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

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Abstract

The currently available morphological and molecular diagnostic techniques for *Fusarium redolens* and the three phylogenetic clades of *Fusarium oxysporum* are problematic. Aligned translation elongation factor 1α (TEF-1α) gene sequences from these species and their close relatives were used to design *F. redolens*-specific primers, and to identify restriction sites that discriminate among the three clades of *F. oxysporum*. The *F. redolens*-specific primers distinguished this species from all others included in the study. There were three TEF-1α-RFLP patterns among *formae speciales* of *F. oxysporum*. These PCR-RFLP patterns corresponded with the three clades. These techniques provide simple and inexpensive diagnostic methods for the identification of *F. redolens* and members of the three clades of *F. oxysporum*.

Introduction

The species composition of section *Elegans* in the genus *Fusarium* has been the subject of considerable debate. This is mainly due to the application of different taxonomic systems for these fungi. Wollenweber (1913) placed six species, namely, *Fusarium lycopersici*, *Fusarium oxysporum*, *Fusarium niveum*, *Fusarium redolens*, *Fusarium*

tracheiphilum and *Fusarium vasinfectum*, in this section, whereas Gerlach & Nirenberg (1982) confined this section to *F. oxysporum*, *F. redolens* and *Fusarium udum*. Snyder & Hansen (1940), however, recognized the six taxa in Wollenweber's (1913) section *Elegans* and Gerlach & Nirenberg's (1982) *F. udum* as synonymous or varieties of *F. oxysporum*. Nelson *et al.* (1983) treated the taxa in Wollenweber's (1913) section *Elegans* in a manner similar to Snyder & Hansen (1940), but recognized Gerlach & 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 DNA-based 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 lack a sister group relationship.

The morphological distinction 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 & Gams, 1988). The two species are currently most easily diagnosed based on restriction fragment length polymorphism (RFLP) patterns of their rRNA internal transcribed spacer (ITS) regions (Waalwijk *et al.*, 1996). But this ITS-RFLP technique does not differentiate *F. redolens* from its close relative *Fusarium hostae* (Baayen *et al.*, 2001). The technique is also expensive and technically demanding as it calls for the use of three restriction enzymes.

Fusarium oxysporum is known for its pathogenic members that are specialized into more than 120 forms and races (Armstrong & Armstrong, 1981). A large number of *formae speciales* have thus been identified based on the fact that they infect only one host species. For example, *formae speciales tulipae*, *crocus* and *apii* only infect tulips, crocus and celery, respectively, (Correll *et al.*, 1986). Some *formae speciales* are known to be monophyletic; e.g. *formae speciales spinaciae*, *lilii*, *radicis-cucumerinum*, *oppontiarum* and *tulipae* (Baayen *et al.*, 2000a). However, *F. oxysporum* is regarded as a complex of morphologically similar fungi with multiple phylogenetic origins residing in three well-supported clades (O'Donnell *et al.*, 1998b; Baayen *et al.*, 2000a; Bogale *et al.*, 2006).

The grouping of *F. oxysporum* isolates into the three clades requires DNA sequence information and the inclusion of representative isolates for comparative purposes. DNA

sequencing is not commonly available to plant pathologists and it is also expensive. The use of fingerprint-based methods such as amplified fragment length polymorphism (AFLP) is also limited by the need to include isolates representing each of these clades (e.g. Bogale *et al.*, 2006). Consequently, a more rapid and less costly technique that does not require DNA sequencing or the inclusion of representative isolates would be useful for grouping *F. oxysporum* isolates into these distinct clades.

Typically, morphology-based diagnoses of *Fusarium* spp. such as *F. redolens* and *F. oxysporum* are hugely challenging. This is mainly due to the application of different taxonomic systems, the lack of mycologists with adequate experience to identify these fungi and the absence of sufficiently informative morphological features. Morphology-based diagnoses also preclude the differentiation of phylogenetic lineages of morphologically uniform species complexes. The objective of this study was, therefore, 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 phylogenetic clades of *F. oxysporum* defined by O'Donnell *et al.* (1998b) was also developed. For both of these techniques, sequence information from the widely used and taxonomically informative translation elongation factor 1α (TEF-1α) gene (Geiser *et al.*, 2004) was used.

