

**Current status of the taxonomic position of *Fusarium oxysporum* formae
specialis cubense within the *Fusarium oxysporum* complex**

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Abstract

Fusarium oxysporum is an asexual fungal species that includes human and animal pathogens and a diverse range of nonpathogens. Pathogenic and nonpathogenic strains of this species can be distinguished from each other with pathogenicity tests, but not with morphological analysis or sexual compatibility studies. Substantial genetic diversity among isolates has led to the realization that *F. oxysporum* represents a complex of cryptic species. *Fusarium oxysporum* f. sp. *cubense* (*Foc*), causal agent of Fusarium wilt of banana, is one of the more than 150 plant pathogenic forms of *F. oxysporum*. Multi-gene phylogenetic studies of *Foc* revealed at least eight phylogenetic lineages, a finding that was supported by random amplified polymorphic DNAs, restriction fragment length polymorphisms and amplified fragment length polymorphisms. Most of these lineages consist of isolates in closely related

vegetative compatibility groups, some of which possess opposite mating type alleles, *MAT-1* and *MAT-2*; thus, the evolutionary history of this fungus may have included recent sexual reproduction. The ability to cause disease on all or some of the current race differential cultivars has evolved convergently in the taxon, as members of some races appear in different phylogenetic lineages. Therefore, various factors including co-evolution the plant host and horizontal gene transfer are thought to have shaped the evolutionary history of *Foc*. This review discusses the evolution of *Foc* as a model *formae specialis* in *F. oxysporum* in relation to recent research findings involving DNA-based studies.

Keywords: taxonomy, species diversity, co-evolution, horizontal gene transfer

Introduction

Fusarium is regarded as one of the most adaptive and versatile genera in the Eumycota. One of its economically more important members is *F. oxysporum* Schlechtendahl emend. Snyder and Hansen, which consists of both pathogenic and nonpathogenic strains (Gordon and Martyn, 1997). The plant pathogenic strains are divided into special forms or *formae speciales* according to the plant species on which they cause disease, and into races according to crop cultivar specificity. To date more than 150 *formae speciales* have been described (Baayen et al., 2000; Hawksworth et al., 1995; O'Donnell et al., 2009; O'Donnell and Cigelnik, 1999). Although nonpathogenic strains of *F. oxysporum* are widespread and genetically more diverse than their pathogenic counterparts (Gordon and Okamoto, 1992), they are not as well studied (Edel et al., 2001). However, after the discovery that nonpathogenic strains play a role in *Fusarium* wilt suppressive soils (Alabouvette, 1990), interest in these strains increased dramatically (Elias et al., 1991; Katan et al., 1994).

The great diversity in *F. oxysporum* raises questions about its evolutionary biology (Does the species have one or several phylogenetic origins?), and whether the fungus represents a single species or a species complex (How distinct are populations and groups of isolates in the species?) (Kistler, 1997; O'Donnell and Cigelnik, 1997). Various techniques/attributes have been utilized to gain insight into these issues. The aim of this review is to critically review the contemporary information and ideas on the evolution and biology of *F. oxysporum* and its *formae speciales*, with specific reference to strains that attack bananas. For this purpose we first consider the taxonomy of these taxa and the specific methods that have been used to characterize them. We then discuss the evolution of *F. oxysporum* by referencing recent DNA-based studies on the members of this species.

The vascular wilt pathogen, *Fusarium oxysporum*

Taxonomic classification

The genus *Fusarium* was erected by Link in 1809 for fungi with canoe- or banana-shaped conidia (Leslie and Summerell, 2006). Approximately 1000 species had been described before Wollenweber and Reinking (1935) thoroughly reclassified the genus into 16 sections and 65 species. Since 1935, various attempts have been made to improve the Wollenweber and Reinking classification system. Snyder and Hansen (1940) compressed the 16 sections into nine species, and the species in section Elegans into a single species, *F. oxysporum*. Snyder and Hansen's classification system was eventually replaced by a system proposed by Nelson et al. (1983), which attempted to bridge the previous systems. The only species that remained unchanged from Snyder and Hansen's (1940) treatment were *F. oxysporum* and *Fusarium solani* (Martius) Appel & Wollenweber emend. Snyder & Hansen, although recent work has proposed that both taxa represent species complexes (O'Donnell et al., 2009; O'Donnell et al., 2008). Leslie and Summerell (2006) summarized information for 70 species

of *Fusarium*, and this monographic publication was the first for the genus to integrate morphological and phylogenetic information.

The morphological taxonomy of species in the genus *Fusarium* is based primarily on the structure and abundance of asexual reproductive structures (chlamydoconidia, phialides, microconidia and macroconidia) and on cultural characteristics (colony texture, colour and cultural aroma) (Booth, 1971; Edel et al., 2000; Gordon and Martyn, 1997; Lorens et al., 2006; Nelson, 1991; Nelson et al., 1983). *Fusarium oxysporum* is characterized mainly by non-septate microconidia formed in false heads on short monophialides, 3-septate macroconidia formed from monophialides on branched conidiophores in sporodochia, and chlamydoconidia with a smooth or rough wall appearance formed singly or in pairs (Fig 1). However, identification of this and other species of *Fusarium* can be challenging, because of variation between isolates (Gaudet et al., 1989; Snyder and Hansen, 1940), which reflects both genetic and environmental effects on the phenotypic expression of morphological characters (Nelson, 1991). In addition to lacking stability, morphological characters may not provide the resolution needed to properly circumscribe newly identified species.

