (Applied Biosystems) were used. All amplification and sequencing reactions were performed on a GeneAmp PCR System 9700 (Applied Biosystems).

Gene sequences were assembled using Sequence Navigator (Version 1.0.1, Applied Biosystems), and aligned using ClustalX (Version 1.8, Thompson *et al.*, 1997). Sequence navigator was also used to infer amino acid sequences from nucleotide sequences. The amino acid sequences were then compared with relevant amino acid sequences in the NCBI (National Center for Biotechnology Information; www.ncbi.nih.gov) database (GenBank) to determine the presence and positions of introns.

Sequence analyses

EF-1α sequences of strains representing the three clades of the *F. solani-H. haematococca* species complex (O'Donnell, 2000), as well as other *F. solani* isolates, were downloaded from GenBank and included in this study (Table 2). PAUP (Phylogenetic Analysis Using Parsimony, Version 4.0b 10, Swofford, 2002) was used to estimate phylogenetic relationships. For heuristic searches, 1,000 random additions of sequences and tree bisection-reconstruction were used with branch swapping only on best trees. All characters were weighted equally and alignment gaps were treated as missing data. Bootstrap analyses (Hillis and Bull, 1993) were performed using 1,000 replications.

AFLP analyses

For AFLP analyses (Vos *et al.*, 1995), genomic DNA (~100 ng) was digested overnight at 37°C with 0.07 U/µL each of endonucleases *Eco*RI (Roche) and *Mse*I (New England Biolabs, Mass.), using a restriction-ligation buffer composed of 10 mM Tris-acetate (pH 7.5), 10 mM magnesium acetate, 50 mM potassium acetate, 5 mM dithiothreitol, and bovine serum albumin at 50 ng/µL. *Eco*RI adaptor (5'-CTC GTA GAC TGC GTA CC-3'/3'-CAT CTG ACG CAT GGT TAA-5') and *Mse*I adaptor (5'-GAC GAT GAG TCC TGA G-3'/3'-TA CTC AGG ACT CAT-5') were ligated to ends of the digested DNA fragments at 37°C for 3 hrs. The ligation mixture contained 30 µL of the digested DNA, 10 pmol *Mse*I adaptor, 1 pmol *Eco*RI adaptor, 1 mM ATP (pH 7), and 0.05 U/µL T4 DNA ligase (Roche).

Pre-selective amplification was done using adaptor-specific primers *Eco*RI (5'-GAC TGC GTA CCA ATT C-3') and *Mse*I (5'-GAT GAG TCC TGA GTA A-3'). The PCR reaction (30 µL) contained 10 µL of adaptor-ligated mixture, PCR buffer (10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl), 0.2 mM of each dNTP, 0.3 µM of each primer, and 0.6 U *Taq* DNA polymerase (Roche). The PCR cycle consisted of 25 cycles of 30 sec at 94°C, 30 sec at 56°C, and 1 min at 72°C. PCR was terminated after a 2-min hold at 72°C. Selective amplification was done using *Eco*RI and *Mse*I adaptor-specific primers, E+2 and M+2 primers, respectively, where '+2' indicates the two selective nucleotides at their 3'-ends. The PCR mixture (20 µL) consisted of 0.5 µL pre-selective PCR product, PCR buffer (10 mM Tris-HCl, 2.0 mM $MgCl_2$, 50 mM KCl), 0.2 mM of each dNTP, 0.04 µM of E+2, 0.25 µM of M+2 and 0.06 U/µL *Taq* polymerase (Roche). Each E+2 primer was 5'-labelled with either Cy5.5 (Inqaba Biotechnologies, RSA) or infrared dyes (LI-COR, Lincoln, USA). The PCR consisted of a touchdown phase of 13 cycles (10 sec at 94°C, 30 sec at 65°C [reduced by 0.7°C /cycle], 1 min at 72°C). This was followed by 23 cycles consisting of 10 sec at 94°C, 30 sec at 56°C, 1 min (increased by 1 sec/cycle) at 72°C. PCR was terminated after a 1-min hold at 72°C. All restriction digestions, ligation reactions, pre-selective and selective PCR amplifications were done on the GeneAmp PCR System 9700.

Selective amplification products were mixed with 10 µL formamide dye (98% [w/v] formamide, 10 mM EDTA, 0.1% [w/v] bromophenol blue, 0.1% [w/v] xylene cyanol) and denatured at 90°C for 3 min. The denatured fragments were resolved on 8% denaturing polyacrylamide gels (Long Ranger, FMC Scientific, Rockland, ME) by electrophoresis at 1500 V for 4 hrs in 0.8x TBE (8.6 mg/ml

Tris-base, 4.4 mg/ml boric acid, 1.6 mM EDTA) at pH 8.3 using a LI-COR IR^2 DNA sequencer (Biosciences, USA).

Images of gels were saved electronically and scored for presence/absence (+/-) of bands using QuantarPro 1.0 (KeyGene Products, The Netherlands). Bands with similar migration patterns were considered identical, and ambiguous bands that were very intense or very faint were not scored. From the binary matrix obtained, the pair-wise distance between all the isolates was calculated using the simple mismatch coefficient that is recommended for dominant markers in haploid organisms (Kosman and Leonard, 2005). Accordingly, pair-wise distances were calculated as follows: pair-wise distance = $1 - [(n - b - c)/n]$, where n is the number of loci, b is the number of bands unique to one individual, and c is the number of bands unique to the other individual in the pair. The resulting distance matrix was used to cluster the isolates by UPGMA (Unweighted Pair-Group Method using Arithmetic means) as implemented in the Molecular Evolutionary Genetic Analysis (MEGA, Version 2.1, Kumar *et al.*, 2001). The goodness of fit of dendrograms and the respective distance matrices were determined using the cophenetic correlation analysis (Sneath and Sokal, 1973).

The Polymorphic Information Content (PIC) of a marker in a data matrix was calculated as: $PIC = 1 - [f^2 + (1 - f)^2]$,where f is the frequency of the marker in the dataset (De Riek *et al.*, 2001). The average PIC value of all markers in a dataset was used as an estimate of the PIC value of the particular primer combination that was used to generate the dataset. Genotypic diversity among isolates was estimated from allelic frequencies using Nei's (1973) equation: $H = 1 - \Sigma x_i^2$,where, x_i is the frequency of the i^{th} allele. Population differentiation was calculated using the equation $\delta_T = (n/n-1)(1-\Sigma p_i^2)$, where p_i is the frequency of the i^{th} allele, and n is the number of individuals sampled (Gregorious, 1987). The coefficient of population subdivision (G_{st}) was computed as $(H_t - H_s)/H_t$, where, H_t is the total genetic diversity, and H_s is the average gene diversity over all subgroups (Nei, 1973). Genetic identity (I) was computed as $I_{xy} / \sqrt{I_x I_y}$ where, I_{xy}, I_x, and I_y are the averages over all loci of $\Sigma x_i y_i$, Σx_i^2, and Σy_i^2, respectively, where x_i and y_i are the frequencies of allele i for populations x and y (Nei, 1973).

RESULTS

Sequence analyses

All of the sequences that were generated during this study have been deposited in GenBank under accession numbers DQ220207 – DQ220247 (for β-tubulin) and DQ220248 – DQ220288 (for EF-1α). The aligned EF-1α dataset contained 705 characters, of which 461 were constant. Among the 244 variable characters, 128 were parsimony informative. Phylogenetic analyses of this dataset separated the Ethiopian isolates and the taxa included from GenBank into two major groups (Fig. 1). The first major group (75% bootstrap) consisted all of the Ethiopian *F. solani* isolates. The taxa that represented one of the three clades (Clade 3) previously described in *F. solani* (O'Donnell, 2000) were also in this group. Within this major group, the Ethiopian isolates resolved into six well-supported lineages (Lineages 1 – 6), where FCC3815 was the only Ethiopian isolate residing in Lineage 6. The second major group (71% bootstrap) consisted exclusively of isolates that represented the remaining two clades (Clades 1 and 2) of *F. solani* (O'Donnell, 2000). This group also consisted of other mitotic species of the *F. solani-H. haematococca* complex. No isolate of *F. solani* from Ethiopia was placed in this second group.

The aligned β-tubulin dataset contained 292 characters, of which only 12 were parsimony informative. Consequently, it did not provide a good resolution. It only resolved Lineage 3 of the Ethiopian *F. solani* isolates as distinct (tree not shown). There is also no β-tubulin sequence information in the GenBank for *F. solani* isolates. For these reasons, the β-tubulin dataset was excluded from the phylogenetic analyses. However, this dataset was useful as the fixed nucleotide sties that it contained enabled discrimination between the two clusters that emerged from the AFLP analyses (see below).

Inspection of the β-tubulin sequences revealed the presence of three nucleotide sites, which were differentially fixed (Fig. 3, positions 146, 152 and 263). These three polymorphisms separated the *F. solani* isolates into two distinct groups, which corresponded to the major AFLP Clusters A and B (Fig. 2). These polymorphisms were all situated at third codon positions within exons and resulted in synonymous substitutions. Although other polymorphic sites were also present in the β-tubulin gene sequences (*e.g.* position 167 in Fig. 3), as well as in the EF-1α sequences, none of them uniquely identified Clusters A and B.

AFLP analyses

Using eight randomly selected *F. solani* isolates, eight Primer Combinations (PCs) were evaluated for the number of polymorphic bands that the PCs generated and for the clearness of the gel images. Out of the eight PCs, E-AC/M-AG (PC1) and E-TC/M-AG (PC2) were chosen for the study. PC1 and PC2 generated a total of 65 distinctly polymorphic bands, which were scored for the 43 Ethiopian isolates included in this study. Although the number of polymorphic bands scored for PC2 (23 bands) was almost half as many as those for PC1 (43 bands), the PIC generated with PC2 (0.393) was slightly higher than that generated with PC1 (0.389). Both dendrograms had high cophenetic correlation values (0.89 for PC1, and 0.88 for PC2) indicating the goodness of fit of the dendrograms to the respective datasets.

Genetic relationships among the isolates based on the datasets obtained from the two AFLP PCs were analyzed as separate and as combined datasets. Based on the individual and combined datasets, all of the Ethiopian *F. solani* isolates, except FCC3185, separated into two major clusters (Clusters A and B) at a similarity of about 75% (Fig. 2, dendrograms for individual PCs not shown). The isolates in these clusters further resolved into five AFLP-based lineages, which corresponded to Lineages 1-5 (Fig. 1) that emerged from the sequence data. Cluster A contained Lineages 1 and 2, while Cluster B contained Lineages 3 – 5. Isolate FCC3815, which was the only Ethiopian isolate in Lineage 6 in the trees that emerged from the EF-1α sequence data, was not included in either of the clusters.

The dendrogram generated from the combined AFLP dataset (Fig. 2) had a similar topology to that based on PC1. The PC2 dendrogram differed slightly in that Cluster A contained isolates from Lineages 1 – 3. Isolates FCC3631, FCC4635 and FCC3776 were also characterized by inconsistent placement in the dendrograms generated from the individual PCs. There was a higher genotypic diversity in Cluster B ($H = 0.747$, $\delta_T = 78.4\%$) than in Cluster A ($H = 0.676$, $\delta_T = 71.1\%$). However, the genetic differentiation between the two clusters was low ($G_{ST} = 0.064$), which was also reflected in the high genetic identity among all the isolates ($I = 0.737$).

DISCUSSION

Analyses of EF-1α and AFLP data separated the Ethiopian isolates of *F. solani* into six well-supported and concordant lineages. The AFLP analyses further grouped five of these lineages into two clusters. The separation of these clusters was also supported by the presence of β-tubulin

nucleotide sites that were fixed differentially between the two clusters. Isolates residing in these clusters or lineages were in no way correlated to geographic origin or source of the isolates. Results of this study also showed that the *F. solani* isolates from Ethiopian soils and plant tissues reside in distinct lineages within the so-called "Clade 3" (O'Donnell, 2000) of the *F. solani-H. haematococca* complex.

The specific relationships of the three clades inferred from the data in this study are not entirely congruent with those of O'Donnell (O'Donnell, 2000). Based on data from the present study, isolates representing the *F. solani-H. haematococca* complex are split into two groups, one containing Clades 1 and 2 while the other consisted entirely of Clade 3. However, based on the combined EF-1α, ITS (ribosomal RNA [rRNA] internal transcribed spacer) and 28S rRNA, O'Donnell (2000) reported that Clade 1 represents the most ancient clade while Clades 2 and 3 are more closely related. This incongruence between findings in the two studies may be due to differences in the taxon sets used or because of other phenomena such as long branch attraction (Lyons-Weiler and Hoelzer, 1997). For example O'Donnell (2000) showed that the 28S rRNA sequences for taxa in Clade 1 were associated with unusually long branches, whereas those associated with EF-1α did not display unusually fast substitution rates. The basal position of this clade emerging from the EF-1α + ITS + 28S rRNA dataset (O'Donnell, 2000) may therefore be the result of long-branch attraction.

The composition of the three *F. solani-H. haematococca* clades emerging from the present study corresponded well with those reported previously (O'Donnell, 2000). Clade 1 included *F. illudens* and *N. plagianthi*, which are suggested to have originated in New Zealand (O'Donnell, 2000). Clade 2 included five *F. solani* lineages, two of which have recently been re-described as *F. virguliforme* (synonym = *F. solani* f.sp. *glycines*), and *F. phaseoli* (synonym = *F. solani* f.sp. *phaseoli*) (Aoki *et al.*, 2003). The remaining three have not been formally described, but their sequences appear in the GenBank database under the binomials *F. cuneirostrum*, *F. brasiliense* and *F. cuneirostrum*. In addition to various *F. solani formae speciales*, Clade 3 (O'Donnell, 2000) included the *N. haematococca* MPs and *Neocosmospora* spp. This clade also included all of the *F. solani* isolates obtained from Ethiopian plant tissues and soils and treated in the present study.

The EF-1α sequence data separated the 43 *F. solani* isolates into six well-supported lineages, where Lineage 6 was distantly related to the remaining five lineages. However, the relationship among Lineages 1 – 5 remains unclear because the sequence data do not harbor sufficient information to determine the phylogenetic affinity of Lineage 3. Although it is difficult to conclude whether this

lineage is more closely related to Lineages 1 and 2 or to Lineages 4 and 5, the AFLP results suggest that Lineage 3 is more closely related to Lineages 4 and 5.

Analyses of EF-1α sequences in this study showed that a single isolate (FCC3815) was different from all other Ethiopian isolates, and that it resides in Lineage 6. This isolate also did not group within either one of the clusters that emerged from the AFLP analyses. The EF-1α data further showed that isolate FCC3185 grouped with the *N. haematococca* MP V (*F. solani* f.sp. *cucurbitae* [Jon.] Snyd. & Hans. race 2). Whether or not this isolate forms part of MP V requires mating tests, which could not be undertaken as part of this study. It also suggests that more comprehensive sampling of Ethiopian soils need to be undertaken to find additional isolates of this type.

The six lineages emerging from the sequence analyses, and those based on the combined AFLP data were closely concordant. AFLP analyses sample the entire genome, whereas gene genealogies examine specific nuclear or mitochondrial genes (Vos *et al.*, 1995; Majer *et al.*, 1996). As such, the AFLP analyses provide independent measures of the evolutionary history of the organism. Consequently, phylogenies supported by the two techniques are likely to reliably reflect the evolutionary history of the *F. solani* isolates included in this study.

AFLP analyses revealed two major clusters within the Ethiopian isolates of *F. solani*. Separation of these clusters was also supported by the presence of three β-tubulin nucleotide sites that were fixed differently between the two clusters. The genetic diversity of isolates within the AFLP Cluster A was greater than that in Cluster B. However, the genetic differentiation between isolates in the two AFLP-based clusters appeared to be small. This suggests that all the Ethiopian isolates represent a single species. Nonetheless, the AFLP data, and β-tubulin sequences to a lesser extent suggest that this single species is either in the process of separating into two different species or that the two clusters might have recently converged into one species.

The *F. solani* isolates in the two AFLP-based clusters shared ~75% similarity. Lineage 6, which is represented by FCC3815, also shared ~75% similarity with isolates in the two clusters. This indicates that the minimum of 70% AFLP-based similarity observed among isolates of some *Fusarium* species (Abdel-Satar *et al.*, 2003; Zeller *et al.*, 2003; Leslie *et al.*, 2004) may be somewhat low for use as a cutoff point for AFLP-based species recognition in the *F. solani-H. haematococca* species complex. This would be especially true if isolate FCC3815 represents *N. haematococca* MP V, which is a unique biological species. Nevertheless, such an AFLP-based tool for recognizing species among the morphologically cryptic *Fusarium* spp. will be of great value.

The Ethiopian isolates treated in this study were obtained from agricultural soils, stem samples of wheat and barley plants, and roots of banana plants collected from different locations in the country. However, the grouping observed among these isolates did not correlate in any way with the source (host or substrate) or geographical origins of the isolates. These strains have not been tested for pathogenicity, and nothing is known regarding their ecological relevance. However, in *F. oxysporum* for example, several genetic diversity studies have shown that some pathogenic strains could have arisen from presumably non-pathogenic strains, and *vice versa,* through mutations involving a few genes (Woo *et al.*, 1996; Baayen *et al.*, 2000). Future studies should consider the pathogenicity of the isolates included in this study and it might then be possible to show a relationship between pathogenic and non-pathogenic strains.

Table 1. Strain numbers, source and geographic origin of the *F. solani* isolates used in this study.