Materials and methods

Isolates

The strains used in this study included isolates of *F. oxysporum, F. redolens* and some of their close relatives such as *F. hostae, Fusarium commune* and *Fusarium foetens*. Strains of other *Fusarium* spp. were also used as these were available in our collection. All the isolates used in this study (Table 1) are maintained in the *Fusarium* Culture Collection (FCC) of the Tree Protection Co-operative Programme (TPCP), Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

Sequence alignment

TEF-1α gene sequences for all isolates of *F. redolens*, *F. hostae*, *F. commune*, *Fusarium miscanthi* and *Fusarium nisikadoi*, and for isolates representing the three clades of *F. oxysporum* (O'Donnell *et al.*, 1998b; Baayen *et al.*, 2000a) were downloaded from the nucleotide database of the National Center for Biotechnology Information (NCBI, http://www.ncbi.nih.gov) and aligned using CLUSTALX (Version 1.8, Thompson *et al.*, 1997). TEF-1α sequences that were determined for *F. oxysporum* and *F. redolens* isolates

obtained from various sources in Ethiopia (Bogale, 2006; Bogale *et al.*, 2006) were also included in the alignment. This alignment was used to develop *F. redolens*-specific PCR primers and to identify restriction sites that could differentiate among the three clades of *F. oxysporum*. The latter involved *in silico* restriction analyses of *F. oxysporum* TEF-1α sequences using VECTOR NTI (Version 9.0.0, InforMax). SEQUENCE NAVIGATOR (Version 1.0.1, Applied Biosystems) was used to predict the amino acid sequences encoded by each of the nucleotide sequences. The presence of introns and their positions in these nucleotide sequences were determined by comparing the predicted amino acid sequences with the TEF-1α amino acid sequence (GenBank accession number AY450432) of a *F. oxysporum* strain.

Extraction of DNA and PCR amplification

DNA was extracted using *N*-cetyl-*N*,*N*,*N*-trimethyl-ammonium bromide (CTAB) (Murray & Thompson, 1980). The partial TEF-1α gene was PCR-amplified for each isolate using the primers EF1 and EF2, and the conditions described by O'Donnell *et al.* (1998b).

For PCR-based identification of *F. redolens* isolates, the *F. redolens*-specific primers that we developed (Redolens-F and Redolens-R, see below) were used in a multiplex PCR. This multiplex PCR also contained a second set of primers (LR3: 5'-CCGTGTTTCAAGACGGG-3', White *et al.*, 1990; and CS33: 5'-CGAATCTTTGAACGCACATTG-3', Visser *et al.*, 1995) that amplify an ~900 bp region of the large subunit (LSU) of the rRNA. The LSU set of primers was included as an internal positive control for a successful PCR. The multiplex PCR contained reaction buffer [10 mM Tris-HCl (pH 8.3), 2.5 mM $MgCl_2$, 50 mM KCl], 3.0 µM of each dNTP, 0.20 µM of each primer, 0.1 U μL^{-1} *Taq* Polymerase (Roche) and ~4 ng μL^{-1} template DNA. The PCR cycling conditions consisted of an initial denaturation at 94°C for 4 min, followed by a touchdown phase of 10 cycles where the annealing temperature was reduced by 0.2°C per cycle starting from 62°C, and a second phase of 30 cycles where the annealing temperature was maintained at 60°C. During both phases of the PCR, the annealing steps were preceded by a denaturation step (94°C for 30 s) and followed by an extension step (72°C for 30 s). PCRs were terminated after a final extension for 10 min. All PCR amplifications were performed on a GenAmp PCR system 9700 (Applied Biosystems).