To a large extent, limitations imposed by reliance on morphological criteria have been superseded by the use of DNA sequences for phylogenetic analyses (Sites and Marshall, 2004; Taylor et al., 2000). This approach has shown that *F. oxysporum* is comprised of a number of distinct lineages (Baayen et al., 2000; Fourie et al., 2009; Kistler, 1997; O'Donnell and Cigelnik, 1997; O'Donnell et al., 1998). It is, therefore, not surprising that many researchers regard *F. oxysporum* as a complex of several different species (Baayen et al., 2000; Kistler, 1997; O'Donnell and Cigelnik, 1997).

The biological species concept has been used to identify species in the *Gibberella fujikuroi* (Sawada) Wollenw. and *Haemanectria heamatococca* (*F. solani*) species complexes (Leslie and Summerell, 2006). However, this approach cannot be used for species that do not produce a teleomorph, such as *F. oxysporum*. In ascomycetes, two mating type (*MAT*) idiomorph alleles, namely *MAT-1* and *MAT-2*, must be present for mating to occur (Kronstad and Staben, 1997; Menzenburg and Glass, 1990). Both *MAT* idiomorphs have been reported for *F. oxysporum* and these idiomorphs were found to have high sequence similarity to those of sexually reproducing *Fusarium* species, and according to reverse transcription PCR were functionally expressed (Arie et al., 2000; Yun et al., 2000). The absence of sexual reproduction could therefore be due to mutations in these genes (Arie et al., 2000; Yun et al., 2000) or mutations within pheromones and receptors not encoded at the *MAT* locus. These pheromones and receptors are presumably important for recognition among strains of opposite mating types (Bölker and Kahmann, 1993), although their specific role in *Fusarium* is not yet fully understood (Lee et al., 2006).

Formae speciales – strain classification using pathogenicity towards specific host species:

Pathogenic strains of *F. oxysporum* cause wilt diseases on a number of agronomically important crops. Isolates that attack the same crop are included in the same *forma specialis*. Most *formae speciales* are pathogenic to a single crop (e.g. *Foc* on banana, *F. oxysporum* f. sp. *dianthi* on carnation, and *F. oxysporum* f. sp. *vasinfectum* on cotton). Some, however, attack more than one crop. For instance, Caffri et al. (2005) reported that *F. oxysporum* f. sp. *cucumerinum* affected both cucumber and melon.

The underlying genetic basis of host specificity in *F. oxysporum* is unknown (Baayen et al., 2000). Thus, it cannot be assumed that individuals in a given *forma specialis* are closely

related or evolved from a common ancestor (O'Donnell et al., 1998). In fact, the available evidence indicates that monophyletic *formae speciales* are the exceptions, rather than the rule, for *F. oxysporum* (Leslie and Summerell, 2006). Koenig et al. (1997) showed that some *Foc* isolates were more closely related to isolates of *F. oxysporum* f. sp. *niveum*, a pathogen of watermelon, than to other isolates from banana. Baayen et al. (2000) also reported a similar, close association between *F. oxysporum* f. sp. *tulipae* and *F. oxysporum* f. sp. *asparagi*, as well as between *F. oxysporum* f. sp. *gladioli* and *F. oxysporum* f. sp. *dianthi*. Presently, *F. oxysporum* f. sp. *albedinis* (Tantaoui et al., 1996), *F. oxysporum* f. sp. *ciceris* (Jimenez-Gasco et al., 2002) and *F. oxysporum* f. sp. *canariensis* (Plyler et al., 2000), are among the few *formae speciales* that appear to be monophyletic. Therefore, the majority of the *forma specialis* designations for this species are pathologically useful, but not phylogenetically informative (Gordon and Martyn, 1997).

Most isolates of *F. oxysporum* are not pathogenic. Nonpathogenic strains of *F. oxysporum* are cosmopolitan and can be found in native soils, water and plant residues. They have also been found in the roots and rhizospheres of diverse plant species (Armstrong and Armstrong, 1948; Wilson, 1995). Individuals that colonize living roots and other plant tissues but that do not necessarily cause disease are known as endophytes. Gordon and Okamoto (1992) speculated that pathogenic strains may arise from endophytic or nonpathogenic strains of *F. oxysporum* and, in support of this hypothesis, Apple and Gordon (1994) reported a close phylogenetic relationship between isolates of *F. oxysporum* f. sp. *melonis* and nonpathogenic, soil-inhabiting *F. oxysporum*. In practice, however, the differentiation between pathogenic and nonpathogenic isolates is not straightforward. They can only be differentiated from one another with the use of pathogenicity trails, and due to the host specificity of *F. oxysporum*,

large numbers of host species need to be tested in order to confirm that the isolate is truly nonpathogenic (Alabouvette et al., 2009).

Race – strain classification using pathogenicity towards specific host cultivars:

Most *formae speciales* of *F. oxysporum* are comprised of two or more races, whereas a small proportion are monotypic (e.g. *F. oxysporum* f. sp. *radicis-lycopersici*) (Di Primo et al., 2001). Races in *F. oxysporum* are defined by their impact on differential host cultivars, although these classifications can be problematic. For example, complete sets of differentials may no longer be available, as is the case with the *F. oxysporum* f. sp. *dianthi*:carnation pathosystem (Migheli et al., 1995). Also, because pathogenicity tests can be influenced by temperature, host age, method of inoculation and other variables, those conducted in different laboratories may generate incongruent results (Correll, 1991; Davis et al., 1996). Furthermore, field testing is time-consuming, expensive, and appropriate test sites may not be available for a given race or for the needed length of time.