FCC Number	Source (host/substrate) [a]	Location
3631	Banana root	Arbaminch
3681	Banana root	Arbaminch
3734	Banana root	Arbaminch
3782	Banana root	Arbaminch
3789	Banana root	Arbaminch
3794	Banana root	Arbaminch
3809	Banana root	Arbaminch
4359	Banana root	Arbaminch
4632	Banana root	Arbaminch
3749	Barley stem	North Shoa
2926	Soil (Barley)	Bale
2934	Soil (Barley)	West Shoa
3723	Soil (Barley)	Bale
3735	Soil (Barley)	Gondar
3810	Soil (Barley)	Sidamo
4636	Soil (Barley)	Bale
3695	Soil (Maize)	East Shoa
3814	Soil (Maize)	Wollo
3820	Soil (Maize)	Zeway
4633	Soil (Maize)	Zeway
3612	Soil (Pulses)	Gondar
3639	Soil (Pulses)	Bale
3689	Soil (Pulses)	Gondar
3649	Soil (Sorghum)	Wollo
3650	Soil (Sorghum)	Wollo
4634	Soil (Sorghum)	Gondar
2930	Soil (tef)	Gondar
3613	Soil (tef)	Sidamo
3623	Soil (tef)	East Shoa
3632	Soil (tef)	Gojjam
3661	Soil (tef)	Gondar
3686	Soil (tef)	Gojjam
3736	Soil (tef)	Wollo
3776	Soil (tef)	Shoa
3815	Soil (tef)	East Shoa
3816	Soil (tef)	East Shoa
4631	Soil (tef)	Wollega
3625	Soil (Wheat)	Bale
3670	Soil (wheat)	North Shoa
4635	Soil (Wheat)	Wollega
3727	Wheat stem	North Shoa
3748	Wheat stem	North Shoa
4448	Wheat stem	West Shoa

[a] The crops in parentheses reflect those planted on the respective farms during the cropping season before the soil samples were collected in the subsequent off-season. The pulses were predominantly broad bean and peas.

Table 2. Strain information for the various EF-1α sequences obtained from GenBank and included in this study to represent known species and clades of the *F. solani-H. haematococca* complex .

Strain Number	Species	Mating Population	Clade	GenBank Accession Number
NRRL31779 [a]	*F. brasiliense*	-	2	AY320150
NRRL31949 [a]	*F. cuneirostrum*	-	2	AY320161
NRRL22090 [c]	*F. illudens*	-	1	AF178326
NRRL31156 [b]	*F. phaseoli*	-	2	AY220187
NRRL22402 [c]	*F. solani* f.sp. *batatas*	II	3	AF178344
NRRL22142 [c]	*F. solani* f.sp. *cucurbitae*	V	3	AF178347
NRRL22153 [c]	*F. solani* f.sp. *cucurbitae*	I	3	AF178346
NRRL22157 [c]	*F. solani* f.sp. *mori*	III	3	AF178359
NRRL22570 [c]	*F. solani* f.sp. *piperis*	-	3	AF178360
NRRL22820 [c]	*F. solani* f.sp. *pisi*	VI	3	AF178355
NRRL22586 [c]	*F. solani* f.sp. *robiniae*	VII	3	AF178353
NRRL22277 [c]	*F. solani* f.sp. *xanthoxyli*	IV	3	AF178336
NRRL31950 [a]	*F. tucumaniae*	-	2	AY320157
NRRL22823 [b]	*F. virguliforme*	-	2	AF395647
NRRL31041 [b]	*F. virguliforme*	-	2	AY220193
NRRL22632 [c]	*Nectria plagianthi*	-	1	AF178354
NRRL22436 [c]	*Neocosmospora africana*	-	3	AF178348

[a] Unpublished, species not formally described.

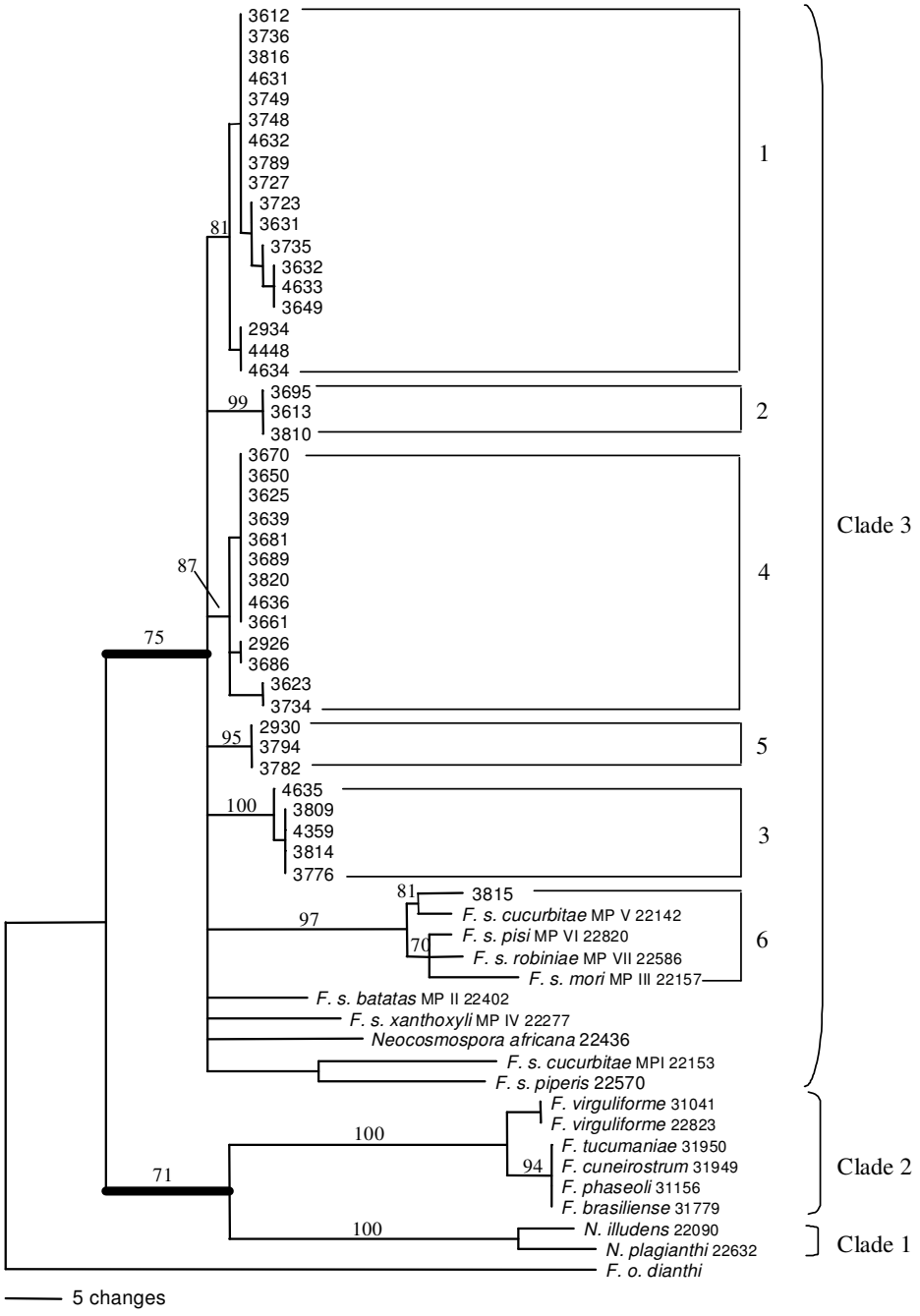
[b] (Aoki *et al.*, 2003).

[c] (O'Donnell, 2000).

Fig. 1. One of 373 most parsimonious trees inferred from the EF-1α sequences of the Ethiopian isolates, as well as 17 GenBank sequences representing previously described Clades 1 – 3 of the *F. solani-H. haematococca* species complex (O'Donnell, 2000). Thick horizontal lines indicate the branches leading to the two major *F. solani* groups. *F. oxysporum* f.sp. *dianthi* was used as an outgroup. Bootstrap values of 70% and higher are indicated above the internodes. The single digits (1 – 6) indicate Lineages 1 – 6 of Clade 3. The tree scores were: length, 428; consistency index, 0.71; retention index, 0.815; rescaled consistency index, 0.579. [a] Unpublished, species not formally described.

Fig. 2. UPGMA dendrogram generated from the combined AFLP data for the 43 Ethiopian *F. solani* isolates included in this study. The scale bar shows the percentage dissimilarity and the single digits (1 – 6) indicate Lineages 1 – 6 as in Fig. 1.

Fig. 3. The partial alignment (sites 146 – 265) of the β-tubulin haplotypes representing Cluster A (isolates FCC3748, FCC3613, and FCC3695) and Cluster B (isolates FCC3774, FCC3625, FCC3686, FCC3794, FCC3782, and FCC2930). The shaded sites show nucleotides that have been fixed differently between the two clusters. The dots indicate nucleotides that are identical to the ones in the first sequence at the respective sites.


3612
3736
3816
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3649
2934
4448
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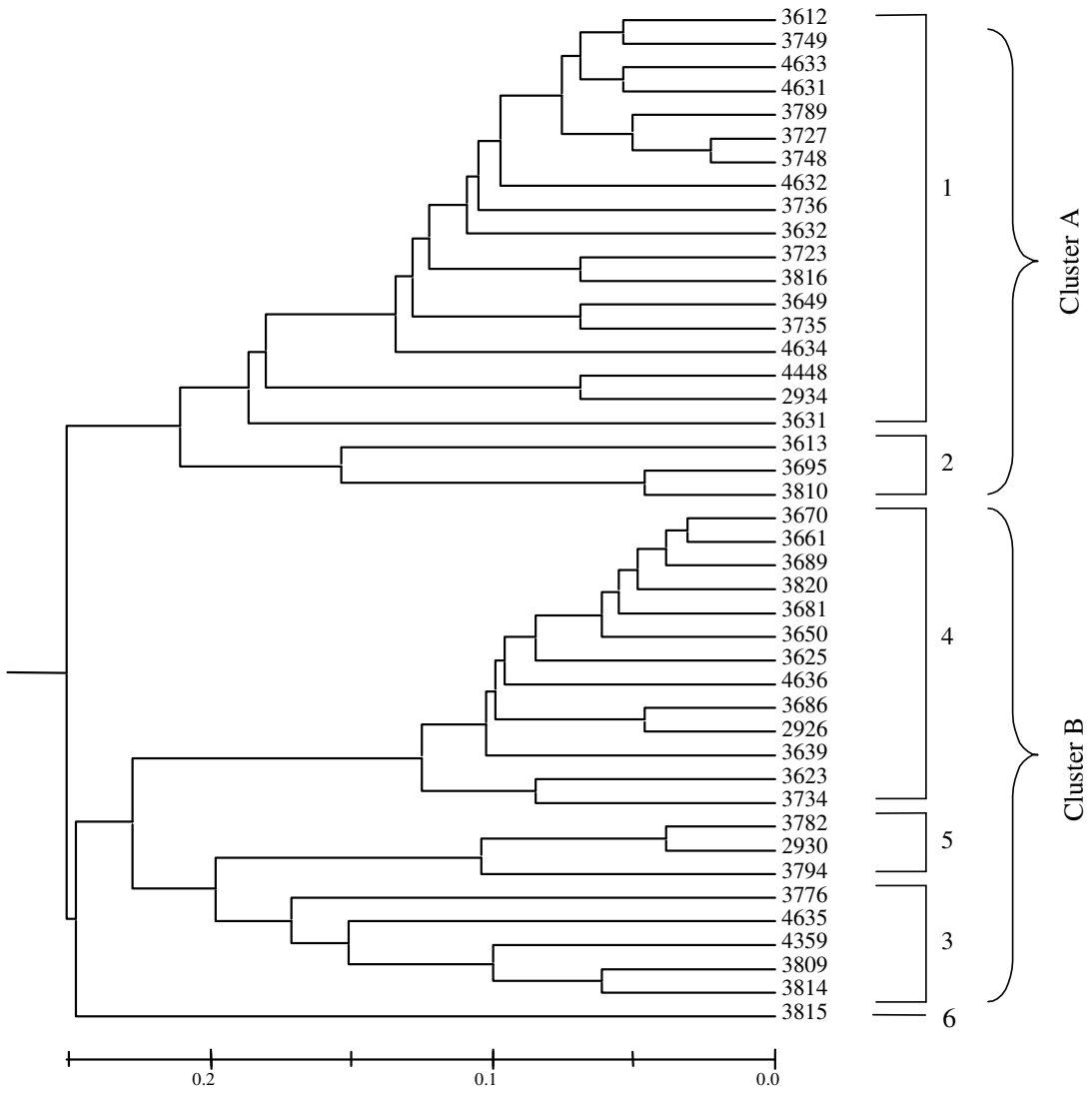
4636
3661
2926
3686
3623
3734
2930
3794
3782
4635
3809
4359
3814
3776
3815
F. s. cucurbitae MP V 22142
F. s. pisi MP VI 22820
F. s. robiniae MP VII 22586
F. s. mori MP III 22157
F. s. batatas MP II 22402
F. s. xanthoxyli MP IV 22277
Neocosmospora africana 22436
F. s. cucurbitae MPI 22153
F. s. piperis 22570
F. virguliforme 31041
F. virguliforme 22823
F. tucumaniae 31950
F. cuneirostrum 31949
F. phaseoli 31156
F. brasiliense 31779
N. illudens 22090
N. plagianthi 22632
F. o. dianthi
81
99
87
75
95
100
81
97
70
100
94
71
100
1
2
4
5
3
6
Clade 3
Clade 2
Clade 1
5 changes

Fig. 1.


3612
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3681
3650
3625
4636
3686
2926
3639
3623
3734
3782
2930
3794
3776
4635
4359
3809
3814
3815
1
2
4
5
3
6
Cluster A
Cluster B
0.2
0.1
0.0

Fig. 2.

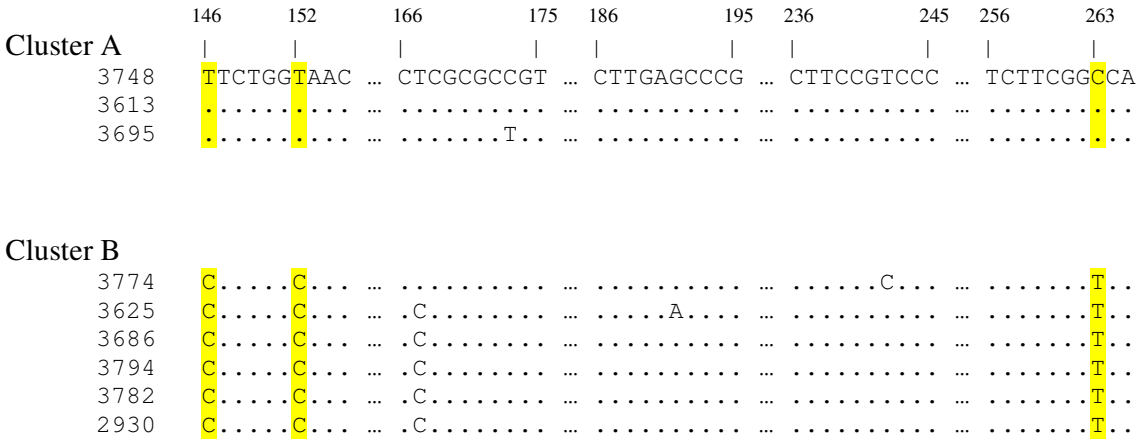
146 152 166 175 186 195 236 245 256 263
Cluster A
3748 TTCTGGTAAC … CTCGCGCCGT … CTTGAGCCCG … CTTCCGTCCC … TCTTCGGCCA
3613 .... … .... … .... … .... … ....
3695 .... … ....T.. … .... … .... … ....
Cluster B
3774 C.....C... … .... … .... … ......C... … ....T..
3625 C.....C... … .C........ … .....A.... … .... … ....T..
3686 C.....C... … .C........ … .... … .... … ....T..
3794 C.....C... … .C........ … .... … .... … ....T..
3782 C.....C... … .C........ … .... … .... … ....T..
2930 C.....C... … .C........ … .... … .... … ....T..

Fig. 3.

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CHAPTER 4

Simple Sequence Repeat (SSR) Markers for the Study of Species in the *Fusarium oxysporum* Complex

ABSTRACT

Pathogenic *Fusarium oxysporum* isolates cause vascular wilt, crown rot and root diseases in a wide variety of plant species. The traditional pathogenicity-based classification of isolates in this species complex is practical in terms of forestry and agriculture, but has several limitations. It is unwieldy, time consuming and inconclusive as pathogenicity is affected by factors such as the environment. It also precludes the classification of non-pathogenic isolates. The use of vegetative compatibility tests and various protein- and DNA-based techniques also has not provided an adequate solution, mainly because of lack of correspondence among groups based on these techniques and *formae speciales*. To serve as an additional tool in the study of species in the *F. oxysporum* complex, nine polymorphic Simple Sequence Repeat (SSR, also called microsatellite) markers were developed based on the Random Amplified Microsatellites (RAMS) technique using Inter-Simple Sequence Repeat (ISSR) primers. These SSR primers amplified a total of 71 alleles among 64 isolates encompassing more than 20 *formae speciales*. The number of alleles amplified at each locus ranged from two to 15, with an average of 7.889 alleles per locus. The allelic diversity at these loci ranged from 0.003 to 0.895, with an average of 0.588. The high degree of polymorphism revealed using these SSR markers should facilitate the further study of the *F. oxysporum* species complex.

INTRODUCTION

Fusarium oxysporum Schlecht. (emend. Snyd. and Hans.) is a ubiquitous soil-borne fungus, with mainly saprophytic members (Burgess, 1981; Correll *et al.*, 1986; Katan and Katan, 1988; Gordon *et al.*, 1990; Gordon and Okamoto, 1989). Some members, however, are pathogenic that cause destructive vascular wilts, crown rots and root rots in a wide variety of crop species, often limiting crop production (Nelson *et al.*, 1981; Correll *et al.*, 1986; Martyn and Bruton, 1989; Kim, 1991). As a species, *F. oxysporum* probably causes more economic damage to agricultural crops than any other pathogen (Correll, 1991).

Although pathogenic *F. oxysporum* affect a wide variety of crop species, individual members are limited to particular hosts or a group of host species. This host specificity of individual isolates has led to the classification of the species into *formae speciales* and races (Snyder and Hansen, 1940). To date, more than 120 *formae speciales* and races have been described based on their virulence (Armstrong and Armstrong, 1981). Given the difference among *formae speciales* and races in terms of their virulence towards plant species, identification based on pathogenicity has a practical significance in forestry and agriculture.