Restriction digestion

TEF-1α PCR products amplified with primers EF1 and EF2 (O'Donnell *et al.*, 1998b) were used in restriction digestion reactions without prior purification. For digestions using endonuclease MseI (BioLabs, England), the reaction mixture (30 µL) consisted of 0.15 U μL^{-1} of the enzyme, 0.1 µg μL^{-1} bovine serum albumin, 3.0 µL NEB2 buffer

(BioLabs, England) and 25 µL of the PCR product. For restriction digestions using AluI (Roche) the reaction mixture (30 µL) consisted of 0.15 U μL^{-1} 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) at 37°C for 4 h to ensure complete digestion. Restriction fragments were resolved by electrophoresis at 4 v cm^{-1} on 3% agarose gels containing ethidium bromide (0.25 µg mL^{-1}) and visualized using a UV transilluminator.

Results

Inspection of the aligned TEF-1α sequences revealed several substitutions and insertions/deletions (indels) among sequences from the various species examined. The presence of these regions in *F. redolens* isolates was used to design *F. redolens*-specific primers (Redolens-F: 5'-ATC GAT TTT CCC TTC GAC TC-3'; Redolens-R: 5'-CAA TGA TGA TTG TGA TGA GAC-3'; Fig. 1a).

Multiplex PCR using the *F. redolens*-specific primers and the LSU primers resulted in two fragments only in *F. redolens* isolates (Fig. 1b). The larger fragment corresponded to the amplification product of the LSU primers, whereas the smaller fragment (386 bp) corresponded to that of the *F. redolens*-specific primers. For isolates of other *Fusarium* spp. included in this study, the multiplex PCR resulted in a single fragment, which corresponded in size with the expected LSU amplicon (Fig. 1b). The only exception was *F. 'oxysporum'* f. sp. *dianthi* (FCC3172), where the multiplex PCR resulted in two fragments as in the *F. redolens* isolates (Fig. 1b).

RFLP patterns of the TEF-1α products for the 15 *formae speciales* of *F. oxysporum* corresponded with the three phylogenetic clades of *F. oxysporum* described previously by O'Donnell *et al.* (1998b). Isolates representing Clade 3 were distinguished from those in Clades 1 and 2 in that only Clade 3 isolates harboured an MseI restriction site, resulting in a TEF-1α-RFLP profile consisting of two fragments (Fig. 2a). Isolates of Clades 1 and 2 were differentiated using the restriction enzyme AluI. Clade 2 isolates harbour two AluI restriction sites, whereas Clade 1 isolates harbour three restriction sites for this enzyme, resulting in distinct RFLP profiles for these two clades (Figs 2b and 3). Isolates of Clade 1 and Clade 2 could easily be distinguished based on the differing sizes of their largest fragments (386 and 452 bp, respectively, Fig. 3).

Discussion

In this study, species-specific primers were developed to identify *F. redolens* isolates by PCR. In *F. redolens*, the multiplex PCR approach using the species-specific primers and the LSU primers consistently yielded two products, one of which was *F. redolens*-specific. This *F. redolens*-specific amplification product differentiated *F. redolens* from other *Fusarium* spp. These *F. redolens*-specific primers thus allow rapid and simple diagnoses of *F. redolens* isolates. A PCR-RFLP technique that was diagnostic for the three clades of *F. oxysporum* previously designated for this species complex was also developed. This TEF-1α-RFLP diagnostic technique will provide a reasonable alternative for grouping *F. oxysporum* isolates into the three clades.

The *F. redolens*-specific primers amplified a fragment of the TEF-1α gene only in *F. redolens* isolates. These primers did not amplify PCR products in the remaining *Fusarium* spp. considered. The significance of the *F. redolens* primers lies particularly in the fact that these primers 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 specific primers allow for differentiation between *F. redolens* and *F. oxysporum*, without the need for RFLP analyses. These primers also amplified a fragment from *F. 'oxysporum'* f.sp. *dianthi* (FCC 3172, Fig. 1). This isolate was originally collected from the Netherlands, and 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 reidentified as *F. r. dianthi* (Baayen *et al.*, 1997). It is therefore believed that isolate FCC3172 represents *F. r. dianthi* and not *F. o. dianthi*. The TEF-1α sequence from this isolate is also identical to those obtained from the *F. redolens* isolates and the *F. redolens* sequences in GenBank and the *Fusarium* Identification database (http://fusarium.cbio.psu.edu; Geiser *et al.*, 2004).