The genetic interactions between phytopathogenic races and resistant and susceptible host cultivars vary greatly among pathosystems. For example, race-specific, monogenic resistance exists in tomato at the *I*, *I2* and *I3* loci, which correspond, respectively, to resistance to races 1, 2 and 3 of *F. oxysporum* f. sp. *lycopersici* (Kawabe et al., 2005; Mes et al., 1999).

However, many *formae speciales*, such as *Foc*, are more complex and have poorly understood host-pathogen genetic interactions.

Four races of *Foc* have been described (Moore et al., 1993; Su et al., 1986; Waite and Stover, 1960). Race 1 attacks Gros Michel, Silk, Pome and Latundan cultivars; race 2 attacks Bluggoe and other plantain (ABB genome) bananas; and race 3 attacks *Heliconia* spp. (Ploetz

and Correll, 1988; Su et al., 1977). Race 3 does not cause disease on banana and is, therefore, no longer considered part of the *Foc* race structure (Ploetz, 2005b). Race 4 is pathogenic to Cavendish bananas and all cultivars that are susceptible to races 1 and 2. Isolates in race 4 are further subdivided into *Foc* 'tropical' and *Foc* 'subtropical', based on whether or not they cause disease on Cavendish bananas under tropical environmental conditions (Ploetz, 2006; Viljoen, 2002). Races of *Foc* are not necessarily closely related to one another (Fourie et al., 2009; Koenig et al., 1997; O'Donnell et al., 1998) and several studies have shown that some *Foc* 'subtropical' race 4 isolates are more closely related to individuals associated with other *formae speciales* of *F. oxysporum* than they are to race 1 or 2 isolates of *Foc* (Baayen et al., 2000; Groenewald et al., 2006).

Characterization of formae speciales based on vegetative compatibility:

Vegetative compatibility has often been exploited in fungal diversity studies. It relies on heterokaryon formation, and in practice may be determined with complementation assays between auxotrophic nutritional mutants. Buxton (1962) was the first to study heterokaryon formation in *Fusarium*, although the method he used to produce and utilize mutants was so laborious that information on populations of these fungi could not be obtained (Katan, 1999). Puhalla (1985) revolutionized these studies by developing a more efficient technique for determining compatibility. He modified a procedure described by Cove (1976) that utilizes nitrate-non utilizing auxotrophic (*nit*) mutants that are readily recovered and stable (Katan, 1999).

Vegetative compatibility is controlled in *Fusarium* spp. and other fungi by several vegetative (*vic*) or heterokaryon (*het*) incompatibility loci (Leslie, 1993). Although the numbers of *vic* loci in *F. oxysporum* cannot be determined using conventional genetic approaches, at least six

are present in *F. circinatum* (Nirenburg & O'Donnell emend. Britz, Coutinho, Wingfield & Marasas) (Gordon et al., 2006) and at least ten are found in *F. verticillioides* (Saccardo) Nirenburg (syn. *F. moniliforme*) (Leslie, 1991). For two individuals to be vegetatively compatible and form a stable heterokaryon, they need to share a common allele at each *vic* locus (Correll, 1991). One would, therefore, expect that the rest of the genomes of individuals in one vegetative compatibility group (VCG) of an asexual species would also be very similar (Leslie, 1990; Leslie, 1993). Although individuals in VCGs of *F. oxysporum* are related by clonal descent (Gordon and Martyn, 1997; Leslie, 1993), a mutation at a single *vic* locus would place closely related individuals in different VCGs (Bentley and Dale, 1995; Bentley et al., 1998). Thus, VCGs represent good phenotypic characters for assessing diversity within populations, but genetic relationships among VCGs must be assessed by other means.

In most cases, the relationship between VCG and race is complex, with a single race being associated with multiple VCGs. For example, Katan et al. (1993) found that VCG 0138 of *F. oxysporum* f. sp. *melonis* in Israel was associated with races 0, 1 and 1-2, whereas VCG 0135 included isolates of races 0 and 2. Similar findings have also been reported for *F. oxysporum* f. sp. *lycopersici* (Cai et al., 2003; Elias and Schneider, 1991; Marlatt et al., 1996; Mes et al., 1999), *F. oxysporum* f. sp. *dianthi* (Aloi and Baayen, 1993; Kalc Wright et al., 1996; Migheli et al., 1998) and *F. oxysporum* f. sp. *melonis* (Elena and Pappas, 2006; Jacobson, 1991; Jacobson and Gordon, 1990; Katan et al., 1993). A notable exception is *F. oxysporum* f. sp. *vasinfectum* that has a one-on-one relationship between VCG and race (Assigbetse et al., 1994; Katan and Katan, 1988).