Classification solely based on pathogenicity, however, is an expensive and labour-intensive task (Ploetz and Correll, 1988). Moreover, pathogenicity tests do not always provide a robust means of classification as the results are affected by a range of factors, such as host genetic composition (Armstrong and Armstrong, 1981), host growth condition (Correll, 1991), stage of host (Hart and Endo, 1981), and mode of inoculation (Kraft and Haglund, 1978). Classification based on virulence alone also precludes the classification of non-pathogenic isolates, which make up a significant component of the species complex (Correll, 1991). To circumvent these problems, vegetative compatibility tests and an array of protein- and DNA-based techniques have been used. These techniques, however, have not provided an adequate solution to the problem associated with the taxonomy of the *F. oxysporum* complex (see Chapter I of this thesis for a review).

Simple Sequence Repeats (SSRs, also known as microsatellites) are tandemly repeated nucleotide motifs embedded in unique DNA stretches (Tautz, 1989). Their hypervariability, abundance in eukaryotic genomes, and co-dominant nature, makes SSR markers a powerful tool for taxonomic and population genetic studies (Tautz, 1989; Kistler *et al.*, 1991; Lehmann *et al.*, 1996; Goldstein *et al.*, 1999; Burgess *et al.*, 2001; Jimenez-Gasco *et al.*, 2004). Consequently, SSRs have been used in the study of several fungal species (Groppe and Boller, 1997; Longato and Bonfante, 1997; Moon *et*

al., 1999). In *F. oxysporum*, DNA fingerprinting involving hybridisation with SSR-containing probes has been used in the classification of some *formae speciales* (Barve *et al.*, 2001). However, no SSR marker has been developed for their study. The objective of this study was, therefore, to develop SSR markers for the study of species in this complex.

MATERIALS AND METHODS

Isolates and DNA extraction

F. oxysporum isolates FCC3175 (obtained from Centraalbureau voor Schimmelcultures [CBS], the Netherlands) and FCC3799 (isolated from an agricultural soil sample from Ethiopia) were used in the development of the SSR markers. The developed markers were tested for polymorphism on 64 isolates, which were obtained from various sources and geographical origins (Table 1). Twenty-one of these isolates, which were originally from different geographical origins, were obtained from the CBS. These represented 21 *formae speciales* of *F. oxysporum*. The remaining 43 strains were isolated from agricultural soil samples and plant materials collected from Ethiopia (Chapter 2 of this thesis).

DNA extraction

DNA was extracted using the CTAB (N-cetyl-N,N,N-trimethyl-ammonium bromide) method (Murray and Thompson, 1980). Mycelium was scraped from the surface of cultures and transferred to Eppendorf tubes containing sterile sand and 700 µL extraction buffer (5% [w/v] CTAB, 1.4 M NaCl, 0.2% [v/v] 2-mercaptoethanol, 20 mM ethylene diamine tetraacetate, 10 mM Tris-HCl [pH 8.0], and 1% [w/v] polyvinylpyrrolidone). The mixture was pulverized; incubated for 1 hr at 60°C, and centrifuged at 9, 300 *g* for 10 min. DNA was extracted from the aqueous phase by repeated phenol-chloroform (1:1, v/v) extractions followed by a final chloroform extraction to remove residual phenol. The DNA was then precipitated with 0.1 volume of 3 M sodium acetate (pH 5.4) and 0.6 volume of 2-propanol, and incubated at –20°C overnight. Precipitated DNA was pelleted by centrifugation at 15, 700 *g* for 30 min at 4°C, washed with 70% ethanol, air-dried, and dissolved in deionised water.

Development of SSR primers

Random Amplified Microsatellites (RAMS) PCR (Hantula *et al.*, 1996) with Inter-Simple Sequence Repeat (ISSR) primers (Lieckfeldt *et al.*, 1993; Buscot *et al.*, 1996; Hantula *et al.*, 1996) was used

to develop SSR markers (Van Der Nest *et al.*, 2000). ISSR primers were used singly or in various combinations (Table 2). The RAMS PCR mixture contained PCR buffer (10 mM Tris-HCl [pH 8.3], 2.5 mM $MgCl_2$, 50 mM KCl), 2.5 µM of each dNTP, 0.20 µM of each primer, 0.05 U/µL *Taq* Polymerase (Roche), and approximately 4 ng/µL template DNA. The PCR cycle consisted of an initial denaturation for 4 min at 94°C, followed by 35 cycles of 30 sec at 94°C, 45 sec at 48°C, and 2 min at 72°C; and a final extension at 72°C for 10 min.

RAMS PCR products were resolved by electrophoresis on 2 % (w/v) agarose gels containing 0.1 µg/ml ethidium bromide, and visualized by UV illumination. RAMS PCR products with distinct bands were then purified using the QIAquick PCR purification kit (QIAGEN, GmbH, Germany) and cloned in *Escherchia coli* cells using Promega's pGEM®-T Easy Vector System and recommendations (Promega, USA). Inserts were amplified from *E. coli* cells containing recombinant plasmids by colony PCR using vector-specific primers (M13 or SP6 and T7). Composition of the colony PCR was the same as that for the RAMS PCR except that a few cells taken from the surface of a bacterial colony using a sterile pipette tip constituted the DNA template. The PCR cycle was also different in that a 53°C annealing temperature, and 30 sec each of annealing and extension periods were used here. PCR-amplified inserts were purified and sequenced in both directions using the BigDye terminator sequencing kit (Version 3.2, Applied Biosystems) and ABI PRISM^{TM} 3100 DNA sequencer (Applied Biosystems). All PCRs and sequencing reactions were performed on a GeneAmp PCR System 9700 (Perkin Elmer).

Genome walking

Sequences of inserts were checked for the presence of SSRs using Sequence Navigator (Version 1.0.1, Perkin Elmer). For SSRs in the middle of inserts, primers flanking the repeats were designed directly using Primer3 (www.broad.mit.edu), whereas for SSRs at the ends of inserts, genome walking (Siebert *et al.*, 1995) was required. For genome walking, approximately 2.0 µg DNA was digested separately with endonucleases *Sca*I, *Hae*III, *Eco*RV and, *Dra*I (GmbH, Germany). Digestion was done overnight at 37°C in 50 µL reaction volume with 0.8 U/µL of restriction enzyme using the appropriate restriction buffer. The digested DNA was extracted once with phenol-chloroform (1:1, v/v), and once with chloroform. It was then precipitated with 0.6 volume of 2-propanol and 0.3 volume of 3 M sodium acetate (pH 5.4), and pelleted by centrifugation at 15, 700 *g*. The pellet was washed with 70% ethanol, air dried and re-suspended in 20 µL sterile distilled water.

Ten-µL of the digested DNA was ligated with the adaptor (5'-CTA ATA CGA CTC ACT ATA GGG CTC GAG CGG CCG CCC GGG CAG GT-3'/3'-H_2N-CCC GTC CA-P-5') overnight at 4°C. The ligation reaction consisted of 50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 0.5 mM ATP, 10 mM dithiothretitol, 5 µM adaptor, and 0.5 U/µL T4 DNA ligase (Roche, USA). Ligation was terminated by incubating the reactions at 75°C for 5 min. The ligation mixture was then diluted 10-fold in sterile deionised water, and used as template for genome walking PCRs using adaptor-specific and gene-specific primer sets.

For each insert that required genome walking, distal and nested primers (with reference to the positions of their binding sites relative to that of the SSRs in the sequence of the insert) were designed. Primary genome walking PCR was done using a gene-specific distal primer and the outer primer specific to the adaptor (5'-GGA TCC TAA TAC GAC TCA CTA TAG GGC-3'). The primary PCR product was used as a template in the secondary genome walking PCR using the corresponding gene-specific nested primer and the inner primer specific to the adaptor (5'-AAT AGG GCT CGA GCG G-3'). The genome walking PCR had the same composition as that of the colony PCR. Its cycle consisted of a denaturation step at 94°C for 1 min followed by 30 cycles of denaturation at 94°C for 30 sec and annealing/extension at 68°C for 6 min, followed by a final annealing/extension step at 68°C for 15 min. The number of cycles was reduced to 20 in the secondary PCR. Genome walking PCR products were examined by electrophoreses using 1% agarose gels (w/v); and those with discreet bands were purified, cloned and sequenced as described above.

Evaluation for polymorphism and GeneScan analyses

The developed primers were evaluated for polymorphism using 64 *F. oxysporum* isolates (Table 1). PCR conditions were the same as that for the colony PCR, except that appropriate annealing temperatures were used for each set of SSR primers. Polymorphism was tested by electrophoreses on 3% (w/v) agarose gels.

For polymorphic primer sets, allele sizes were determined using the 3100 DNA sequencer by comparison with a LIZ 500 internal size standard (Applied Biosystems) with the aid of GeneMapper (Version 3.0, Applied Biosystems). For this reason, one primer from each of the sets was 5'-end labelled with a fluorescent dye (Applied Biosystems) (Table 2), and PCRs were performed as with the respective unlabelled primer sets. The PCR products were then diluted by a hundred-fold in sterile distilled water, and denatured by mixing 1 µL of the diluted product with 10

µL formamide, which also contained 0.14 µL of the LIZ 500 size standard. Denaturation was ensured by incubating the mixture at 95°C for 2 min, and the fragments were then separated on the DNA sequencer.

Determination of allelic diversity

Allelic diversity was calculated for each locus as: $1-\sum p_i^2$, where p_i is the frequency of the *i*th allele (Nei, 1973).

Linkage determination

A nucleotide sequence from each of the nine polymorphic loci has been deposited in the GenBank database (Table 2). Linkage among the markers was assessed using BLASTN (www.ncbi.nih.gov) analysis of these sequences against the genomes of *Gibberella zea* and *Neurospora crassa*.

RESULTS

A total of nine polymorphic SSR primer sets were developed in this study (Table 2). Three of the sets (MB11, MB13, and MB14) were designed directly as the SSRs were in the middle of inserts. For the remaining six primer sets, genome walking was required because the SSRs were at the ends of inserts. Each of the primer sets amplified a single band in all the 64 isolates.

GeneScan with labelled PCR products revealed that the nine primer sets amplified a total of 71 alleles (Table 3) across the 64 isolates. The number of alleles amplified with these primers ranged from two (locus FO10) to 15 (locus FO2). A range of allelic diversities was also evident at each locus. Locus FO13 and FO2 had the highest allelic diversities, 0.895 and 0.891, respectively, whereas Locus FO10 had the least allelic diversity, 0.003.

DISCUSSION

Nine polymorphic SSR primer sets were developed using the RAMS PCR technique. Each of these primer sets amplified a single band in all the isolates tested. At each of the nine loci that the primers amplified, a high degree of polymorphism was revealed among the isolates tested. I believe that this high degree of polymorphism would be sufficient for studying species in the *F. oxysporum* complex.

The RAMS PCR technique using ISSR primers (singly or in various combinations) (Van Der Nest *et al.*, 2000) has enabled us to develop nine polymorphic SSR markers. Since most of the markers were developed using pairs of ISSR primers, however, the use of ISSR primers in pairs, rather than individually, may be more efficient to develop SSR markers using this technique.

A total of 71 alleles were amplified using the nine polymorphic markers among the 64 *F. oxysporum* isolates. The number of alleles amplified with these markers ranged from two (locus FO10) to 15 (locus FO2). A range of allelic diversities was also observed. Locus FO10 had the least allelic diversity (0.003), whereas loci FO2 and FO13 had the highest allelic diversities, 0.891 and 0.895, respectively. The average number of alleles per locus was 7.889, with an average allelic diversity of 0.588. This indicated that the primers revealed a high degree of polymorphism.

The variation among some of the alleles in terms of size does not appear to be a function of the repeating units. For instance, the difference in increasing order of size of the three alleles at locus FO14 is only 1 base pair (bp, Table 3). However, the repeating unit involves at least three nucleotides (Table 2). This suggests that insertions/deletions also play a role in the polymorphism observed among some of the alleles.

The SSR sequences were compared with the *Gibberella zea* and *Neurospora crassa* genome sequences in the NCBI database (National Center for Biotechnology Information; www.ncbi.nih.gov) using the BLASTN search tool, to determine if there was linkage between the markers. This is pertinent because *F. oxysporum* is an asexual fungus and linkage cannot be determined by mating tests. However, none of the sequences, except that from locus FO2, were significantly homologous to those in the NCBI nucleotide database. It was consequently not possible to assess linkage between the markers.

The traditional pathogenicity-based classification of isolates in the *F. oxysporum* species complex has several limitations. The use of vegetative compatibility tests, and various protein- and DNA based techniques also has not solved the problem associated with the taxonomy of isolates of *F. oxysporum*. SSR markers are advantageous in that they are hypervariable, abundantly found in eukaryotic genomes, and co-dominant. These characters make SSRs very useful in taxonomic and population genetic studies. The high degree of polymorphism revealed using the nine SSR markers developed in this study should be sufficient for studies focussed on understanding the genetic diversity amongst isolates residing in this species complex. The SSR primers should be particularly useful because the fungus is one of the most common *Fusarium* spp. residing in the soil environment and that it includes pathogens, biological control agents and saprophytes. Their application should also enhance understanding relatedness of *formae speciales* in the *F. oxysporum* complex.

Table 1. Strains of the *Fusarium oxysporum* species complex that were used to test the SSR markers developed in this study. CBS numbers are shown for isolates that were obtained from the CBS.

FCC No.	*Forma specialis*	CBS No.	Host or substrate	Origin
2929	-	-	Soil	Ethiopia
2932	-	-	Soil	Ethiopia
2937	-	-	Soil	Ethiopia
3171	*conglutinans*	186.53	*Brassica oleraceae*	USA
3172	*dianthi*	491.97	-	-
3173	*gladioli*	137.97	-	Netherlands
3174	*lini*	259.51	*Linum usitatissimum*	Canada
3175	*lycopersici*	413.90	*Lycopersicon esculentum*	Israel
3176	*tulipae*	195.65	-	-
3177	*niveum*	419.90	*Citrullus lanatus*	Israel
3178	*phaseoli*	935.73	*Phaseolus* sp.	USA
3179	*pisi*	127.73	*Pisum sativum*	UK
3180	*radicis-lycopersici*	101587	*Lycopersicon* sp.	-
3181	*raphani*	488.76	*Raphanus sativus*	Germany
3183	*vasinfectum*	411.90	*Gossypium barbadense*	Israel
3184	*elaedis*	783.83	-	-
3186	*nicotianae*	179.32	-	-
3187	*passiflorae*	744.79	-	-
3188	*perniciosum*	794.70	-	-
3189	*lupini*	101.97	*Lupinus* sp.	-
3458	*melonis*	424.90	-	-
3459	*redolens*	489.97	*Dianthus* sp.	-
3460	*chrysanthemi*	129.81	-	-
3461	*cucurbitacearum*	680.89	-	-
3616	-	-	Soil	Ethiopia
3618	-	-	Soil	Ethiopia
3620	-	-	Soil	Ethiopia
3622	-	-	Soil	Ethiopia
3633	-	-	Soil	Ethiopia
3635	-	-	Soil	Ethiopia
3636	-	-	Soil	Ethiopia
3640	-	-	Soil	Ethiopia
3641	-	-	Soil	Ethiopia
3647	-	-	Soil	Ethiopia
3671	-	-	Soil	Ethiopia
3677	-	-	Soil	Ethiopia
3692	-	-	Soil	Ethiopia
3697	-	-	Soil	Ethiopia
3718	-	-	Banana roots	Ethiopia
3718	-	-	Soil	Ethiopia
3725	-	-	Soil	Ethiopia
3745	-	-	Soil	Ethiopia

FCC No.	*Forma specialis*	CBS No.	Host or substrate	Origin
3746	-	-	Soil	Ethiopia
3750	-	-	Wheat stem	Ethiopia
3753	-	-	Soil	Ethiopia
3755	-	-	Soil	Ethiopia
3759	-	-	Soil	Ethiopia
3760	-	-	Soil	Ethiopia
3784	-	-	Banana roots	Ethiopia
3786	-	-	Banana roots	Ethiopia
3788	-	-	Banana roots	Ethiopia
3792	-	-	Banana roots	Ethiopia
3796	-	-	Banana roots	Ethiopia
3799	-	-	Banana roots	Ethiopia
3801	-	-	Banana roots	Ethiopia
3802	-	-	Banana roots	Ethiopia
3803	-	-	Banana roots	Ethiopia
3807	-	-	Lentil	Ethiopia
3812	-	-	Wheat stem	Ethiopia
3813	-	-	Banana roots	Ethiopia
3819	-	-	Banana roots	Ethiopia
4332	-	-	Soil	Ethiopia
4414	-	-	Banana roots	Ethiopia
4439	-	-	Banana roots	Ethiopia

Table 2. Primer sequences, SSR motifs in amplicons obtained using the respective SSR primers, and GenBank accession numbers of sequences of the amplicons from the *Fusarium oxysporum* isolates FCC3175 and FCC3799.

Isolate[a]	ISSR primer	Primer	Microsatellite primer sequence (5'→3')[b]	T_a[c]	SSR motif	Dye	Locus[d]	GenBank Accession No[e].
FCC3175	$(CA)_5$	MB2	F: TGCTGTGTATGGATGGATGG R[f]: CATGGTCGATAGCTTGTCTCAG	57	$(GT)_{11}(GA)_6$	NED	FO2	AY931024
FCC3175	$(CA)_5 + (CAG)_5$	MB5	F[f]: ACTTGGAGGAAATGGGCTTC R: GGATGGCGTTTAATAAATCTGG	54	$(TG)_9$	PET	FO5	AY931030
FCC3175	$(CA)_5 + (GACA)_4$	MB9	F: TGGCTGGGATACTGTGTAATTG R[f]: TTAGCTTCAGAGCCCTTTGG	51	$(CA)_9$	VIC	FO9	AY931029
FCC3175	$(CAA)_5 + (CAC)_5$	MB10	F[f]: TATCGAGTCCGGCTTCCAGAAC R: TTGCAATTACCTCCGATACCAC	48	$(CAA)_5$	PET	FO10	AY931028
FCC3799	$(CAA)_5 + (CCA)_5$	MB11	F: GTGGACGAACACCTGCATC R: AGATCCTCCACCTCCACCTC	68	$(GGC)_7$	6-FAM	FO11	AY931025
FCC3799	$(CAC)_5 + (CAG)_5$	MB13	F: GGAGGATGAGCTCGATGAAG R: CTAAGCCTGCTACAACCTCG	68	$(CTTGGAAGTGGTAGCGG)_{15}$	6-FAM	FO13	AY931026
FCC3799	$(CAC)_5 + (CT)_8$	MB14	F: CGTCTCTGAACCACCTTCATC R: TTCCTCCGTCCATCCTGAC	57	$(CCA)_5$	VIC	FO14	AY931027
FCC3799	$(CT)_8 + (GT)_5$	MB17	F: ACTGATTCACCGATCCTTGG R[f]: GCTGGCCTGACTTGTTATCG	57	$(CA)_{21}$	NED	FO17	AY931023
FCC3175	$(CT)_8 + (GT)_5$	MB18	F[f]: GGTAGGAAATGACGAAGCTGAC R: TGAGCACTCTAGCACTCCAAAC	57	$(CAACA)_6$	PET	FO18	AY931031

[a]FCC = *Fusarium* culture collection, FABI, University of Pretoria, South Africa. [b]5'-end of one primer of each set was labelled with a fluorescent dye. [c]Annealing temperature in °C. [d]Each locus is indicated by an FO-number (FO = *F. oxysporum*). [e]GenBank accession number for sequence of microsatellite PCR product. [f]Primer was developed after genome walking.