The PCR-RFLP scheme developed in this study provides a simple means to distinguish among the three clades of *F. oxysporum*. The presence of an MseI recognition site in the TEF-1α PCR products of Clade 3 (O'Donnell *et al.*, 1998b) isolates uniquely distinguishes this clade. Isolates in Clades 1 and 2 are easily differentiated with the

restriction enzyme AluI. Restriction analyses of the TEF-1α PCR product from *F. oxysporum* isolates using MseI and then using AluI (in isolates where the MseI recognition site is lacking) facilitate grouping of the isolates into the three clades. This TEF-1α-RFLP approach was found to be robust as restriction maps and gel patterns generated in Vector NTI using TEF-1α sequences of *F. oxysporum* from GenBank (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. For example, selection and breeding for resistance in cereals are facilitated by the use of species-specific markers to diagnose the various fungi implicated in *Fusarium* ear blight, some of which are difficult to differentiate morphologically (Parry *et al.*, 1995; Schilling *et al.*, 1996). The situation for *F. redolens* and *F. oxysporum* is equally complex as both these species are associated with a wide range of diseases of diverse plant species (Booth, 1971). The present *F. redolens*-specific primers should be useful in this regard as they allow unambiguous and easy identification of *F. redolens* isolates. Furthermore, the polyphyletic nature of the *F. oxysporum* complex is well documented (e.g. O'Donnell *et al.*, 1998b; Baayen *et al.*, 2000a, b; Wong & Jeffries, 2006). Isolates within a single *forma specialis* of *F. oxysporum* are often unrelated, with separate evolutionary origins in the three clades of the complex. The present TEF-1α-RFLP technique should facilitate diagnoses of this character.

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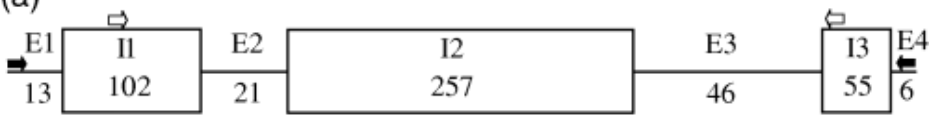
Figures and Tables

Fig. 1. Map showing binding sites of primers on the TEF-1α gene fragment (a), and multiplex PCR products obtained using the LSU and *Fusarium redolens*-specific primer sets on 22 *Fusarium* isolates representing 11 species (b). (a) Exons are indicated as lines (E1–E4), below which are shown the lengths of the corresponding amino acids; introns are indicated as boxes (I1–I3), inside which are shown the nucleotide lengths (as in our alignments including gaps). Solid arrows indicate binding sites for primers EF1 and EF2, whereas open arrows indicate binding sites for the *F. redolens*-specific primers. (b) Lanes 1–7, *F. redolens* isolates NRRL25600, NRRL28381, FCC4640, FCC4641, FCC4642, FCC4643 and FCC4644, respectively. Lane 8, *F. o. dianthi* (FCC3172). Lanes 9 and 25, 100-bp ladder marker (Roche). Lanes 10–24, respectively, isolates NRRL29642 (*Fusarium hostae*), NRRL29889 (*F. hostae*), NRRL31076 (*Fusarium commune*), NRRL28387 (*F. commune*), NRRL31852 (*Fusarium foetens*), FCC3174 (*Fusarium oxysporum* f.sp. *lini*), FCC3181 (*F. o. raphanai*), FCC3186 (*F. o. nicotianae*), FCC4631 (*Fusarium solani*), FCC4637 (*Fusarium lactis*), FCC4645 (*Fusarium avenaceum*), FCC4647 (*Fusarium equiseti*), FCC4639 (*Fusarium dlamini*) and FCC4638 (*Fusarium acutatum*). 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.