Twenty-four VCGs have been described for *Foc* (Bentley and Dale, 1995; Katan, 1999; Katan and Di Primo, 1999; Moore et al., 1993; Ploetz and Correll, 1988). A few of these, such as

VCGs 0123 and 0126, are found in several different countries, whereas others have narrower distributions and are limited to a single country [e.g. 0122 (Philippines), 01211 (Australia), 01212 (Tanzania), 01214 (Malawi), and 01217 (Malaysia)] or a region [01218 (Asia)]. Individuals in some VCGs are cross-compatible (bridge) with those in other VCGs (e.g. the 0120-01215, 0124-0125-0128-01220, and 01213-01216 complexes) (Ploetz, 1990). Isolates in VCGs 0120, 0129, 01211, and 01215 that affect Cavendish cultivars in the subtropics comprise *Foc* ‘subtropical’ race 4. Isolates from these VCGs normally do not attack Cavendish in the tropics (Bentley et al., 1998; Brake et al., 1990; Moore et al., 1993; Ploetz and Correll, 1988). In contrast, isolates in the VCG 01213-01216 complex affect Cavendish in the tropics without predisposing factors, and are referred to *Foc* ‘tropical’ race 4 (Bentley et al., 1998).

The evolution of *Fusarium oxysporum*

Fungi, like all living organisms, have the ability to adapt in response to changing or new environments. Environmental changes exert selection pressure on an organism (McDonald, 1997), and only individuals that adjust to change are able to succeed. The capacity of populations of pathogens to adapt is determined, in part, by their diversity (McDonald and McDermott, 1993). As the gene pool that a population can sample increases, so too does its adaptability to a changing environment or a new host genotype.

It is generally assumed that the dynamics of the evolution of fungi are determined by five evolutionary forces: mutation, natural selection, genetic drift, gene flow and mating system (McDonald and Linde, 2002). Mutations are changes that take place in DNA base sequences, which are thought to be rare and change the genetic constitution of a population slowly.

Natural selection typically favours genotypes with a reproductive advantage, while genetic

drift refers to changes in gene frequency within a population that take place due to chance alone. Gene flow represents the movement of gametes, individuals or populations, from one area to another, potentially expanding the range of novel pathotypes. The final evolutionary force that brings about change in *F. oxysporum* and its populations is reproductive strategy. Since no teleomorph is known for this fungus, meiotic recombination is not believed to be involved in generating new genetic combinations. Rather, asexual organisms like *F. oxysporum* are thought to evolve by means of mutations only, and potentially through the processes of parasexual recombination (*i.e.* a non-sexual mechanism for creating new genetic combinations) (Buxton, 1962; Kuhn et al., 1995). Parasexual recombination, however, would be limited to fungi that are vegetatively compatible (Menzenburg and Glass, 1990). Thus, parasexual opportunities to generate novel combinations in *F. oxysporum* would be limited by the degree of genetic similarity between the recombining strains (e.g. Fourie et al., 2009).

Various phenotypic traits have been used to assess diversity in phytopathogenic *F. oxysporum* (Ploetz, 1999). These include vegetative compatibility (Buxton, 1962; Puhalla, 1985), pathogenicity on different crops (Brake et al., 1990; Pegg et al., 1993; Pegg et al., 1994; Ploetz, 1994; Su et al., 1986; Waite and Stover, 1960), isozyme profiling (Ploetz, 1990), volatile compound production (Moore et al., 1991), and chromosome number and genome size (Boehm et al., 1994). A major disadvantage of these characters is their limited variation and that some, such as pathogenicity, are under strong selection pressure. This will lead to an underestimation of diversity and biased results (McDonald and McDermott, 1993). In general, these problems can be avoided with techniques that assess genetic variation more directly. Genetic markers that measure variation in both coding and non-coding regions of DNA can be quite variable (McDonald and McDermott, 1993). Those that have been used to study *F. oxysporum* and *Foc* include DNA sequences (Fourie et al., 2009; O'Donnell et al., 1998),

restriction fragment length polymorphisms (RFLPs) (Koenig et al., 1997), random amplified polymorphic DNAs (RAPDs) (Alves-Santos et al., 1999; Bentley et al., 1994; Bentley et al., 1998), amplified fragment length polymorphisms (AFLPs) (Groenewald et al., 2006), and microsatellites or simple sequence repeats (SSRs) (Bogale et al., 2006; Brave et al., 2001).

DNA sequence analysis:

Assessing relationships between individuals at the nucleotide level has become common place because DNA sequence information is now relatively easy and inexpensive to generate.

Depending on the genomic region that is targeted, such data can be used for studies at all taxonomic levels. Sequence data can be explored for indels (insertions or deletions) or single nucleotide polymorphisms (SNPs) for use in species- or group-specific diagnostics.

Alternatively, sequence information can be used directly for similarity-based DNA comparisons and/or phylogenetic analyses. For a given genomic region, closely related individuals are expected to have more similar sequences, albeit that sequence similarity may not always be a true reflection of phylogeny (Goldstein et al., 1995; Goldstein et al., 1999). Phylogenies based on DNA sequences are usually well-resolved when data for a number of genes or regions are congruent and combined (Lutzoni et al., 2004; Soltis et al., 1999); they generally provide better resolution than any of the other techniques that are discussed below. For example, Baayen et al. (2000) utilized AFLPs and DNA sequences to examine phylogenies for eight *formae speciales* of *F. oxysporum*, and found higher levels of homoplasy in their AFLP data.

Multi-gene phylogenetic approaches have resolved many species of *Fusarium* (Nirenberg and O'Donnell, 1997; Nirenberg and O'Donnell, 1998; O'Donnell et al., 2004b; Zeller et al., 2003), as well as groups within the *F. oxysporum* species complex (O'Donnell et al., 2009).