Table 3. SSR allele sizes, number of alleles and allelic diversities based on 43 *F. oxysporum* strains from Ethiopia and 21 *F. oxysporum formae speciales* from CBS (numbers of isolates tested are indicated in parentheses).

Locus	Microsatellite allele sizes in base pairs (bp)			Total number of alleles	Allelic diversity
	Ethiopian isolates from soil (27)	Ethiopian isolates from plant tissues (16)	CBS isolates (21)		
FO2	237, 238, 250, 252, 254, 260, 264, 271, 275	246, 248, 254, 257, 260, 264	234, 237, 240, 242, 246, 248, 252, 254, 257, 264	15	0.891
FO5	252, 254, 267, 269	254, 256, 267	252, 254, 256, 267, 274, 344	7	0.768
FO9	126, 238	105, 126	126, 130, 141, 234, 237, 240, 254	9	0.693
FO10	206	206	206, 208	2	0.003
FO11	172, 175, 180, 182	175, 177, 180	172, 175, 180, 182, 186	6	0.707
FO13	264, 296, 382, 345, 395, 400, 422, 476, 483, 492, 500	296, 376, 395, 400, 476, 483, 492, 500	144, 264, 296, 345, 376, 395, 422, 476	13	0.895
FO14	183, 184	184	184, 186	3	0.351
FO17	303, 312, 317, 319, 320, 339	299, 312, 317, 319	299, 301, 308, 312, 317, 321, 331, 334, 337	13	0.719
FO18	284, 288	284, 289, 293	284, 289, 293	3	0.327

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CHAPTER 5

Characterization of *Fusarium oxysporum* isolates from Ethiopia using AFLP, SSR and DNA sequence analyses

ABSTRACT

Fusarium oxysporum is one of the world's most important fungal pathogens of agricultural crops. Yet the significance of this fungus in Ethiopian agriculture has not been well investigated. Amplified Fragment Length Polymorphism (AFLP), Simple Sequence Repeat (SSR) markers and DNA sequence analyses were used to study 32 strains of *F. oxysporum* from Ethiopia and to compare them with characterised isolates from a fungal culture collection. Sequences from GenBank representing the three known phylogenetic clades of *F. oxysporum* were also included for comparative purposes. All the three methods used in this study separated the Ethiopian strains into three lineages, which corresponded with the three well-known clades previously characterized for large numbers of isolates of *F. oxysporum*. Inspection of the sequence data revealed five fixed nucleotide sites in the translation Elongation Factor-1α (EF-1α) partition, which uniquely distinguished the three lineages. Thirty of the Ethiopian isolates grouped in Lineage 2, whereas the remaining two isolates grouped in Lineages 1 and 3. Eighteen *formae speciales* included in this study for comparative purposes did not separate according to host with any of the three DNA-based techniques used. This confirmed that pathogenicity of isolates does not necessarily correlate with phylogenetic grouping. AFLP and SSR complimented DNA sequence analyses, suggesting that DNA sequence analyses could be used together with AFLP and/or SSR to study the population biology and evolution of this important species complex. The genetic diversity observed among the Ethiopian isolates was low. This reflects the nature of the Ethiopian agricultural system that heavily relies on local crop varieties, thereby restricting the introduction of new genotypes of the fungus via infected seeds.

INTRODUCTION

Fusarium oxysporum Schlecht. (emend. Snyd. and Hans.) is a widely distributed soil inhabiting fungus, which is known to be phylogenetically diverse. Most strains assigned to this species are saprophytic, commonly colonizing senescent or damaged plant tissues (Katan and Katan, 1988; Gordon *et al.*, 1989; Gordon and Okamoto, 1990). Some non-pathogenic strains have been used as biological control agents against soil borne plant pathogens (Mandeel and Baker, 1991; Postma and Rattink, 1992), while others have veterinary and/or medical significance (Gupta *et al.*, 2000; Pereiro *et al.*, 2001). However, *F. oxysporum* is best known for the plant pathogenic strains, which cause wilt, root rot and crown rot diseases on a wide variety of crops, often limiting crop production (Nelson *et al.*, 1981; Martyn and Bruton, 1989).

The significance of *F. oxysporum* in Ethiopian agriculture has not been carefully investigated. The only reports of this fungus are those of Bekele (1985) and Bekele and Karr (1997). These studies represented crop disease inventories (Bekele, 1985) and a diagnostic survey for causal agents of *Fusarium* Head Blight (FHB) of wheat (Bekele and Karr, 1997). This is interesting because a recent survey (Chapter 2 of this thesis) has shown that *F. oxysporum* is one of the two *Fusarium* spp. most commonly isolated from banana roots, wheat and barley stems, and agricultural soils from Ethiopia.

The taxonomy of *F. oxysporum* has been extensively debated over many years. Wollenweber (1913) placed this species in the Section *Elegans* together with six other *Fusarium* species and forms. Snyder and Hansen (1940) recognized the six taxa as synonymous with *F. oxysporum*. However, *F. redolens*, which is very difficult to distinguish from *F. oxysporum* based on morphological characteristics, has been recognised as a discrete taxon using DNA-based techniques (Waalwijk *et al.*, 1996; O'Donnell *et al.*, 1998a). Currently, *F. oxysporum* is regarded as a complex of morphologically similar fungi with multiple phylogenetic origins consisting of isolates residing in at least three well-supported clades (O'Donnell *et al.*, 1998b; Baayen *et al.*, 2000a).

Most studies of *F. oxysporum* have focused on the plant pathogenic isolates (Plyler *et al.*, 2000; Sivaramakrishnan *et al.*, 2002; Mohammadi *et al.*, 2004; Pasquali *et al.*, 2004). However, the non-pathogenic members makeup a significant proportion of the isolates encountered, and harbour most of the genetic diversity within this species complex (Gordon and Okamoto, 1992; Appel and Gordon, 1994; Edel *et al.*, 1997; 2001; Bao *et al.*, 2002). Several studies involving pathogenic and non-pathogenic strains have revealed that there is a great deal of genetic relatedness between the two groups (Appel and Gordon, 1995; Fiely *et al.*, 1995; Alves-Santos *et al.*, 1999; Baayen *et al.*,

2000a; Skovgaard and Rosendahl, 2002). This has led some researchers to conclude that particular pathogenic isolates might have evolved from non-pathogenic strains by mutations involving a few loci (Appel and Gordon, 1995; Skovgaard and Rosendahl, 2002). Some non-pathogenic isolates have also been considered to have evolved from pathogenic strains through loss of virulence (Skovgaard and Rosendahl, 2002). Consequently, assessment of genetic diversity within the non-pathogenic populations is important.

DNA-based techniques have increasingly become the tool of choice for understanding the genetic diversity and phylogeny of *Fusarium* spp. For example, DNA sequence analysis has been used to reveal a large number of phylogenetic species in the *F. solani-Nectria haematococca* (O'Donnell *et al.*, 2000), *F. graminearum* (O'Donnell *et al.*, 2000), and the *F. oxysporum* species complexes (O'Donnell *et al.*, 1998b). Amplified Fragment Length Polymorphisms (AFLPs) have been used to distinguish species and phylogenetic groups in a number of *Fusarium* species (Baayen *et al.*, 2000a; Marasas *et al.*, 2001; Zeller *et al.*, 2003; Belabid *et al.*, 2004; Leslie *et al.*, 2004) including *F. oxysporum* (Baayen *et al.*, 2000a; Belabid *et al.*, 2004). Simple Sequence Repeats (SSRs, also known as microsatellites) have also been used in other fungal species because of the high resolution they provide (Jarne *et al.*, 1996; Barnes *et al.*, 2005; Enjalbert *et al.*, 2005).

The objective of this study was to consider the diversity of *F. oxysporum* strains isolated from agricultural soil and plant samples from Ethiopia, and furthermore assess how these isolates compare with those representing known phylogenetic groups previously characterized in this species complex. This was achieved using AFLP, SSR and DNA sequence comparisons.

MATERIALS AND METHODS

Isolates

The *F. oxysporum* isolates that formed the basis of this study (Table 1) were obtained from agricultural soils and plant tissues collected from various parts of Ethiopia. Some of these isolates have also previously been used in the development of SSR markers in *F. oxysporum* (Bogale *et al.*, 2005). Eighteen isolates representing different *formae speciales* (Table 1) of *F. oxysporum* were obtained from the Centraalbureau voor Schimmelcultures (CBS), the Netherlands and were used for comparative purposes. Sequences representing the three clades of *F. oxysporum* described by O'Donnell *et al.* (1998b) were obtained from GenBank and included in the sequence analyses. Also, relevant sequences of a *Fusarium* sp. (NRRL25184) were obtained from GenBank and used as an

outgroup in the sequence analyses. This isolate was chosen as an outgroup because it was used for the same purpose in the study (O'Donnell *et al.*, 1998b) where groupings in *F. oxysporum* were first described.

DNA extraction

DNA was extracted from isolates using the CTAB (N-cetyl-N,N,N-trimethyl-ammonium bromide) method (Murray and Thompson, 1980). For this purpose, isolates were grown on malt extract agar medium (2% [w/v] malt extract and 1.5% [w/v] agar) in Petri dishes for 7-10 days at 25°C. Mycelium was then scraped from the surface of the cultures and transferred to Eppendorf tubes containing sterile sand and 700 µL extraction buffer (5% [w/v] CTAB, 1.4 M NaCl, 0.2% [v/v] 2-mercaptoethanol, 20 mM EDTA [ethylene diamine tetraacetate], 10 mM Tris-HCl [pH 8.0], and 1% [w/v] polyvinylpyrrolidone). The mixture was pulverized; incubated for 1 hr at 60°C, and centrifuged at 9,300 *g* for 10 min. DNA was extracted from the aqueous phase by repeated phenol-chloroform (1:1) extractions followed by a final chloroform extraction to remove residual phenol. DNA was then precipitated with 0.1 volume of 3 M sodium acetate (pH 5.4) and 0.6 volume of 2-propanol, and incubated at –20°C overnight. Precipitated DNA was pelleted by centrifugation at 15,700 *g* for 30 min at 4°C, washed with 70% ethanol, air-dried, and dissolved in deionised water.

Sequence analyses

Fragments of the genes encoding translation Elongation Factor 1α (EF-1α), mitochondrial Small Subunit ribosomal RNA (mtSSU rRNA) and β-tubulin were amplified by PCR using primer pairs EF1 and EF2 (O'Donnell *et al.*, 1998b), MS1 and MS2 (White *et al.*, 1990), and 2A and 2B (Glass and Donaldson, 1995), respectively. PCR mixtures contained reaction buffer (10 mM Tris-HCl [pH 8.3], 2.50 mM $MgCl_2$, 50 mM KCl), 2.50 µM of each dNTP, 0.20 µM of each primer, 0.05 U/µL *Taq* Polymerase (Roche, USA), and approximately 4 ng/µL template DNA. The PCR cycling conditions consisted of an initial denaturation (94°C) for 4 min, followed by 35 cycles of denaturation for 30 sec, annealing for 30 sec (56°C for EF1 and EF2, 51°C for MS1 and MS2, 68°C for 2A and 2B), and extension at 72°C for 30 sec. PCRs were terminated after a final extension for 10 min. PCR products were purified using the QIAquick PCR purification kit (QIAGEN, GmbH, Germany), and sequenced in both directions using the respective PCR primers. For this purpose, the BigDye terminator sequencing kit (Version 3.2, Applied Biosystems) and an ABI PRISM™ 3100 DNA sequencer (Applied Biosystems) were used. All PCRs and sequencing reactions were performed on a GeneAmp PCR System 9700 (Applied Biosystems).

Gene sequences were assembled using Sequence Navigator (Version 1.0.1, Applied Biosystems), and aligned using ClustalX (Version 1.8, Thompson *et al.*, 1997), after which the alignments were manually corrected where needed. Sequence navigator was also used to predict amino acid sequences for nucleotide sequences. The predicted amino acid sequences were then compared with the corresponding amino acid sequences in GenBank to determine the possible positions of introns. PAUP (Phylogenetic Analysis Using Parsimony, Version 4.0b 10, Swofford, 2002) was used to estimate phylogenetic relationships and to test combinability of datasets. For this purpose, heuristic searches based on 1,000 random addition sequences and tree bisection-reconstruction were used with the branch swapping option set on best trees only. To test the combinability of datasets, a partition homogeneity test with heuristic searches was employed using 10,000 replications. All characters were weighted equally and alignment gaps were treated as missing data. Bootstrap analysis (Hillis and Bull, 1993) was based on 1,000 replications.

AFLP and SSR analyses

AFLP analysis was performed as previously described (Chapter 3 of this thesis), except that different Primer Combinations (PCs), E-CC/M-TA (PC1), E-TC/M-AA (PC2), and E-TC/M-AG (PC3), were used. Amplification and resolution of SSR loci was performed with the labelled SSR primers and conditions described by Bogale *et al.* (2005). For both the AFLP and SSR analyses, the presence/absence of an allele at a particular locus was scored as 1/0, and the pair-wise distance among the isolates was calculated from the binary matrix using the simple mismatch coefficient (Sneath and Sokal, 1973): pair-wise distance = $1 - [(n - b - c)/n]$, where n is the number of loci, b is the number of bands unique to one individual, and c is the number of bands unique to the other individual in the pair. The resulting distance matrices were used to cluster the isolates by the UPGMA (Unweighted Pair-Group Method using Arithmetic means) method implemented in the Molecular Evolutionary Genetics Analysis (Version 2.1, Kumar *et al.*, 2001). The goodness of fit of dendrograms and the respective distance matrices were determined using the cophenetic correlation analysis (Sneath and Sokal, 1973). The Polymorphic Information Content (PIC) of a marker in a data matrix was calculated as $1 - [f^2 + (1 - f)^2]$, where f is the frequency of the marker in the dataset (De Riek *et al.*, 2001). The average PIC value of all markers in a dataset was used as an estimate of the PIC value of the PC that was used to generate the dataset.

Genotypic diversity (H) among isolates was estimated from allelic frequencies using the equation $H = 1 - \Sigma x_i^2$, where, x_i is the frequency of the i^{th} allele (Nei, 1973). Genetic identity (I) was computed as $I_{xy} / \sqrt{I_x I_y}$ where, I_{xy}, I_x, and I_y are the averages over all loci of $\Sigma x_i y_i$, Σx_i^2, and Σy_i^2,

respectively, where x_i and y_i are the frequencies of allele i for populations x and y (Nei, 1973). Population differentiation was calculated using the equation $\delta_T = (n/n-1)(1-\Sigma p_i^2)$, where p_i is the frequency of the i^{th} allele, and n is the number of individuals sampled (Gregorious, 1987). The coefficient of population subdivision (G_{ST}) was computed as $(H_t - H_s)/H_t$, where, H_t is the total genetic diversity, and H_s is the average gene diversity over all subgroups (Nei, 1973). In all calculations, alleles with frequencies of 0.95 and higher were excluded from the analyses.

RESULTS

Sequence analyses

One mitochondrial (mtSSU rRNA) and two nuclear (EF-1α and β-tubulin) genes were sequenced for each isolate. All of these sequences have been deposited in GenBank under accession numbers DQ220021 – DQ220082 (for β-tubulin), DQ220083 - DQ220144 (for EF-1α) and DQ220145 – DQ220206 (for mtSSU). Although a 656 base pair (bp) mtSSU fragment was sequenced from each of the Ethiopian isolates and the *formae speciales*, only a 213 bp sequence at the 3'-end of these sequences was used in the analyses. This was to match the size of these sequences to those obtained from GenBank. Inclusion of the remaining 5'-end portion of the mtSSU sequence in the analyses of the isolates from this study and some GenBank sequences, for which the complete sequence information was obtained, resulted in trees with the same topology as those based on the smaller fragment, but with slightly higher bootstrap values (data not shown). Sequences for the three genes were analysed individually and in all possible combinations since they represented homogeneous partitions (p = 0.033). Sequence alignment characteristics and scores of the various phylogenetic trees generated are summarized in Table 2; but only the tree inferred from the combined sequence data is presented here (Fig. 1).

Trees inferred from the individual EF-1α and the combined pairs of datasets had the same topology as those inferred from the combined EF-1α + mtSSU + β-tubulin dataset. The combined sequence data separated all the taxa analysed into two well-supported groups consisting of three lineages. One of these groups (93% bootstrap) exclusively consisted isolates of Lineage 1, and corresponded to one of the three previously recognised phylogenetic clades for *F. oxysporum* (O'Donnell *et al.*, 1998b). The second main group (92% bootstrap) consisted of a number of unresolved taxa as well as two strongly supported lineages (Lineage 2 and Lineage 3). Lineage 2 and Lineage 3 corresponded to the other two phylogenetic clades of *F. oxysporum* (O'Donnell *et al.*, 1998b). The unresolved taxa included three Ethiopian isolates (FCC3813, FCC3792, and FCC3797), *F. o.*

redolens, F. o. cubense, and one of the taxa (NRRL25607) representing Clade 2 of O'Donnell *et al.* (1998b). Most of the Ethiopian isolates grouped within Lineage 2. The isolates representing different *formae speciales* of *F. oxysporum* had positions in all the three lineages showing no obvious pattern of grouping.