(a)



(b)

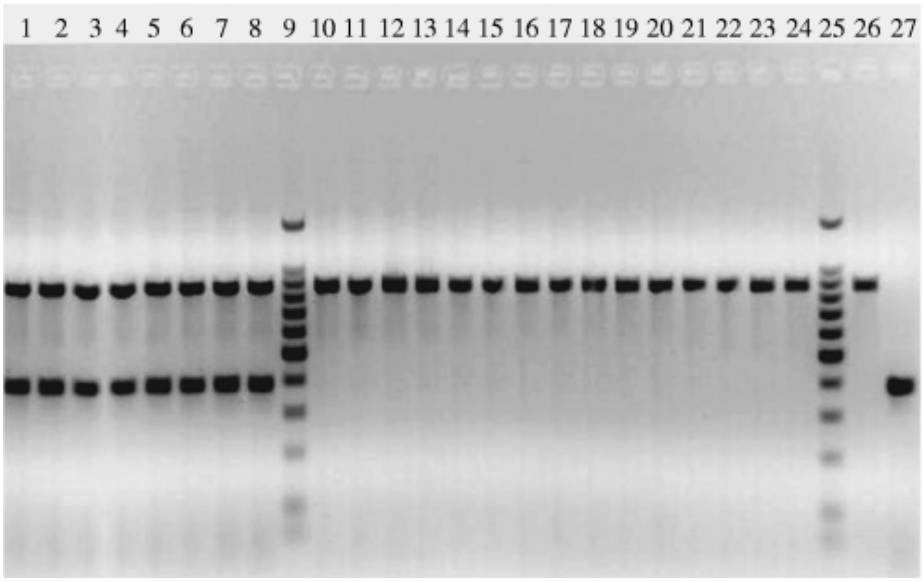


Fig. 2. Restriction maps of the partial TEF-1α gene region for isolates representing the three clades of *Fusarium oxysporum* generated with restriction enzymes MseI (a) and AluI (b). Arrows and numbers indicate restriction sites and fragment sizes (bp), respectively.

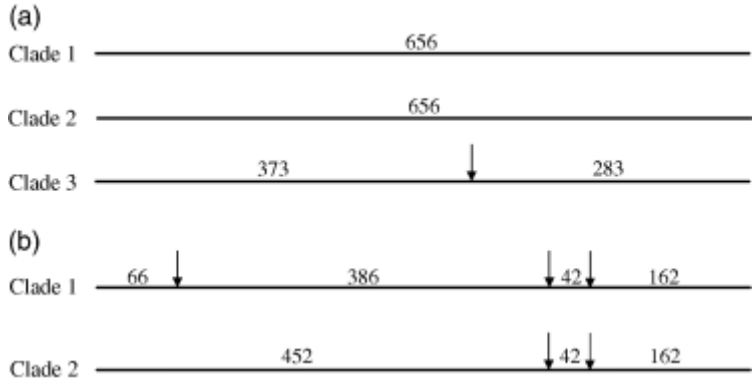


Fig. 3. TEF-1α-RFLP profiles for *Fusarium oxysporum* isolates representing the three clades of *F. oxysporum* using AluI (lanes 1, 2, and 4–9) and MseI (lanes 11–17). Lanes 3 and 10, 100-bp ladder marker. Lanes 1 and 2, isolates FCC3174 and FCC3188, respectively, both representing Clade 1. Lanes 4–9, isolates FCC3171, FCC3177, FCC3181, FCC3184, FCC3189 and FCC3461, respectively, all representing Clade 2. Lanes 11–17, isolates FCC3173, FCC3175, FCC3178, FCC3180, FCC3186, FCC3187 and FCC3460, respectively, all representing Clade 3. Note that the 42-bp fragment in lanes 1, 2 and 4–9 is not visible because it is too small and/or very faint.