By making use of a multi-gene approach, O'Donnell et al. (1998) first showed conclusively that strains within a given *formae speciales* are not necessarily monophyletic. Based on the combined analysis of four gene regions, Fourie et al. (2009) recently showed that *Foc* represents at least eight distinct lineages. Bogale et al. (2006) sequenced the TEF and mtSSU region of 32 *F. oxysporum* isolates from Ethiopia and separated these isolates into three groups that corresponded with the three major clades of *F. oxysporum* that were defined by O'Donnell et al. (1998). Skovgaard et al. (2002) divided a global population of *F. oxysporum* f. sp. *vasinfectum* into four lineages with TEF, mtSSU, nitrate reductase and phosphate permase gene sequences, and determined that this *forma specialis* had at least two independent evolutionary origins, with isolates in races 3 and 5 evolving independently from the other races of this pathogen. Kawabe et al. (2005) confirmed the polyphyletic nature of *F. oxysporum* f. sp. *lycopersici* with IGS, *MAT* and endopolygalacturonase sequences. Their worldwide collection of isolates formed three evolutionary lineages, each of which contained single or closely related VCGs.

RAPDs:

RAPD analyses rely on amplification of various regions of a genome via PCR with short oligonucleotide sequences that are used as primers. The resulting fragments are then separated using electrophoresis to obtain fingerprints that can be compared among individuals (Welsh and McClelland, 1990; Williams et al., 1990). Advantages of this technique are that no prior knowledge of sequence data is needed, amplification sites are distributed throughout the genome, and low quantities of DNA template are sufficient for amplification. Disadvantages include poor reproducibility between laboratories, and the amplification of non-target DNA (McDonald, 1997). It is, therefore, of great importance that precautions are taken to avoid

contamination of template DNA. In addition, RAPDs are dominant markers and co-migrating bands cannot be assumed to be homologous (McDonald, 1997).

RAPDs have been used extensively to study populations of various *formae speciales* of *F. oxysporum*. Bentley et al. (1994), for instance, found identical or near identical haplotypes for VCGs of *Foc* in Australia. In 1998, Bentley et al. (1998) also used DNA amplified fingerprinting (DAF) (Caetano-Anolles et al., 1991) analysis, a technique closely related to RAPDs, to identify 33 genotypes among 241 *Foc* isolates, and separated these into nine clonal lineages. VCGs of *F. oxysporum* f. sp. *gladioli* were separated into three groups using RAPDs (Mes et al., 1999), while Manualis et al. (1994) used RAPDs to distinguish *F. oxysporum* f. sp. *dianthi* from nonpathogenic *F. oxysporum* isolates. Assigbetse et al. (1994) also grouped a global collection of *F. oxysporum* f. sp. *vasinfectum* isolates according to race and geographic origin using RAPDs.

RFLPs:

RFLPs utilize restriction enzymes to cut DNA at specific nucleotide recognition sites. The resulting DNA fragments can be separated by electrophoresis and the bands compared directly when a specific region is targeted with, for example, PCR (PCR-RFLP), or the resulting DNA fragments may be transferred to nylon membranes and subsequently hybridized with DNA probes to identify polymorphisms. For both types of RFLP no prior knowledge of sequence data is usually needed as restriction sites are thought to be distributed throughout the genome. For PCR-RFLPs low quantities of DNA template are required. However, the generation of hybridization-based RFLPs is technically demanding, and requires high quality DNA and in some cases prior sequence information to develop DNA probes.

RFLPs or PCR-RFLPs have been used for diverse applications. For example, Bogale et al. (2007) distinguished isolates in the three clades of the *F. oxysporum* complex with PCR-RFLPs of TEF sequences, and Attitalla et al. (2004) differentiated isolates in two *formae speciales* that affect tomato, *F. oxysporum* f. sp. *lycopersici* and *F. oxysporum* f. sp. *radicis-lycopersici*, with mtDNA RFLP haplotypes. Zambounis et al. (2007) identified specific members of *F. oxysporum* f. sp. *vasinfectum* from Australia with PCR-RFLPs of IGS sequences. Koenig et al. (1997) used RFLPs and cDNA probes to characterize a worldwide collection of *Foc* and suggested that some *formae speciales* of *F. oxysporum* were not monophyletic. Most recently, Fourie et al. (2009) utilized the IGS region to develop a PCR-RFLP diagnostic method for assigning isolates of *Foc* to its various evolutionary lineages.

AFLPs:

For AFLP analysis, two restriction enzymes, usually a frequent and a rare cutter (*EcoRI* and *MseI*), are used to digest genomic DNA. Target-specific adapters are then ligated to the digested genomic DNA, followed by two individual rounds of PCR. For the pre-selective amplification, standard *EcoRI* and *MseI* adapter primers are used for the amplification reaction, however, for the final or selective amplification, standard *EcoRI* and *MseI* adapter primers with additional nucleotides are used to reduce the number of fragments amplified. These amplified products are then subjected to polyacrylamide gel electrophoresis and the absence and presence of bands are scored (Vos et al., 1995). This method can be used for population diversity studies by comparing the fingerprints of individual isolates, is amenable to automation, and no sequence data for primer construction is required. Since more loci are screened and longer primers are used, AFLPs are more reproducible than RAPDs (McDonald,

1997). However, purified, high molecular weight DNA is required and, as is the case with RAPDSs, co-migrating bands may not be homologous.