In trees inferred from the individual EF-1α dataset, the bootstrap support for Lineage 3 was weak, and some isolates from this lineage formed a separate group. There was only a weak bootstrap support for Lineage 2 in trees inferred from the mtSSU partition. The β-tubulin partition did not resolve the isolates, as the groups inferred from this dataset did not have strong bootstrap supports. Because of the higher number of parsimony informative sites the combined pairs of datasets contained, the trees inferred from these datasets had higher bootstrap values than trees inferred from the individual partitions (Table 2).

Inspection of the sequence partitions revealed the presence of EF-1α sites that uniquely differentiated each lineage from the other two as well as from the unresolved taxa (Fig. 4). Two positions (129 and 345) distinguished Lineage 1, one position (397) distinguished Lineage 3, and two positions (68 and 225) distinguished Lineage 2. None of these characters uniquely distinguished the unresolved taxa (FCC3813, FCC3459, FCC3179 and NRRL25607, shown in figure 4). These taxa shared characters 129 and 345 with Lineage 2 and Lineage 3, and character 397 with Lineage 1 and Lineage 2. Comparison of predicted amino acid sequences coded by the EF-1α sequences with relevant amino acid sequences in the GenBank showed that all these unique nucleotide sites were located in intron positions, and did not encode for amino acids. Such distinguishing nucleotide sites were absent from the mtSSU and β-tubulin partitions.

AFLP and SSR analyses

The combined AFLP data separated the Ethiopian isolates and the *formae speciales* of *F. oxysporum* into two major groups (91% bootstrap values each). These groups were the same as those that emerged from the DNA sequence analyses (Fig. 1). One of these major AFLP-based groups corresponded to Lineage 1 that emerged from the sequence data. The second major group included four unresolved taxa, Lineage 2 and Lineage 3. Only isolate FCC3812 grouped in a different lineage to that indicated by DNA sequence comparisons. This isolate grouped in Lineage 2 in the AFLP-based dendrogram (Fig. 2), whereas it had a position in Lineage 3 in the phylogram based on sequence data (Fig. 1).

The individual AFLP datasets differed with regard to the three Ethiopian isolates (FCC3813, FCC3792 and FCC3797) and to *F. o. redolens* and *F. o. pisi*, which did not resolve with respect to Lineage 2 and Lineage 3 in the sequence analyses. These isolates grouped close to Lineage 2 in the PC2 dendrogram, whereas they were distinct from both Lineage 2 and Lineage 3 in the PC1 and PC3 dendrograms (results not shown). Some isolates also changed their positions in all dendrograms. For example, isolates FCC3812 and FCC3183 that resided in Lineage 3 in the sequence analyses, grouped with isolates in Lineage 1 in the PC1 dendrogram. Dendrograms based on the combined pairs of AFLP datasets also showed some discrepancies (results not shown). For example, isolate FCC3461 that resided in Lineage 2 in the sequence analyses, was in Lineage 1 in the PC1 + PC2 dendrogram. The individual PC1 and PC3 datasets, and the combined PC1 + PC3, and PC2 + PC3 datasets had the same topology as the combined PC1 + PC2 + PC3 dendrogram (Fig. 2).

The SSR analysis resolved the Ethiopian isolates and the *formae speciales* into three groups, which broadly corresponded with the three lineages that emerged from the sequence and AFLP analyses. However, the taxa that did not resolve with respect to Lineage 2 and Lineage 3 in the sequence and AFLP analyses, grouped in Lineage 2 in the SSR analyses (Fig. 3). A higher similarity was observed among the isolates using SSR (Fig. 3, ~90% similarity; I = 0.892) than AFLP (Fig. 2, ~75% similarity; I = 0.749). There was a low genotypic diversity among the Ethiopian isolates. However, this estimate was higher using SSR (H = 0.550) than AFLP (H = 0.268). There was a higher genetic diversity among isolates in Lineage 2 than among isolates in Lineage 3 (Table 3). However, the genetic differentiation between the two lineages was low, which was also reflected in the high genetic identity among all the isolates of the two lineages (Table 3).

In the AFLP analysis, PC1 generated the highest number of polymorphic bands and PIC (Table 4). PC2 generated more polymorphic bands than PC3, which suggested that PC2 was more polymorphic (Table 4). However, the information content generated using PC2 was smaller, as measured by the PIC. A similar situation was observed with the combined PC1 + PC2 and PC1 + PC3 datasets. The combined PC1 + PC2 dataset had more polymorphic bands, suggesting that it was more informative than the combined PC1 + PC3 dataset. However, the PIC value indicated otherwise (Table 4). The polymorphism generated using the individual or combined AFLP datasets was much higher than that generated using the SSR dataset, as determined from the PIC analysis.

The datasets generated using the three AFLP PCs were analysed individually and in all possible combinations (Table 4), but only the dendrogram generated using the combined PC1 + PC2 + PC3

data is shown (Fig. 2). The polymorphism generated using the nine SSR primer sets is given in Table 4, and the dendrogram generated using the SSR dataset is shown in Fig. 3.

DISCUSSION

All the three techniques used in this study separated the Ethiopian *F. oxysporum* isolates and the *formae speciales* into three lineages. These lineages that emerged from the SSR, AFLP and DNA sequence analyses were also concordant. Most of the Ethiopian isolates resided in a single lineage, whereas the isolates that represented different *formae speciales* and included in this study for comparative purposes were widely separated in the three lineages. Therefore, the three lineages did not correspond with host or geographical origin of the isolates studied. Sequence analyses also showed that the three lineages emerging from this study were the same as the three clades previously described for *F. oxysporum* by O'Donnell *et al*. (1998b).

None of the three techniques applied in this study provided host-related resolution for the *formae speciales*. The sequence analyses, which included a larger number of different *formae speciales,* also indicated the lack of resolution among the different *formae speciales*. This suggests that phylogenetic groups do not necessarily correlate with virulence groups. Other studies also reported a similar finding (Skovgaard and Rosendahl, 1998; Baayen *et al.*, 2000a; Bao *et al.*, 2002). This result is perhaps not surprising as *formae speciales* are based on a phenotypic character (pathogenicity to specific hosts), which is influenced by a range of factors and not linked to phylogenetic placement.

The results of the phylogenetic analyses correspond largely to those of O'Donnell *et al*. (1998b) and Baayen *et al*. (2000a), since three strongly-supported lineages (Lineages 1 – 3) were also recovered in this study. These lineages corresponded with the three clades (Clades 1 - 3) reported by O'Donnell *et al*. (1998b). However, within the larger Lineage 2-Lineage 3 assemblage, a number of isolates remained unresolved. These results suggest that the *F. oxysporum* species complex might include more than the three main phylogenetic clades designated by O'Donnell *et al*. (1998b). To address this issue many more *F. oxysporum* isolates from diverse sources will need to be evaluated.

Three Ethiopian isolates (FCC3813, FCC3792 and FCC3797), and two isolates representing *F. o. pisi* and *F. o. redolens* did not resolve with respect to Lineage 2 and Lineage 3, based on the sequence and the combined AFLP analyses. However, the SSR analysis placed these isolates within

Lineage 2. Analyses of the individual PC2 dataset and the combined PC1 + PC2 dataset also grouped these isolates close to Lineage 2. An isolate of *F. o. cubense* (NRRL25607) was also amongst the unresolved taxa. This strain represented clone 1 of *F. o. cubense* and was reportedly from *F. oxysporum* Clade 2 (O'Donnell *et al.*, 1998b) based on EF-1α and mtSSU rDNA sequence information. These results suggested that the unresolved taxa may belong to Lineage 2, which corresponds to Clade 2 of O'Donnell *et al.* (1998b).

There were some discrepancies among the results of the three techniques used to resolve the taxonomic position of the Ethiopian *F. oxysporum* isolates. One of these discrepancies was with respect to the position of isolate FCC3812 from Ethiopia. Sequence and SSR analyses placed this isolate in Lineage 3, whereas AFLP placed it in Lineage 2. However, AFLP analysis based on the individual PC2 and PC3 datasets also placed this isolate in Lineage 3. This difference in the position of isolate FCC3812 may be due to the homoplasy associated with AFLP. Homoplasy arises in AFLP because alleles are not easily recognized, allelic fragments are scored as independent where they are not so, and changes in fragment size rather than changes in site are scored (Majer *et al.*, 1996). Similar inconsistencies have been observed in other studies (O'Donnell *et al.*, 1999).

The 32 Ethiopian isolates studied were obtained from various sources and different locations in the country. Consequently, these isolates most probably represent a large proportion of the genetic diversity of the fungus that exists in Ethiopia. The fact that 30 of these isolates grouped in a single lineage is a reflection of the low genetic diversity among the Ethiopian isolates of *F. oxysporum*. The traditional subsistence farming in Ethiopia accounts for most of the crop production in the country, which almost entirely relies on local crop and seed varieties. The low genetic diversity observed among the Ethiopian isolates is, therefore, not surprising since introduction of new genotypes of the fungus via infected seeds from outside is expected to be limited. Edel *et al.* (2001) have also reported low genetic diversities in populations of *F. oxysporum* from soils with similar histories of agricultural practices including tillage, use of pesticides and the type of crop(s) planted. The strains included in this study have not been tested for pathogenicity, and thus cannot be considered relative to this characteristic.

There was a low genetic diversity among the isolates in Lineage 2 and Lineage 3, although the estimate was higher using SSR than AFLP. Only a small amount of the total variation among the isolates was partitioned between the two lineages. This was evident from the low coefficient of genetic differentiation, and the high genetic similarity among all the isolates as well as the small

number of fixed EF-1α sites. The higher genetic variation observed within each of the two lineages using SSR than AFLP may reflect the higher resolution power of SSR at the sub-species level.

The three lineages revealed in this study shared ~74% AFLP-based and close to 90% SSR-based similarity. Considering the minimum AFLP-based similarity of 70% observed within isolates of particular *Fusarium* species (Abdel-Satar *et al.*, 2003; Zeller *et al.*, 2003; Leslie *et al.*, 2004), the three lineages revealed in this study can be considered to constitute the same species. This cut off point for AFLP-based species recognition in *Fusarium*, however, seems to be rather arbitrary. Baayen *et al.* (2000b), for example, have reported AFLP-based similarity of only 40% among some isolates of *F. proliferatum*. Other studies have also revealed that isolates within particular *Fusarium* species may share only 50-55% isozyme-based (Yli-Mattila *et al.*, 1996; Baayen *et al.*, 1997) and RFLP-based (Baayen *et al.*, 1997) similarities. The three lineages revealed in this study, however, represent distinct phylogenetic entities as each had strong bootstrap support. The significance of these lineages and the three clades (O'Donnell *et al.*, 1998b) to which they correspond, remains to be substantiated in terms of agriculture and forestry.

To the best of my knowledge, this is the first report involving analyses of SSR, AFLP and nucleotide sequences, in the study of *F. oxysporum*. The use of all three of the techniques to study all of the isolates made the study demanding in terms of cost, labour and time. A good strategy, especially when working with large number of isolates, would be to use AFLP for an initial assessment of variation. This would allow for a relatively cheap and high throughput genome wide coverage. However, AFLPs overestimate variation. The associated high level of homoplasy also limits their value in phylogenetic studies. To overcome these shortcomings, representative isolates could be studied using DNA sequence analyses. Sequence information from such regions of the DNA as the EF-1α, β-tubulin and mtSSU, accurately reflect evolutionary histories, but care must be taken since sequence information from some regions of the DNA has been shown to be inappropriate and even misleading (Waalwijk *et al.*, 1996; O'Donnell Cigelnik, 1997). SSRs can then be used to assess variation among closely related isolates as their hypervariability provides for a finer resolution. A drawback of SSRs is the high initial cost of development, and the fact that they must be generated *de novo* in each species as the regions that flank the repeats vary considerably among species. When available, however, SSRs are easy to work with, readily transferable, and easy to analyse. This strategy should enable more efficient analyses of genetic diversity in *F. oxysporum*.

F. oxysporum is a complex species composed of non-pathogenic and pathogenic isolates that cause a wide variety of diseases in different plants. Among the techniques that have been used to characterize this important species, the pathogenicity test that is used to group isolates into *formae speciales* and races is practical and important in terms of agriculture and forestry. But, it precludes non-pathogenic isolates. Groups based on vegetative compatibility and the various molecular methods do not always correspond with *formae speciales* or with each other. As such, these groups may not be as meaningful in terms of forestry and agriculture as those based on pathogenicity. A more practical approach would be to use a diversity of techniques supported with pathogenicity tests, since this will allow a more reliable and practical system of classification at the sub-species level.

Table 1. Isolates of the *F. oxysporum* species complex included in this study, their origin and source (host/substrate).

Isolate [a]	*forma specialis*	Origin (host/substrate)	Location
FCC2929	*not determined*	Ethiopia (banana roots)	Arbaminch
FCC3616	"	" (soil)	Wello
FCC3618	"	" (soil)	Wello
FCC3620	"	" (banana roots)	Arbaminch
FCC3624	"	" (banana roots)	Arbaminch
FCC3633	"	" (soil)	East Shoa
FCC3635	"	" (banana roots)	Arbaminch
FCC3636	"	" (soil)	Gondar
FCC3641	"	" (whet stem)	Central Ethiopia
FCC3647	"	" (banana)	Arbaminch
FCC3671	"	" (banana roots)	Arbaminch
FCC3677	"	" (soil)	Gojjam
FCC3692	"	" (banana roots)	Arbaminch
FCC3697	"	" (barley)	Bale
FCC3725	"	" (soil)	Zeway
FCC3746	"	" (soil)	Gojjam
FCC3750	"	" (wheat stem)	Central Ethiopia
FCC3753	"	" (soil)	Gojjam
FCC3755	"	" (banana roots)	Arbaminch
FCC3786	"	" (barley stem)	Central Ethiopia
FCC3788	"	" (soil)	Wello
FCC3792	"	" (soil)	Zeway
FCC3795	"	" (Barley)	Bale
FCC3796	"	" (soil)	West Shoa
FCC3797	"	" (soil)	Zeway
FCC3799	"	" (banana roots)	Arbaminch
FCC3801	"	" (banana roots)	Arbaminch
FCC3802	"	" (pulses)	Gondar
FCC3803	"	" (soil)	West Shoa
FCC3812	"	" (barley)	West Shoa
FCC3813	"	" (soil)	Bale
FCC3819	"	" (soil)	East Shoa
NRRL26035 [b]	*canariensis*	"	-
FCC3460	*chrysanthemi*	CBS	-
FCC3171	*conglutinans*	"	-
NRRL25367 [c]	*cubense*	GenBank	-
NRRL25607 [c]	"	"	-
NRRL25609 [c]	"	"	-
NRRL26029 [c]	"	"	-
NRRL26038 [c]	"	"	-
FCC3461	*cucurbitacearum*	CBS	-
FCC3184	*elaedis*	CBS	-
NRRL26574 [c]	*erytroxyli*	GenBank	-
FCC3173	*gladioli*	CBS	-
FCC3174	*lini*	CBS	-
FCC3189	*lupini*	CBS	-
FCC3175	*lycopersici*	"	-
NRRL26202 [c]	"	GenBank	-
NRRL26203 [c]	"	"	-
NRRL26383 [c]	"	"	-
NRRL26406 [c]	"	"	-

Isolate [a]	*forma specialis*	Origin (host/substrate)	Location
FCC3186	*nicotianae*	CBS	-
FCC3177	*niveum*	"	-
FCC3187	*passiflorae*	CBS	-
NRRL22549 [c]	"	GenBank	-
FCC3188	*perniciosum*	CBS	-
FCC3178	*phaseoli*	CBS	-
FCC3179	*pisi*	"	-
FCC3180	*radicislycopercisi*	CBS	-
NRRL26033 [c]	"	GenBank	-
NRRL26380 [c]	"	"	-
NRRL26381 [c]	"	"	-
FCC3181	*raphanai*	CBS	-
FCC3459	*redolens*	"	-
NRRL22555 [c]	*tuberosi*	"	-
FCC3183	*vasinfectum*	CBS	-

[a] Only the Ethiopian and CBS isolates were studied using the SSR, AFLP and DNA sequence analyses. For GenBank strains, relevant sequences were downloaded from the database and used in the sequence analyses.

[b] Unpublished.

[c] (O'Donnell *et al.*, 1998b).

Table 2. Sequence alignment characteristics and phylogenetic tree scores inferred from the individual EF-1α, mtSSU and β-tubulin partitions and the combined datasets using 98 taxa.

Dataset	Aligned sites[a]	Constant Sites[b]	Pars. Info. Sites[c]	MPTs[d]	Tree scores[e]	Nodes >50% BS[f]
EF-1α	676	590 87.3%	38 5.62%	98	L = 104, CI = 0.865, RI = 0.953, RC = 0.825	21
β-tubulin	278	263 94.6%	2 0.72%	12	L = 54, CI = 0.296, RI = 0.050, RC = 0.015	1
mtSSU	213	189 88.7%	11 5.16%	26	L = 27, CI = 0.926, RI = 0.984, RC = 0.911	5
EF-1α+ β-tubulin	954	848 88.9%	44 4.61%	105	L = 141, RI = 0.924, RC = 0.734, CI = 0.794	22
EF-1α + mtSSU	889	773 87.0%	49 5.51%	116	L = 122, CI = 0.943, RI = 0.984, RC = 0.927	25
β-tubulin + mtSSU	491	451 91.9%	13 2.65	48	L = 61, CI = 0.721, RI = 0.903, RC = 0.651	5
EF-1α + β-tubulin + mtSSU	1167	1036 88.8%	56 4.80%	120	L = 172, CI = 0.824, RI = 0.943, RC = 0.778	27

[a] Number of aligned sites including gaps.

[b] Number of constant sites.

[c] Number of parsimony informative sites.

[d] Number of most parsimonious trees.

[e] Tree scores (L = tree length, CI = consistency index, RI = retention index, RC = rescaled consistency index).

[f] Number of nodes with bootstrap values of 50% or more.