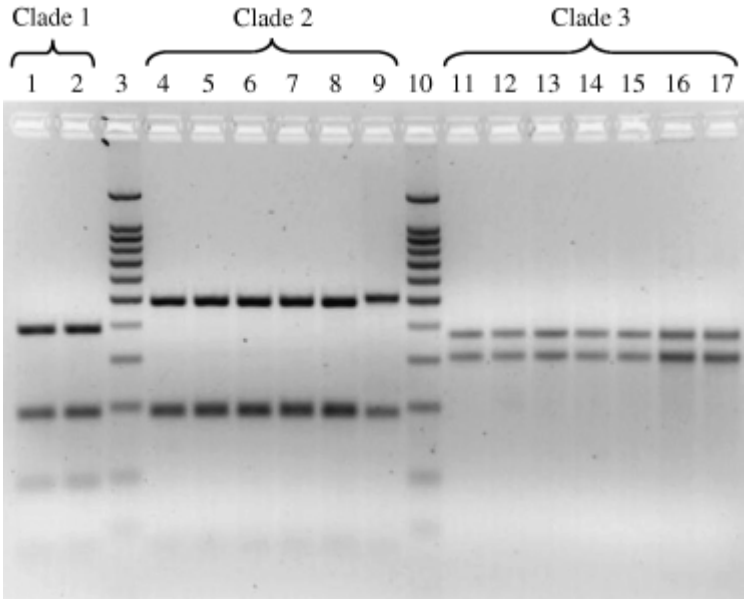


Table 1. Isolates of *Fusarium* used in this study

Species*	Strain number	Source†
F. acutatum	FCC4638	TPCP
F. avenaceum	FCC4645	TPCP
F. commune	NRRL28387	USDA
F. commune	NRRL31076	USDA
F. dlamini	FCC4639	TPCP
F. equiseti	FCC4647	TPCP
F. foetens	NRRL31852	USDA
F. hostae	NRRL29642	USDA
F. hostae	NRRL29889	USDA
F. lactis	FCC4637	TPCP
F. oxysporum f.sp. *chrysanthemi* (3)	FCC3460 or CBS 129.81	CBS
F. oxysporum f.sp. *conglutinans* (2)	FCC3171 or CBS 186.53	CBS
F. oxysporum f.sp. *cucurbitacearum* (2)	FCC3461 or CBS 680.89	CBS
F. oxysporum f.sp. *dianthi*	FCC3172 or CBS 491.97	CBS
F. oxysporum f.sp. *elaedis* (2)	FCC3184 or CBS 783.83	CBS
F. oxysporum f.sp. *gladioli* (3)	FCC3173 or CBS 137.97	CBS
F. oxysporum f.sp. *lini* (1)	FCC3174 or CBS 259.51	CBS
F. oxysporum f.sp. *lupine* (2)	FCC3189 or CBS 101.97	CBS
F. oxysporum f.sp. *lycopersici* (3)	FCC3175 or CBS 413.90	CBS
F. oxysporum f.sp. *nicotianae* (3)	FCC3186 or CBS 179.32	CBS
F. oxysporum f.sp. *niveum* (2)	FCC3177 or CBS 419.90	CBS
F. oxysporum f.sp. *passiflorae* (3)	FCC3187 or CBS 744.79	CBS
F. oxysporum f.sp. *perniciosum* (1)	FCC3188 or CBS 794.70	CBS
F. oxysporum f.sp. *phaseoli* (3)	FCC3178 or CBS 935.73	CBS
F. oxysporum f.sp. *radicis-lycopersici* (3)	FCC3180 or CBS 1101587	CBS
F. oxysporum f.sp. *raphanai* (2)	FCC3181 or CBS 488.76	CBS
F. redolens	FCC4640	TPCP
F. redolens	FCC4641	TPCP
F. redolens	FCC4642	TPCP

Species*	Strain number	Source†
F. redolens	FCC4643	TPCP
F. redolens	FCC4644	TPCP
F. redolens	NRRL25600	USDA
F. redolens	NRRL28381	USDA
F. solani	FCC4631	TPCP

*Numbers in parenthesis indicate the phylogenetic clades (Bogale *et al.*, 2006) of the respective isolates.
†Fungal collection from which isolates were obtained. USDA, Unites States Department of Agriculture, USA; CBS, Centraalbureau voor Schimmelcultures, the Netherlands.