Baayen et al. (2000) studied the evolutionary histories of eight *formae speciales* of *F. oxysporum* with AFLPs and DNA sequences. Both sets of data indicated that *F. oxysporum* f. spp. *lili* and *tulipae* were monophyletic, but that *F. oxysporum* f. spp. *asparagi*, *dianthi*, *gladioli* and *lini* were not. The congruence between DNA sequence and AFLP-based data was also reported by Bogale et al. (2006) in their study of Ethiopian *F. oxysporum*. To analyze clinically important *F. oxysporum* isolates, O'Donnell et al. (2004a) used AFLP and DNA sequence data and concluded that a single clonal lineage of the fungus was recently dispersed among hospitals in the US and Europe. With the use of AFLP and DNA sequence data Stewart et al. (2006) were able to separate nonpathogenic strains of *F. oxysporum* from those of the recently described species *Fusarium commune* (Skovgaard et al; 2003), which are not distinguishable from *F. oxysporum* based on morphology (Skovgaard et al; 2003; Stewart et al., 2006). Groenewald et al. (2006) identified seven genotypic groups from a global collection of *Foc* with AFLPs and confirmed the pathogen's polyphyletic nature.

Microsatellite/Single Sequence Repeat (SSR) markers:

Single Sequence Repeats (SSRs) are used to study the evolution and diversification of pathogen populations that are closely related. Highly polymorphic di-, tri-, tetra-, penta- or hexa-nucleotide repeats that are scattered throughout the genome are compared (Queller et al., 1993; Santana et al., 2009). The up- and downstream regions of these repeated sequences are usually highly conserved, thus allowing their amplification with specific PCR primers. Amplified products are subsequently separated and analysed using a DNA sequencer. SSRs are present in varying frequencies in most organisms (Chambers and MacAvoy, 2000), and

are evenly spaced throughout the genome, highly polymorphic (Jarne and Lagoda, 1996) and reproducible. However, SSR markers/primer pairs usually cannot be used across distantly related species (Barbara et al., 2007). Although they are generally expensive and time consuming to develop, significant time and cost reductions can be achieved with next generation sequencing technologies (Santana et al., 2009).

Brave et al. (2001) used a modified SSR technique to study *F. oxysporum* f. sp. *ciceris* isolates from India and were able to differentiate four races of the pathogen. The modified method involved restriction digestion of total genomic DNA with hexa-, tetra- and penta-nucleotide cutting enzymes, followed by transfer of digested products to nylon membranes and hybridisation with various labeled di-, tri- and tetra-nucleotide probes. They showed that races 1 and 4 were closely related, whereas races 2 and 3 were distantly related to the other races. Bogale et al. (2006) used SSRs in conjunction with DNA sequences and AFLPs to show that genetic diversity was low among isolates of *F. oxysporum* from various different sources and locations in Ethiopia, which the authors attributed to a reliance on local crops and seed sources in Ethiopia and, thus, minimal introductions of new fungal genotypes.

Evolution and diversity of *Foc*

Foc is believed to have co-evolved with its *Musa* hosts in Southeast Asia (Ploetz, 1990; Ploetz and Pegg, 1997; Stover, 1962; Vakili, 1965) and from there it was introduced into new banana-producing countries by the movement of infected planting material (Pegg et al., 1996). In a study of a global collection of *Foc*, Boehm et al. (1994) discerned two major groups based on electrophoretic karyotype; one group generally associated with banana cultivars with partial B genomes (*i.e.* at least one chromosome derived from *M. balbisiana*; e.g. Lady finger AAB and Bluggoe ABB), and the other associated with banana cultivars with

pure A genomes (*i.e.* all chromosomes derived from *M. acuminata*; e.g. Cavendish AAA and Gros Michel AAA). These groups were confirmed with RFLP data (Koenig et al., 1997), RAPD and DAF analysis (Bentley and Bassam, 1996; Bentley and Dale, 1995; Bentley et al., 1998), sequence analysis of the TEF and mtSSU genes (Fourie et al., 2009; O'Donnell et al., 1998) and AFLP analysis (Groenewald et al., 2006).

Ploetz and Pegg (1997) suggested that the two Boehm et al. (1994) groups are evidence for independent evolutionary origins of *Foc* in Southeast Asia, and reported that Wallace's line appeared to be the eastern boundary for the natural distribution of these groups. One VCG of *Foc* (VCG 01214), however, appears to have a different evolutionary history and may have originated outside the Indo-Malayan region (Ploetz, 2005a; 2005b; Ploetz and Pegg, 1997). Isolates of this VCG affect ABB cooking bananas (Bluggoe and Silver Bluggoe) and are genetically distinct from other *Foc* lineages/VCGs (Koenig *et al.*, 1997; O'Donnell *et al.*, 1998). Isolates in VCG 01214 have only been found in a small area in northern Malawi (Ploetz *et al.*, 1992) and, thus, may have evolved on a host other than banana (no *Musa* spp. are native to Africa). One possibility is enset, *Ensete ventricosum* (Welh.) E.E. Cheesman, which is in the Musaceae, endemic to Malawi, and susceptible when inoculated with isolates of VCG 01214 (Ploetz, unpublished data). A recent study by Fourie et al. (2009) confirmed that this VCG is more closely related to non-pathogenic *F. oxysporum* isolates and other *formae speciales* of *F. oxysporum* than to *Foc*, but that it forms part of a bigger clade of *F. oxysporum* that includes *Foc* isolates associated with banana cultivars with partial B genomes.