Table 3. Genetic diversity and population differentiation statistics between Lineage 1 and Lineage 2 as determined from the SSR and AFLP analyses.

	Using AFLP					Using SSR			
	H	δ_T	G_{ST}	I		H	δ_T	G_{ST}	I
Lineage 2	0.608	0.684	0.008	0.799	Lineage 2	0.956	0.981	0.036	0.910
Lineage 3	0.606	0.622			Lineage 3	0.851	0.957		

Table 4. The number of polymorphic bands, effective number of alleles per locus, polymorphic information content, and co-phenetic correlation coefficients of the three AFLP PCs and the SSR markers.

Primer combination	Number of polymorphic bands	Polymorphic information content (*PIC*)	Cophenetic correlation coefficient
E-CC/M-TA (PC1)	31	0.351	0.844
E-TC/M-AA (PC2)	26	0.280	0.899
E-TC/M-AG (PC3)	24	0.290	0.901
PC1 + PC2	57	0.318	0.882
PC1 + PC3	55	0.325	0.878
PC2 + PC3	50	0.285	0.900
PC1 + PC2 + PC3	81	0.310	0.882
SSR (nine primer pairs)	65	0.156	0.879

Fig. 1. One of 133 MPTs inferred from the combined EF-1α-β-tubulin-mtSSU data set using the Ethiopian isolates, *formae speciales*, and *F. oxysporum* sequences from GenBank. FCC numbers followed by Eth indicate Ethiopian isolates, whereas FCC numbers followed by names of *formae speciales* indicate isolates obtained from the CBS. Names of *formae speciales* followed by NRRL numbers indicate strains for which sequences were downloaded from GenBank. *Fusarium* sp. NRRL125184 was used as outgroup. Bootstrap values of 70% and higher are shown above nodes.

Fig. 2. Dendrogram generated using the combined PC1-PC2-PC3 AFLP dataset for the *F. oxysporum* isolates obtained from Ethiopia (indicated by strain numbers) and *formae speciales* obtained from the CBS (names of *formae speciales* indicated). Bootstrap values of 50% and more are indicated above nodes. The scale bar indicates the dissimilarity index.

Fig. 3. Dendrogram generated using the SSR dataset for the *F. oxysporum* isolates obtained from Ethiopia (indicated by strain numbers) and *formae speciales* obtained from the CBS (names of *formae speciales* indicated). Bootstrap values of 50% and higher are indicated above nodes. The scale bar indicates the index of dissimilarity.

Fig. 4. The partial EF-1α sequences (5'→3', sites 60 – 400) of EF-1α haplotypes representing the three lineages and the unresolved taxa (isolates that did not resolve with respect to Lineage 2 and Lineage 3 in the sequence-based phylogram, Fig. 1). The shaded sites show nucleotides that uniquely distinguish particular lineages. The dots indicate nucleotides that are identical to the ones in the first sequence at the respective sites. The numbers at the top of sequences indicate nucleotide sites.

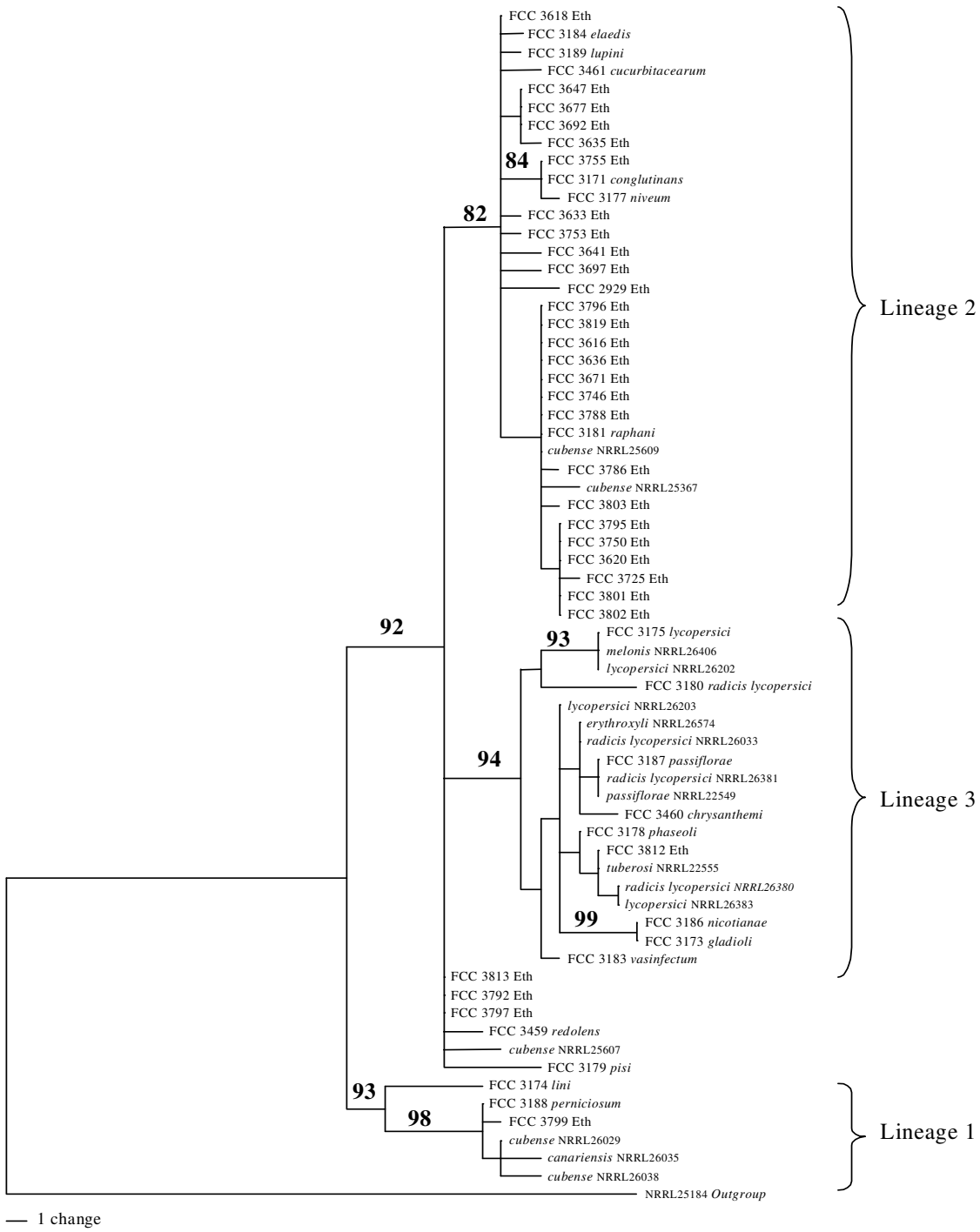
FCC 3618 Eth
FCC 3184 elaedis
FCC 3189 lupini
FCC 3461 cucurbitacearum
FCC 3647 Eth
FCC 3677 Eth
FCC 3692 Eth
FCC 3635 Eth
84
FCC 3755 Eth
FCC 3171 conglutinans
FCC 3177 niveum
82
FCC 3633 Eth
FCC 3753 Eth
FCC 3641 Eth
FCC 3697 Eth
FCC 2929 Eth
FCC 3796 Eth
FCC 3819 Eth
FCC 3616 Eth
FCC 3636 Eth
FCC 3671 Eth
FCC 3746 Eth
FCC 3788 Eth
FCC 3181 raphani
cubense NRRL25609
FCC 3786 Eth
cubense NRRL25367
FCC 3803 Eth
FCC 3795 Eth
FCC 3750 Eth
FCC 3620 Eth
FCC 3725 Eth
FCC 3801 Eth
FCC 3802 Eth
Lineage 2
92
93
FCC 3175 lycopersici
melonis NRRL26406
lycopersici NRRL26202
FCC 3180 radicis lycopersici
lycopersici NRRL26203
erythroxyli NRRL26574
radicis lycopersici NRRL26033
94
FCC 3187 passiflorae
radicis lycopersici NRRL26381
passiflorae NRRL22549
FCC 3460 chrysanthemi
FCC 3178 phaseoli
FCC 3812 Eth
tuberosi NRRL22555
radicis lycopersici NRRL26380
lycopersici NRRL26383
99
FCC 3186 nicotianae
FCC 3173 gladioli
FCC 3183 vasinfectum
Lineage 3
FCC 3813 Eth
FCC 3792 Eth
FCC 3797 Eth
FCC 3459 redolens
cubense NRRL25607
FCC 3179 pisi
93
FCC 3174 lini
FCC 3188 perniciosum
98
FCC 3799 Eth
cubense NRRL26029
canariensis NRRL26035
cubense NRRL26038
Lineage 1
NRRL25184 Outgroup
1 change

Fig. 1

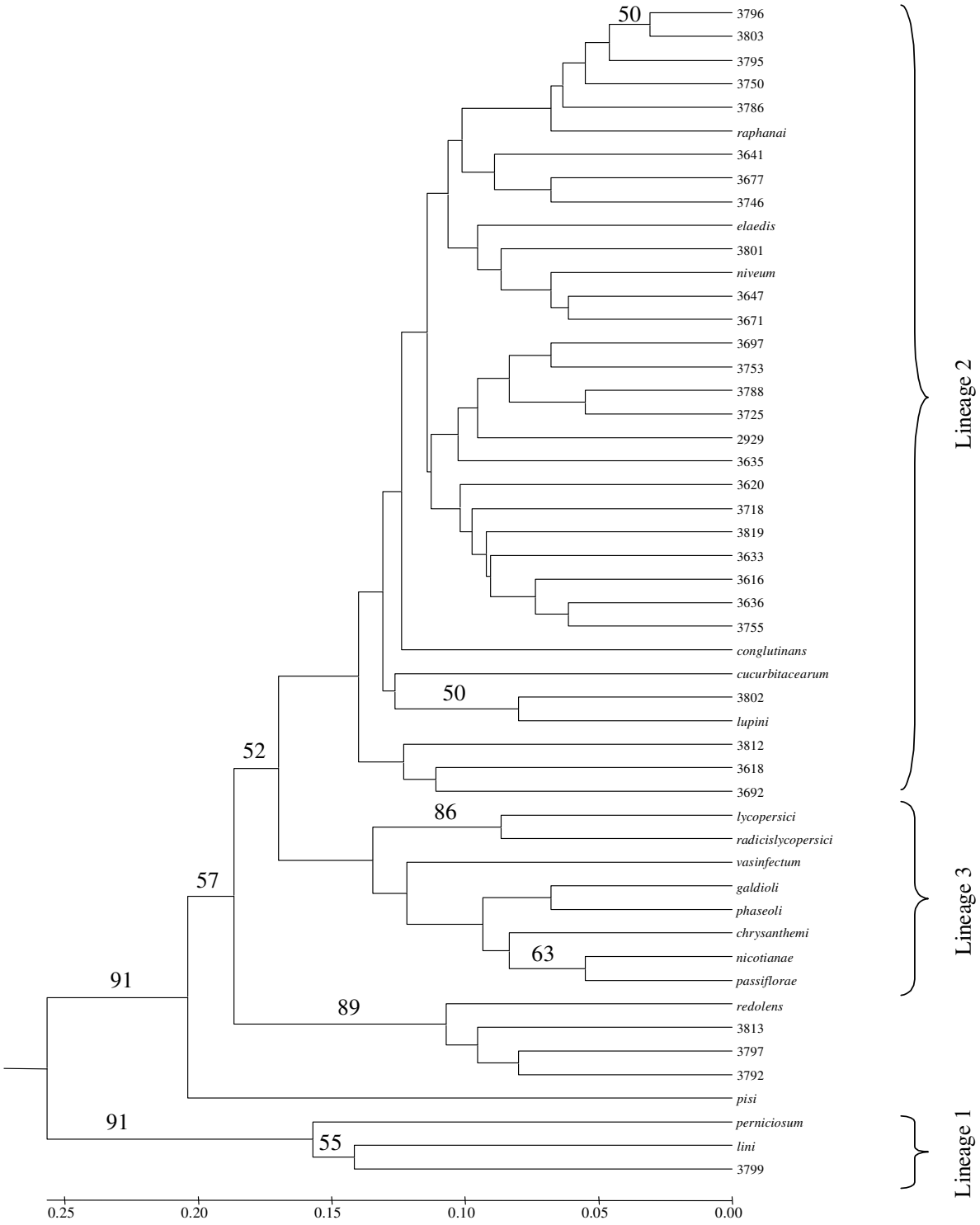
50
3796
3803
3795
3750
3786
raphanai
3641
3677
3746
elaedis
3801
niveum
3647
3671
3697
3753
3788
3725
2929
3635
3620
3718
3819
3633
3616
3636
3755
conglutinans
cucurbitacearum
50
3802
lupini
52
3812
3618
3692
86
lycopersici
radicislycopersici
vasinfectum
57
galdioli
phaseoli
chrysanthemi
63
nicotianae
passiflorae
91
89
redolens
3813
3797
3792
pisi
91
perniciosum
55
lini
3799
0.25
0.20
0.15
0.10
0.05
0.00
Lineage 2
Lineage 3
Lineage 1

Fig. 2

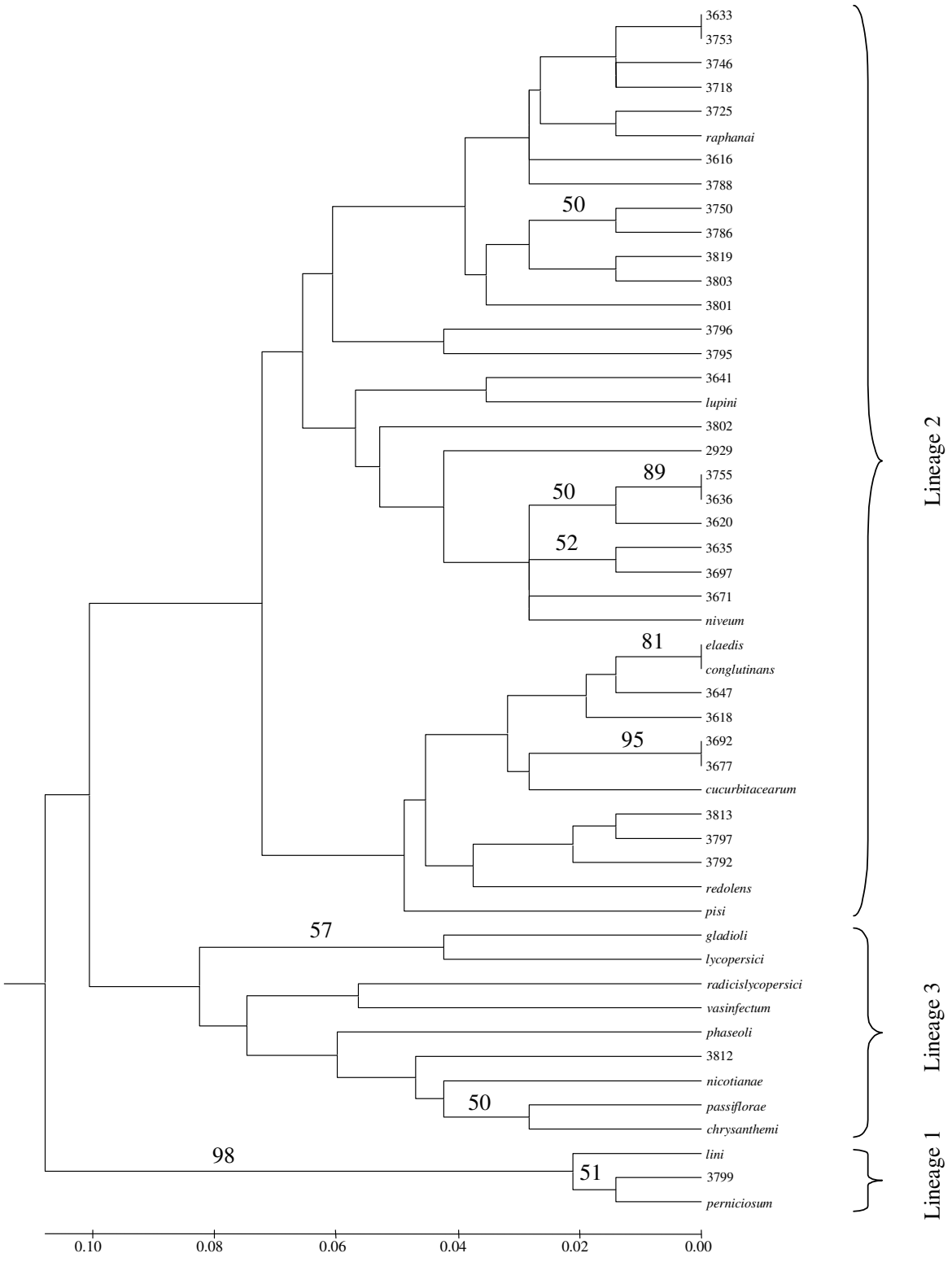
3633
3753
3746
3718
3725
raphanai
3616
3788
50
3750
3786
3819
3803
3801
3796
3795
3641
lupini
3802
2929
89
3755
50
3636
3620
52
3635
3697
3671
niveum
81
elaedis
conglutinans
3647
3618
95
3692
3677
cucurbitacearum
3813
3797
3792
redolens
pisi
57
gladioli
lycopersici
radicislycopersici
vasinfectum
phaseoli
3812
nicotianae
50
passiflorae
chrysanthemi
98
lini
51
3799
perniciosum
0.10
0.08
0.06
0.04
0.02
0.00
Lineage 2
Lineage 3
Lineage 1

Fig. 3

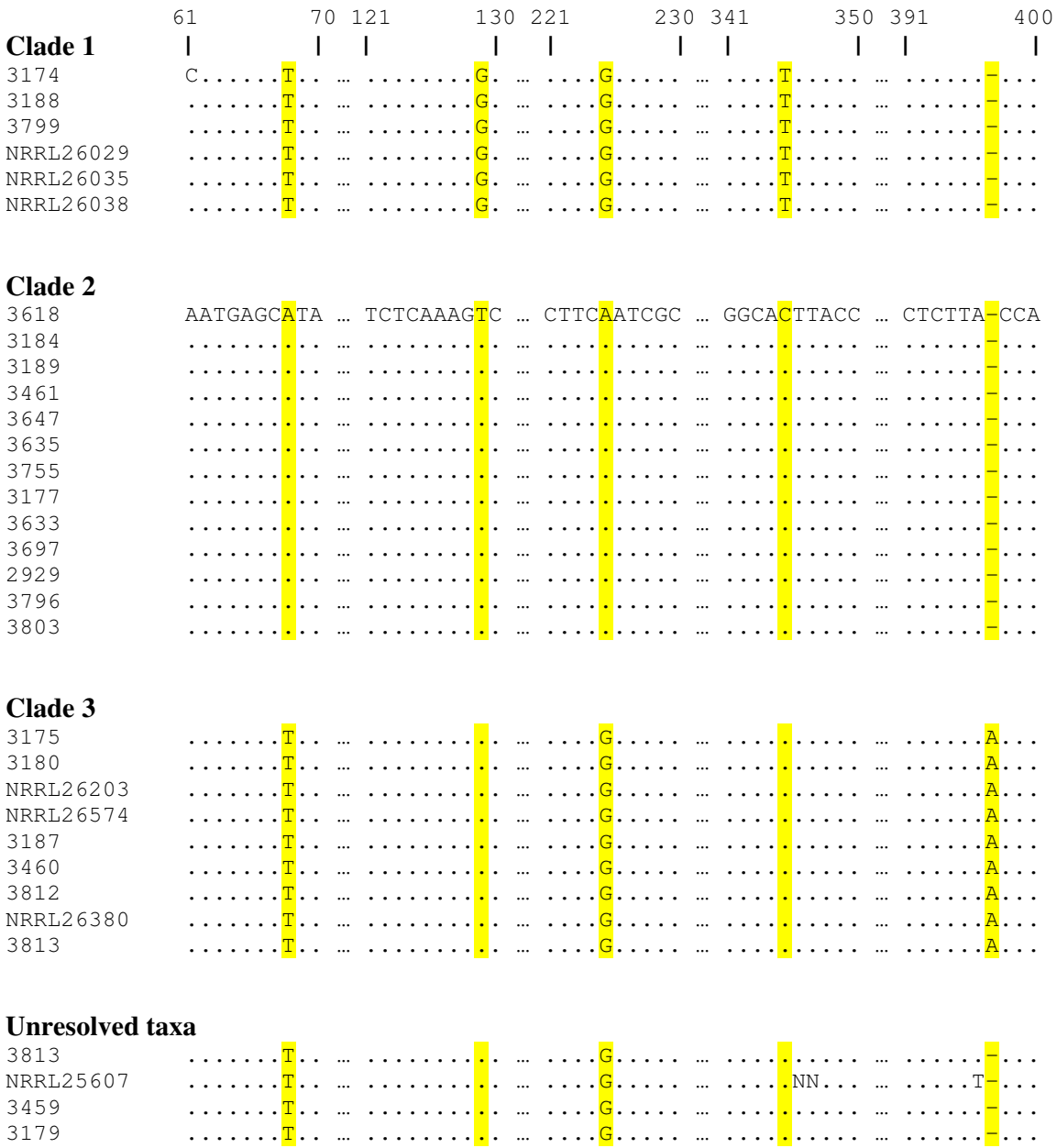
61 70 121 130 221 230 341 350 391 400
Clade 1
3174 C.....T.. … .....G. … ....G..... … ....T..... … .....-...
3188 .....T.. … .....G. … ....G..... … ....T..... … .....-...
3799 .....T.. … .....G. … ....G..... … ....T..... … .....-...
NRRL26029 .....T.. … .....G. … ....G..... … ....T..... … .....-...
NRRL26035 .....T.. … .....G. … ....G..... … ....T..... … .....-...
NRRL26038 .....T.. … .....G. … ....G..... … ....T..... … .....-...
Clade 2
3618 AATGAGCATA … TCTCAAAGTC … CTTCAATCGC … GGCACTTACC … CTCTTA-CCA
3184 .....T.. … .....G. … ....G..... … ....T..... … .....-...
3189 .....T.. … .....G. … ....G..... … ....T..... … .....-...
3461 .....T.. … .....G. … ....G..... … ....T..... … .....-...
3647 .....T.. … .....G. … ....G..... … ....T..... … .....-...
3635 .....T.. … .....G. … ....G..... … ....T..... … .....-...
3755 .....T.. … .....G. … ....G..... … ....T..... … .....-...
3177 .....T.. … .....G. … ....G..... … ....T..... … .....-...
3633 .....T.. … .....G. … ....G..... … ....T..... … .....-...
3697 .....T.. … .....G. … ....G..... … ....T..... … .....-...
2929 .....T.. … .....G. … ....G..... … ....T..... … .....-...
3796 .....T.. … .....G. … ....G..... … ....T..... … .....-...
3803 .....T.. … .....G. … ....G..... … ....T..... … .....-...
Clade 3
3175 .....T.. … .....G. … ....G..... … ....T..... … .....A...
3180 .....T.. … .....G. … ....G..... … ....T..... … .....A...
NRRL26203 .....T.. … .....G. … ....G..... … ....T..... … .....A...
NRRL26574 .....T.. … .....G. … ....G..... … ....T..... … .....A...
3187 .....T.. … .....G. … ....G..... … ....T..... … .....A...
3460 .....T.. … .....G. … ....G..... … ....T..... … .....A...
3812 .....T.. … .....G. … ....G..... … ....T..... … .....A...
NRRL26380 .....T.. … .....G. … ....G..... … ....T..... … .....A...
3813 .....T.. … .....G. … ....G..... … ....T..... … .....A...
Unresolved taxa
3813 .....T.. … .....G. … ....G..... … ....T..... … .....-...
NRRL25607 .....T.. … .....G. … ....G..... … .....NN... … .....T-...
3459 .....T.. … .....G. … ....G..... … ....T..... … .....-...
3179 .....T.. … .....G. … ....G..... … ....T..... … .....-...

Fig. 4.

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CHAPTER 6

Species-specific primers for *Fusarium redolens* and a PCR-RFLP technique to distinguish among the three clades of *Fusarium oxysporum*

ABSTRACT

Differentiation between *Fusarium redolens* and *F. oxysporum* based on morphology is problematic. This is mainly due to isolates of the two species having intermediate forms. *F. oxysporum* is a complex species composed of three phylogenetically distinct clades, which are designated based on DNA sequence information. Currently, *F. redolens* and *F. oxysporum* are differentiated using a DNA-based method. However, this method is expensive, laborious, and lacks robustness, as it does not differentiate *F. redolens* from *F. hostae*. The aligned translation Elongation Factor 1α (EF-1α) gene sequences of *F. redolens, F. oxysporum* and their close relatives, namely, *F. hostae*, *F. foetens, F. commune, F. nisikadoi*, and *F. miscanthi,* were used to design *F. redolens*-specific primers. Restriction enzyme sites that allow differentiation among the three clades of *F. oxysporum* were also identified from the aligned sequences. The *F. redolens*-specific primers did not result in a PCR product in any other *Fusarium* species studied here. More importantly, these primers differentiated *F. redolens* and *F. oxysporum* based only on the presence and absence, respectively, of a PCR amplification product. Restriction Fragment Length Polymorphism (RFLP) analyses of EF-1α products from different *formae speciales* of *F. oxysporum* separated the isolates into three groups. These groups fully corresponded with the three clades of *F. oxysporum*. The PCR-RFLP technique developed here will provide a cheaper alternative for grouping *F. oxysporum* isolates into these clades. The technique also eliminates the need for inclusion of isolates representing these clades for comparative purposes.

INTRODUCTION

The species composition of section *Elegans* in the genus *Fusarium* has been the subject of much debate. This is mainly due to the application of different taxonomic systems. For example, Wollenweber (1913) placed *F. lycopersici, F. oxysporum, F. niveum, F. redolens, F. tracheiphilum*, and *F. vasinfectum* in this section. Gerlach and Nirenberg (1982), confined the section *Elegans* to *F. oxysporum, F. redolens* and *F. udum*. However, Snyder and Hansen (1940) recognized the six taxa in Wollenweber's (1913) section *Elegans* and Gerlach and Nirenberg's (1982) *F. udum* as synonymous with or as varieties of *F. oxysporum*. Nelson *et al*. (1983) also treated the taxa in Wollenweber's (1913) section *Elegans* in a manner similar to Snyder and Hansen (1940), but recognized Gerlach and Nirenberg's (1982) *F. udum* as an insufficiently documented species, which may belong to either section *Elegans* or section *Lateritium*.

The taxonomic position of *F. redolens* has also been problematic. Wollenweber (1913) recognized *F. redolens* as a distinct species. Booth (1971) treated this fungus as a variety of *F. oxysporum*, whereas Nelson *et al*. (1983) recognized *F. redolens* as a synonym of *F. oxysporum*. This controversy continued until the distinction between the two species was defined using molecular methods (Waalwijk *et al.*, 1996; O'Donnell *et al.*, 1998a; Gams *et al.*, 1999; Baayen *et al.*, 2000a). These studies revealed that *F. redolens* and *F. oxysporum* are not only different species, but also that they even lack a sister group relationship. For example, Baayen *et al*. (2001) showed that the *F. oxysporum* complex is more closely related to the *F. nisikadoi-F. miscanthi* complex than it is to the *F. redolens-F. hostae* complex.

Morphological dinstinction between *F. oxysporum* and *F. redolens* is mainly based on the sizes of their macroconidia (Gordon, 1952). This differentiation is, however, complicated due to the presence of intermediate forms of the fungi (Baayen and Gams, 1988). The two species are currently diagnosed based on Restriction Fragment Length Polymorphisms (RFLP) of their Internal Transcribed Spacer (ITS) regions (Waalwijk *et al.*, 1996). But this PCR-RFLP technique does not allow for differentiation of *F. redolens* from its close relative *F. hostae* (Baayen *et al.*, 2001). The technique is also financially and technically demanding as it calls for the use of three restriction enzymes.

The controversy surrounding the taxonomy of *Fusarium* Section *Elegans* extends beyond the question of whether or not the five taxa in Wollenweber's circumscription are synonymous with or varieties of *F. oxysporum*. *F. oxysporum* consists of a wide array of non-pathogenic strains as well

as many important pathogens. The species is best known for its pathogenic members that are specialized into more than 120 forms and races, each infecting particular hosts or a group of host species (Armstrong and Armstrong, 1981). A large number of the *formae speciales* infect only one host species. For example, *formae speciales tulipae, crocus* and *apii* only infect, respectively, tulips, crocus and celery (Correll *et al.*, 1986). Some *formae speciales* are known to have monophyletic origins; *formae speciales spinaciae*, *lilii*, *radicis-cucumerinum*, *oppontiarum* and *tulipae* (Baayen *et al.*, 2000a) are examples of this. Currently, however, *F. oxysporum* is regarded as a complex of morphologically similar fungi with multiple phylogenetic origins consisting of three well-supported clades (O'Donnell *et al.*, 1998b; Baayen *et al.*, 2000a).

The significance of the three *F. oxysporum* clades in terms of pathogenicity, source or geographical origin of the constituting isolates has not been substantiated. The grouping of isolates in these clades requires DNA sequence information and the inclusion of representative isolates for comparative purposes. DNA sequencing is, however, complex, expensive and not commonly available to plant pathologists. The use of AFLP for this purpose is also limited by the need for inclusion of isolates representing each of these clades (*e.g.*, Chapter 5 of this thesis). Consequently, a cheaper technique that does not require the inclusion of representative isolates would be useful for grouping isolates into these distinct clades.

The objective of this study was to develop a simple diagnostic technique for routine identification of *F. redolens*. A PCR-RFLP technique that enables easy and rapid identification of the fungi residing in the three clades of *F. oxysporum* defined by O'Donnell *et al*. (1998b) was also developed.

MATERIALS AND METHODS

Isolates

Some of the *Fusarium* isolates (Table 1) included in this study were obtained from agricultural soils and plant tissues collected in a previous study (Chapter 2 of this thesis). Fifteen isolates representing 15 *formae speciales* of *F. oxysporum* were also obtained from the Centraalbureau voor Schimmelcultures (CBS), Utrecht, Netherlands, and included in this study for comparative purposes. Isolates of *F. redolens, F. hostae, F. commune* and *F. foetens*, obtained from the United States Department of Agriculture (USDA), were also used. All isolates used in this study are also maintained in the *Fusarium* Culture Collection (FCC) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

Sequence alignment

EF-1α (translation Elongation Factor 1α) gene sequences for all *F. redolens*, *F. hostae, F. commune, F. miscanthi*, and *F. nisikadoi* isolates, and for isolates representing the three clades of *F. oxysporum* (O'Donnell *et al.*, 1998b; Baayen *et al.*, 2000a) were downloaded from the NCBI (National Center for Biotechnology Information, www.ncbi.nih.gov) database (GenBank) and aligned using ClustalX (Version 1.8, Thompson *et al.*, 1997). EF-1α sequences that were generated for *F. oxysporum* and *F. redolens* isolates obtained from various sources in Ethiopia in a previous study (Chapter 5 of this thesis) were also included in the alignment. This alignment was used to develop *F. redolens*-specific PCR primers and to identify restriction enzymes that could differentiate among the three clades of *F. oxysporum*. The latter involved *in silico* restriction analyses of *F. oxysporum* EF-1α sequence haplotypes using Vector NTI (Version 9.0.0, InforMax, USA).

Extraction of DNA

DNA was extracted using the CTAB (N-cetyl-N,N,N-trimethyl-ammonium bromide) method (Murray and Thompson, 1980) from isolates grown on malt extract medium (2% [w/v] malt extract, 1.5% [w/v] agar). For this purpose, mycelium was scraped from the surface of 10–15 day-old cultures and transferred to Eppendorf tubes containing sterile sand and 700 µL extraction buffer (5% [w/v] CTAB, 1.4 M NaCl, 0.2% [v/v] 2-mercaptoethanol, 20 mM EDTA [ethylene diamine tetraacetate, pH 8.0], 10 mM Tris-HCl [pH 8.0], and 1% [w/v] polyvinylpyrrolidone). The mixture was pulverized; incubated for 1 hr at 60°C, and centrifuged at 9,300 *g* for 10 min. DNA was extracted from the aqueous phase by repeated phenol-chloroform (1:1) extractions followed by a final chloroform extraction to remove residual phenol. The DNA was then precipitated with 0.1 volume of 3 M sodium acetate (pH 5.4) and 0.6 volume of 2-propanol, and incubated at -20°C overnight. Precipitated DNA was pelleted by centrifugation at 15,700 *g* for 30 min at 4°C, washed with 70% ethanol, air-dried, and dissolved in deionised water.

PCR amplification

For RFLPs, a ~ 656 base pair (bp) fragment of the EF-1α gene was PCR-amplified from each isolate using primers EF1 and EF2 (EF-1α primers, O'Donnell *et al.*, 1998b). PCR mixtures contained reaction buffer (10 mM Tris-HCl [pH 8.3], 2.5 mM $MgCl_2$, 50 mM KCl), 2.5 µM of each dNTP, 0.20 µM of each primer, 0.5 M betaine, 0.05 U/µL *Taq* Polymerase (Roche, USA), and approximately 4 ng/µL template DNA. The PCR cycling conditions consisted of an initial

denaturation at 94°C for 4 min, followed by 35 cycles of denaturation, annealing (58°C), and extension (72°C), each for 30 sec. PCRs were terminated after a final extension for 10 min.

For PCR-based identification of *F. redolens* isolates, the *F. redolens*-specific primers developed in this study (Redolens-F and Redolens-R, see below) were used, in a multiplex PCR where primers that amplify a fragment of the LSU (large subunit, LR3: 5'-CCGTGTTTCAAGACGGG-3' and LR3-RR: 5'-CGAATCTTTGAACGCACATTG-3') were also included. This was done in order to use the amplification product of these primers as an internal control for a successful PCR. The PCR mixture and cycling conditions for this multiplex PCR were the same as in the PCRs, where only the EF-1α primers were used. However, 0.20 µM of each of the *F. redolens*-specific primers was also included in the mixture for the multiplex PCR. The cycling condition for the multiplex PCR also consisted of a touch down phase of 10 cycles, where the annealing temperature was progressively reduced by 0.4°C/cycle starting from 62°C. This was followed by 30 cycles at an annealing temperature of 58°C.

Restriction digestion

Amplified products were used in restriction digestion reactions without prior purification. For digestions using endonuclease *Mse*I (Biolabs, England), the reaction mixture (30 µL, pH 7.9) consisted of 0.15 U/µL of the enzyme, 0.1 µg/µL bovine serum albumin, 3.0 µL NEB2 buffer (Biolabs, Engaland) and 25 µL of the PCR product. For restriction digestions using *Alu*I (Roche, USA), the reaction mixture (30 µL, pH 7.9) consisted of 0.15 U/µL of the enzyme, 3.0 µL of buffer A (Roche), and 25 µL of the PCR product. All restriction digestions were done on a GeneAmp PCR System 9700 (Applied Biosystems, USA) at 37°C for 4 hrs to ensure completion of the digestion. Restriction fragments were resolved by electrophoresis at 4 V/cm on 3% agarose gels containing ethidium bromide (0.25 µg/ml); and visualized by UV illumination.

RESULTS

The aligned EF-1α sequences generated in a previous study (Chapter 2 of this thesis) using primers EF1 and EF2, as well as those obtained from GenBank, revealed several insertions/deletions (indels) among sequences from the various species included. The presence of these regions in *F. redolens* isolates were used to design *F. redolens*-specific primers (Redolens-F: 5'-CTT TCG TCA ATC CCG ACC AAG-3'; Redolens-R: 5'-CAA TGA TGA TTG TGA TGA GAC-3'). Multiplex

PCR using these *F. redolens*-specific primers and the LSU primers, resulted in two fragments only in *F. redolens* isolates (Fig. 1). The larger fragment corresponded to amplification product of the LSU primers, whereas the smaller fragment corresponded to that of the *F. redolens*-specific primers. For isolates of other *Fusarium* spp. included in this study, this multiplex PCR resulted only in a single fragment, which corresponded to amplification product of the LSU primers (Fig. 1). The only exception to this was *F. oxysporum dianthi* (FCC3172), where the multiplex PCR resulted in two fragments as in the *F. redolens* isolates (Fig. 1).