Foc isolates are divided into distinct lineages with clusters of closely related VCGs, even when they are distributed over a broad geographic area. These relationships have been documented with RAPDs, RFLPs, AFLPs, electrophoretic karyotypes and multi-gene

phylogenies (Bentley et al., 1994; Boehm et al., 1994; Fourie et al., 2009; Groenewald et al., 2006; Koenig et al., 1997; O'Donnell et al., 1998; Pegg et al., 1995), and suggest a clonal reproductive strategy for this pathogen. The diversity of *Foc*, therefore, may be due to mutation (Ploetz, 1993) and/or parasexual recombination (Buxton, 1962; Kuhn et al., 1995). On the other hand, based on a reanalysis of data for unique haplotypes reported by Koenig et al. (1997), Taylor et al. (1999) suggested that recombination may have occurred between lineages I (VCG 0124 complex) and VIII (VCG 01212). Also, Fourie et al. (2009) reported the occurrence of both mating type idiomorphs in closely related groups of *Foc* isolates. Whether recombination between these lineages is contemporary or historical, and the mechanism(s) by which it occurred, are not yet known.

Apart from co-evolution and possible sexual recombination, horizontal gene transfer may have also played an important role in the evolutionary history of *Foc*. Multi-gene phylogenies not only revealed the polyphyletic nature of *Foc* but also that pathogenicity towards a specific cultivar evolved convergently (Baayen et al., 2000; Fourie et al., 2009; O'Donnell et al., 2009; O'Donnell et al., 1998). This is, however, not unexpected since this informal taxonomic ranking is based on pathogenicity towards a specific host plant (Kistler, 2000), and thus generally subject to strong selection pressure and possibly horizontal gene transfer events (Nimchuk et al., 2003; Temporini and VanEtten, 2004; Van der Does and Rep, 2007; Yoon et al., 2007). Recently, comparative genomics of *F. graminearum*, *F. verticillioides* and *F. oxysporum* f.sp. *lycopersici* revealed a lineage-specific or mobile pathogenicity chromosome that could be experimentally transferred between genetically isolated *F. oxysporum* isolates (Ma et al., 2010). Sequence analysis indicated that this lineage-specific chromosome have an evolutionary origin different from the rest of the genome. The authors, therefore, concluded that horizontal gene transfer (i.e. the transfer of the entire host specific chromosome) could

explain the polyphyletic origins of host specialization and the emergence of new lineages in incompatible genetic backgrounds.

Four evolutionary models have been proposed for the diverse relationships that have been observed between VCGs and *formae speciales* in *F. oxysporum* (Kistler, 2000; Kistler and Momol, 1990). Two of these models posit that host ranges expanded before populations became vegetatively incompatible, while the other two state that vegetative incompatibility arose before host range expansion (Kistler, 2000; Kistler and Momol, 1990). According to the first model, host specialization is followed by infrequent genetic isolation by way of vegetative incompatibility. In other words, the pathogen and host co-evolve (Burdon, 1992). Based on the second model, host specialization coincides with more frequent genetic isolation, suggesting that the DNA sequence variation within *formae speciales* is predominantly within VCGs. In the third model, infrequent genetic isolation is followed by specialization that could describe the loss of a sexual stage, and would be supported if isolates of different *formae speciales* and VCGs were equidistant genetically, regardless of host relationships. The last model entails frequent genetic isolation followed by speciation. In this model genetic distance between VCG and *forma specialis* would be variable, therefore not equidistant to host relationships. The evolution of *Foc* fits into the first described model, if we presume that the pathogen and host co-evolved. However, if we consider the close relationship between some *Foc* lineages and other *formae speciales* of *F. oxysporum*, the last two models could also describe the evolution of some *Foc* lineages. Therefore, based on our current understanding of this pathosystem, the evolution of *Foc* has been complex, and various factors such as co-evolution, horizontal gene transfer and possible sexual recombination have shaped its evolutionary history.

Conclusions

Studies on the phylogenetics and population biology of *F. oxysporum* over the past decade have facilitated a better understanding of the evolutionary history, genetic structure and diversity of Fusarium wilt pathogens. Although many of these studies have been biased by their focus on pathogens of a single agricultural crop, with a corresponding overestimation of clonality, they provided information that has helped to manage important diseases that these pathogens cause (McDonald, 1997). Understanding the composition of a pathogen population not only contributes to the selection of resistant cultivars, but can also help predict the durability of resistance in a new host genotype (McDonald & Linde 2002). In addition, such information can be used to detect new pathogens or pathogen lineages in an area, to study the epidemiology of diseases that they cause, and to evaluate the effectiveness of a given management strategy.

Koenig et al. (1997), Bentley et al. (1998), O'Donnell et al. (1998), Groenewald et al. (2006) and Fourie et al. (2009) confirmed the polyphyletic origins of *Foc*, divided the taxon into lineages that represent two of the four major clades in *F. oxysporum*, and demonstrated that certain isolates and populations of *Foc* are more closely related to isolates in other *formae speciales* of *F. oxysporum* than to isolates in *Foc*. These studies suggest that both co-evolution and horizontal gene transfer shaped the evolutionary history of this pathogen. Taylor et al. (1999) also showed that recombination might have contributed to the evolution of *Foc*. Although it is not known whether sexual (meiotic) or asexual (parasexual) recombination was involved, the presence of both *MAT* idiomorphs in a lineage would suggest that sex was possible during its evolution (Abo et al., 2005). The origins and nature of genetic variation in *Foc* are important subjects for future study.