The *F. oxysporum* isolates obtained from Ethiopia, and the 15 *formae speciales* obtained from the CBS could be separated into three groups based on RFLP patterns of their EF-1α PCR products. In one group of isolates, digestion of this amplicon using *Mse*I resulted in two fragments (Fig. 2A). This group (Group 3) corresponded to Clade 3 of O'Donnell *et al*. (O'Donnell *et al.*, 1998b). Among the remaining isolates, which lacked an *Mse*I recognition site, restriction of the EF-1α amplicon using *Alu*I revealed two groups (Groups 1 and 2). In the first group of isolates (Group I), three *Alu*I restriction sites were observed; and when the PCR fragment was digested with this enzyme, four fragments resulted (Figs. 2B and 3). This group of isolates (Group 1) corresponded to Clade 1 of O'Donnell *et al*. (1998b). Among the second group of isolates (Group 2), restriction digestion using *Alu*I resulted in three fragments (Figs. 2B and 3). This group (Group 2) corresponded to Clade 2 of O'Donnell *et al*. (1998b). Group 1 and Group 2 isolates could easily be distinguished based on the differing sizes of their largest fragments (386 bp and 452 bp fragments, respectively, Fig. 3).

DISCUSSION

In this study, species-specific primers to identify *F. redolens* isolates by PCR were developed. In *F. redolens* isolates, multiplex PCR using these species-specific primers and the LSU primers resulted in two products, one of which was *F. redolens*-specific. This *F. redolens*-specifc amplification product differentiated *F. redolens* from all the other *Fusarium* spp. included in this study. A PCR-RFLP technique that was diagnostic for the three clades of *F. oxysporum* previously designated by O'Donnell *et al*. (1998b) was also developed .The *F. redolens*-specific primers developed in this study will thus allow rapid and simple diagnoses of *F. redolens* isolates as distinct from isolates of *F. oxysporum*. Also, the PCR-RFLP diagnostic technique will provide a cheaper alternative for grouping *F. oxysporum* isolates into the three well-known clades, previously designated for this species complex.

The *F. redolens*-specific primers developed in this study amplified a fragment of the EF-1α gene only in *F. redolens* isolates. These primers did not amplify PCR products in the remaining *Fusarium* isolates considered. The significance of the *F. redolens* primers lies particularly in the fact that they allow for differentiation between *F. redolens* and *F. oxysporum*, based on the presence or absence, respectively, of PCR amplification products for the two species. Both species reside in *Fusarium* section *Elegans* because of their morphological similarity although they share only ~35% AFLP-based genetic similarity (Baayen *et al.*, 2000b). The *F. redolens*-specific primers developed in this study allow for differentiation between *F. redolens* and *F. oxysporum,* without the need for a further RFLP analysis. The *F. redolens*-specific primers resulted in amplification in *F. oxysporum* f.sp. *dianthi* (FCC3172, Fig. 1) This isolate (CBS 491.97) was originally collected from the Netherlands, and was reported to be pathogenic to *Dianthus* spp. Some isolates of both *F. oxysporum* and *F. redolens* are known to attack *Dianthus* spp. Such isolates of *F. oxysporum* are placed in *F. oxysporum* f.sp. *dianthi*, whereas those of *F. redolens* are placed in *F. redolens* f.sp. *dianthi* (Waalwijk *et al.*, 1996; Baayen *et al.*, 1997). Some *F. r. dianthi* isolates were first misidentified as *F. o. dianthi* isolates. After the development of the ITS-RFLP technique for distinguishing between isolates of the two species (Waalwijk *et al.*, 1996) these isolates were correctly identified as *F. r. dianthi* (Baayen *et al.*, 1997). I therefore believe that isolate FCC3172 represents *F. r. dianthi* and not *F. o. dianthi*. The EF-1α sequence from this isolate is also identical to those obtained from the Ethiopian *F. redolens* isolates and the *F. redolens* sequences in GenBank and the *Fusarium* database (http://fusarium.cbio.psu.edu).

The PCR-RFLP technique developed in this study provides an easy means of distinction between the three clades of *F. oxysporum*. The presence of an *Mse*I recognition site in the EF-1α PCR products of Group 3 (Clade 3, O'Donnell *et al.*, 1998b) isolates uniquely distinguishes this clade. The distinction between isolates of the other two groups (Groups 1 and 2, respectively corresponding to Clades 1 and 2, O'Donnell *et al.*, 1998b) is made based on the sizes of the largest *Alu*I restriction fragments from these isolates. Restriction analyses of the EF-1α PCR product from *F. oxysporum* isolates using *Mse*I and then using *Alu*I (in isolates where the *Mse*I recognition site is lacking) will enable grouping of the isolates into the three clades. This PCR-RFLP approach was found to be robust as restriction maps and gel patterns generated in Vector NTI using relevant GenBank EF-1α sequences (data not shown) corresponded with the results obtained in the laboratory (Figs. 2 and 3).

Accurate identification of *Fusarium* spp. is crucial for the development of control strategies and programmes aimed at breeding for resistance to pathogens. The common approach of using morphology for identification has many limitations. These include morphological instability in many *Fusarium* isolates, the application of different taxonomic systems, and the lack of mycologists with adequate experience to identify these fungi. The *F. redolens*-specific primers developed in this study should be useful in promoting unambiguous and easy differentiation between *F. redolens* and *F. oxysporum* isolates. The PCR-RFLP technique developed for *F. oxysporum* should also make it possible to easily place isolates in one of the three clades for these fungi defined by O'Donnell *et al*., (1998b). Although the significance of fungi residing in these clades has not been clearly shown, it is believed that the fungi in these clades represent phylogenetically discrete entities. It is likely that recognising them will be important in the future and the PCR-RFLP technique developed in this study should facilitate the process.

Table 1. Isolates of *Fusarium* used in this study.

Species	Strain number	Source
F. acutatum	FCC4638	Ethiopia
F. avenaceum	FCC4645	Ethiopia
F. commune	NRRL28387	USDA
F. commune	NRRL31076	USDA
F. dlaminii	FCC4639	Ethiopia
F. equiseti	FCC4647	Ethiopia
F. foetens	NRRL31852	USDA
F. hostae	NRRL29642	USDA
F. hostae	NRRL29889	USDA
F. lactis	FCC4637	Ethiopia
F. oxysporum f.sp. *chrysanthemi*	FCC3460	CBS
F. oxysporum f.sp. *conglutinans*	FCC3171	CBS
F. oxysporum f.sp. *cucurbitacearum*	FCC3461	CBS
F. oxysporum f.sp. *dianthi*	FCC3172	CBS
F. oxysporum f.sp. *elaedis*	FCC3184	CBS
F. oxysporum f.sp. *gladioli*	FCC3173	CBS
F. oxysporum f.sp. *lini*	FCC3174	CBS
F. oxysporum f.sp. *lupini*	FCC3189	CBS
F. oxysporum f.sp. *lycopersici*	FCC3175	CBS
F. oxysporum f.sp. *nicotianae*	FCC3186	CBS
F. oxysporum f.sp. *niveum*	FCC3177	CBS
F. oxysporum f.sp. *passiflorae*	FCC3187	CBS
F. oxysporum f.sp. *perniciosum*	FCC3188	CBS
F. oxysporum f.sp. *phaseoli*	FCC3178	CBS
F. oxysporum f.sp. *radicis-lycopersici*	FCC3180	CBS
F. oxysporum f.sp. *raphanai*	FCC3181	CBS
F. redolens	FCC4640	Ethiopia
F. redolens	FCC4641	Ethiopia
F. redolens	FCC4642	Ethiopia
F. redolens	FCC4643	Ethiopia
F. redolens	FCC4644	Ethiopia
F. redolens	NRRL25600	USDA
F. redolens	NRRL28381	USDA
F. solani	FCC4631	Ethiopia

Fig. 1. Multiplex PCR products obtained using the LSU and the *F. redolens*-specific primer sets from 22 *Fusarium* isolates representing 11 species. Lanes 1-7, respectively, are products from *F. redolens* isolates NRRL25600, NRRL28381, FCC4640, FCC4641, FCC4642, FCC4643 and FCC4644. Lane 8 is *F. o. dianthi* (FCC3172). Lane 9 is a 100-bp ladder marker. Lanes 10-24, respectively, are products from NRRL29642 (*F. hostae*), NRRL29889 (*F. hostae*), NRRL31076 (*F. commune*), NRRL28387 (*F. commune*), NRRL31852 (*F. foetens*), FCC3174 (*F. oxysporum* f.sp. *lini*), FCC3181 (*F. oxysporum* f.sp. *raphanai*), FCC3186 (*F. oxysporum* f.sp. *nicotianae*), FCC4631 (*F. solani*), FCC4637 (*F. lactis*), FCC4645 (*F. avenaceum*), FCC4647 (*F. equiseti*), FCC4639 (*F. dlaminii*), and FCC4638 (*F. acutatum*). Lane 25 is a 100-bp ladder marker. Examples of PCR products obtained using only the LSU primers (Lane 26) and the *F. redolens*-specific primers (Lane 27) from the same *F. redolens* isolate (NRRL25600) are shown for comparative purposes.

Fig. 2. Restriction maps of the EF-1α gene from isolates representing the three groups (Groups 1-3, corresponding to Clades 1-3, respectively, of *F. oxysporum*) generated with restriction enzymes *Mse*I (Fig. 2A) and/or *Alu*I (Fig. 2B). Arrows indicate restriction sites for the respective enzymes.

Fig. 3. RFLP profiles generated by digesting EF-1α PCR amplicons from *F. oxysporum* isolates representing Groups 1-3 (Clades 1-3, respectively, of *F. oxysporum*) using *Alu*I (lanes 2, 3, and 5-10) and *Mse*I (lanes 12-18). Lane 1, a 100-bp ladder marker. Lanes 2 and 3, isolates FCC3174 and FCC3188 both representing Group 1. Lane 4, a 100-bp ladder marker. Lanes 5–10, isolates FCC3171, FCC3177, FCC3181, FCC3184, FCC3189 and FCC3461 all representing Group 2. Lane 11, a 100-bp ladder marker. Lanes 12–18, isolates FCC3173, FCC3175, FCC3178, FCC3180, FCC3186, FCC3187 and FCC3460 all representing Group 3. Lane 19, a 100-bp ladder marker. Note that the two small fragments (42 and 66 bp fragments in lanes 2, 3 and 5-10; 42 bp fragments in lanes 12-18) were not visible because these fragments were too small and/or very faint.

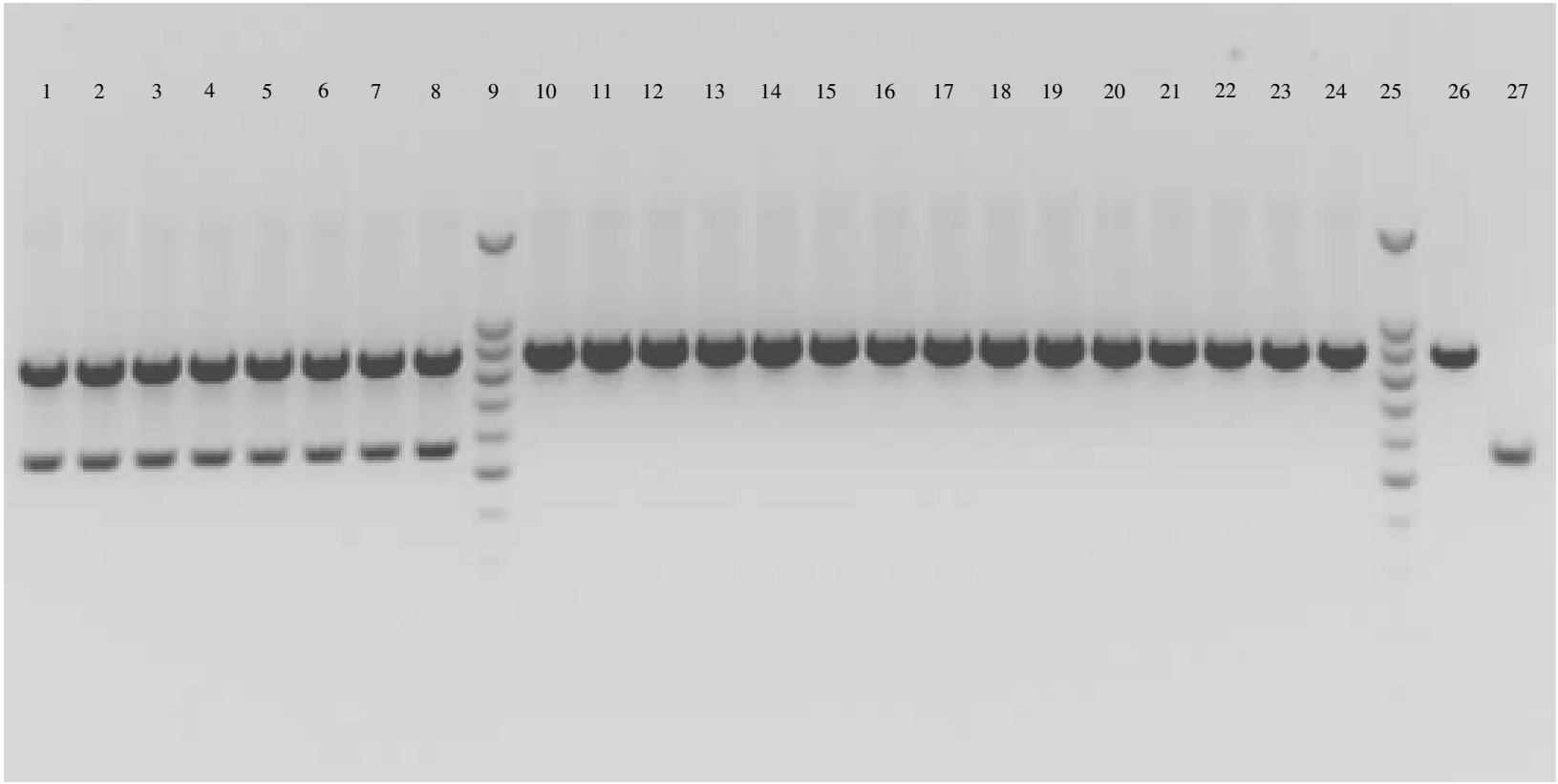

Fig. 1.

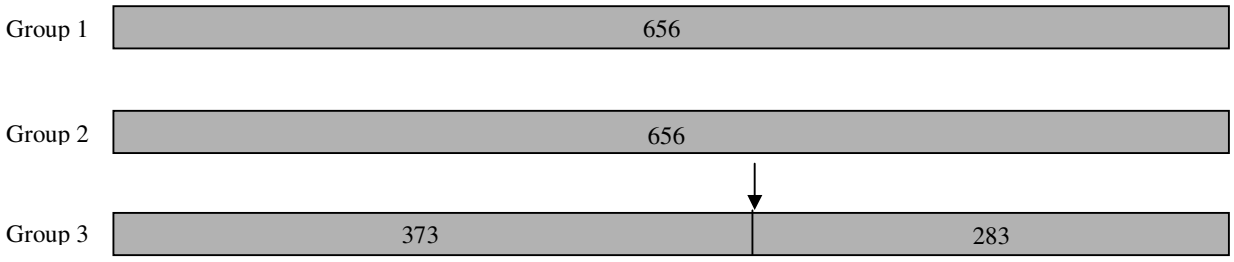
Group 1
656
Group 2
656
Group 3
373
283

Fig. 2A

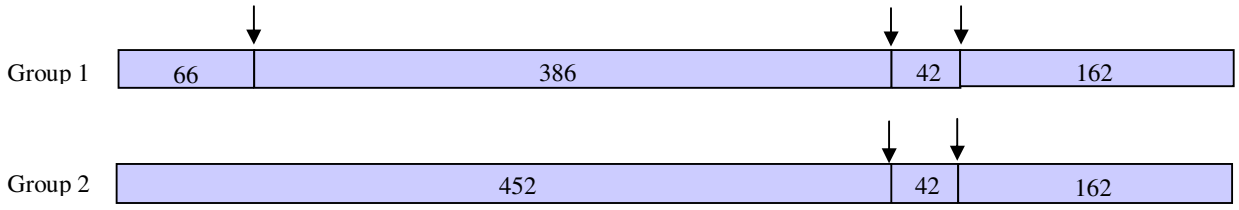
Group 1
66
386
42
162
Group 2
452
42
162

Fig. 2B

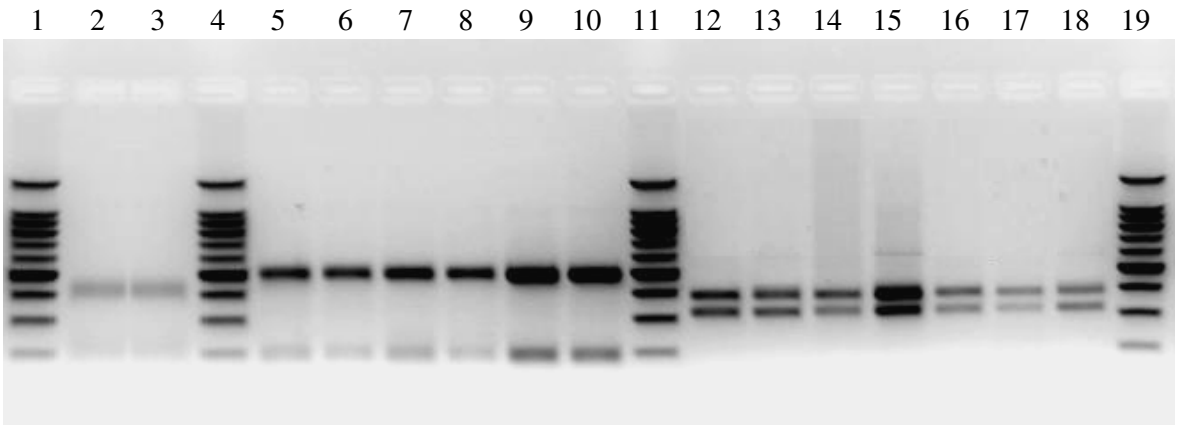

Fig. 3.

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