Future research should further focus on in-depth comparisons between closely related *formae speciales* and nonpathogens of *F. oxysporum*, rather than focusing strictly on pathogens of agricultural crops. Molecular markers should be developed that enable the quick and accurate identification of the pathogen and significant variants, such as the recently developed *Foc* ‘tropical’ race 4 marker (Dita et al., 2010). Additional genome sequencing projects to build on current knowledge regarding pathogenicity genes might eventually contribute to the development of plant varieties with resistance to Fusarium wilt.

Acknowledgements

We would like to acknowledge Suzanne Bullock from the Botanic Gardens Trust Sydney for the images of microconidia, macroconidia and monophialides, and Barbara Nel from the University of Pretoria, FABI for the photos of chlamydospores and symptoms of Fusarium wilt in South Africa.

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Figure 1. Morphological characteristics of *Fusarium oxysporum*. (A) Oval to kidney-shaped microconidia; (B) sickle-shaped, thin-walled and delicate macroconidia; (C) microconidia produced in false heads on short monophialides; and (D) a single, terminal chlamydospore.

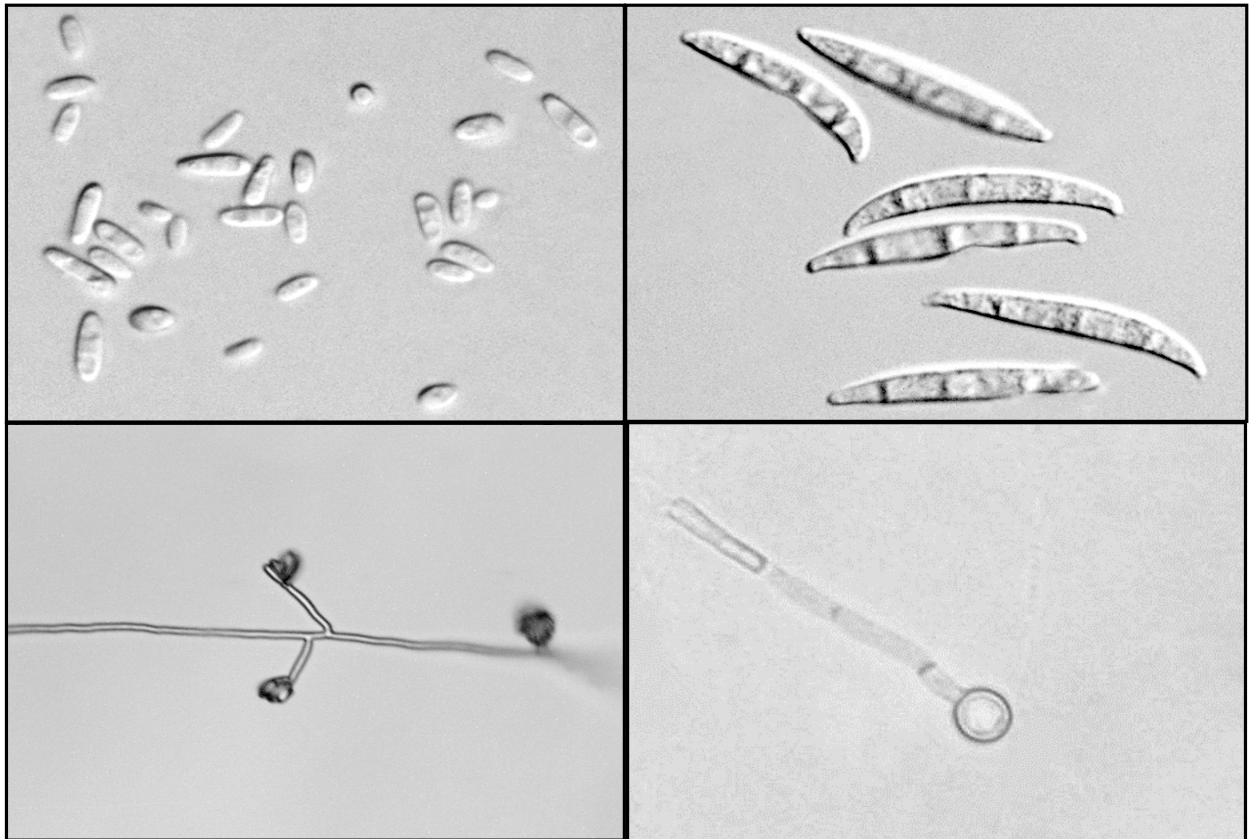


Figure 2. Disease symptoms of Fusarium wilt of banana caused by ‘subtropical’ race 4 of *Fusarium oxysporum* f. sp. *cubense* (*Foc*) in South Africa (**A**) and ‘tropical’ race 4 in Malaysia (**B**). Affected plants wilt rapidly, older and then younger leaves become yellow and brown, and plants eventually die. In some cases, the base of pseudostems split (**C**). Internally, a deep golden discoloration of the inner rhizome develops (**D**), while the vascular bundles in the pseudostem will turn yellow to reddish-brown (**E**).

