Phylogenetic and Biological Characterization of *Fusarium oxysporum* Isolates Associated with Onion in South Africa

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Abstract

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Fusarium oxysporum f. sp. *cepae* causes Fusarium basal rot of onion, a disease of worldwide importance. Limited information is available on the phylogenetic diversity, vegetative compatibility groups (VCGs), mating type idiomorphs, and virulence of *F. oxysporum* isolates associated with onion. Therefore, these characteristics were investigated in 19 *F. oxysporum* f. sp. *cepae* isolates from Colorado, 27 *F. oxysporum* f. sp. *cepae* and 33 *F. oxysporum* isolates nonpathogenic to onion from South Africa. Six *F. oxysporum* f. sp. *cepae* VCGs (0421 to 0426) were identified, of which three were new. The dominant VCGs in Colorado and South Africa were VCG 0421 (47% of isolates) and VCG 0425 (74%), respectively. VCG 0423 was the only VCG that was shared between the two regions. Molecular phylogenies (intergenic spacer region of the rDNA, elongation factor 1 α , and mitochondrial small-

subunit) confirmed the polyphyletic nature of *F. oxysporum* f. sp. *cepae* and showed that some *F. oxysporum* f. sp. *cepae* and nonpathogenic *F. oxysporum* isolates were genetically related. Most *F. oxysporum* f. sp. *cepae* isolates clustered into two distinct, well-supported clades. The largest clade only contained highly virulent isolates, including the two main VCGs (0421 and 0425), whereas the basal clade mostly contained moderately virulent isolates. These groupings along with the VCG data provide an important basis for selection of isolates for use in breeding programs, and for the development of molecular markers to identify VCGs. Mating type genotyping revealed the distribution of both mating type (*MAT1-1* and *MAT1-2*) idiomorphs across phylogenetic clades, and the fact that several isolates contained both idiomorphs.

Onion bulb diseases caused by *Fusarium* spp. can be subdivided into Fusarium basal rot and Fusarium bulb rot caused by *Fusarium oxysporum* f. sp. *cepae* W.C. Snyder & H.N. Hansen and *F. proliferatum* (Matsush.) Nirenberg, respectively (15,19,20). Both of these diseases have been reported from several regions in the world, including the Netherlands, Uruguay, Serbia, and the United States, although bulb rot was reported for the first time in the United States only in 1997 (15,19,20,25,44,57). *F. oxysporum* f. sp. *cepae* causes economically the most important Fusarium disease, because it causes field and storage losses. In contrast, *F. proliferatum* has only been reported as causing storage losses, even though infection takes place in the field (15,19,20).

Doidge et al. (18) first reported *F. oxysporum* f. sp. *cepae* as the causal agent of onion basal rot in the Western Cape Province of South Africa. In 1973, Holz (30) also reported *F. oxysporum* f. sp. *cepae* as the causal agent of serious onion harvest losses on certain farms in the Caledon-Riviersonderend district of the Western Cape (31). Since then, the Koue Bokkeveld region in the Western Cape has increasingly become the location for the production of export-quality Pukekohe-type onion, with the first noticeable losses due to Fusarium basal rot being reported by growers in the early 1990s. The disease continues to be problematic in the Koue Bokkeveld where, in recent years (2004 to 2006), field and storage losses were 15 to 25% (M. J. Southwood, unpublished data). In South Africa, the Koue Bokkeveld suffers the most severe losses from basal rot, with the disease being less prevalent in other regions, including the Limpopo, Boland, and Southern and Northern Cape.

F. oxysporum f. sp. *cepae* is one of more than 120 formae speciales within the *F. oxysporum* species complex, which also includes nonpathogenic isolates (24,35,41). The forma specialis

designation is used to distinguish pathogenic from nonpathogenic strains. Isolates within a forma specialis can further be subdivided into races, which are identified based on virulence reactions on a set of differential host cultivars (3,14). Another method used to define genetic relationships within formae speciales of *F. oxysporum* isolates is vegetative compatibility groups (VCGs), where isolates are grouped into a specific phenotypic class (27,39,50). VCGs are based on the genetics of the fungus, because genotypes will only fuse to form a stable heterokaryon if they have the same heterokaryon or vegetative incompatibility (vic) loci. Strains that form a stable heterokaryon are considered to belong to the same VCG (37,50). Therefore, VCGs are useful for characterizing the genetic diversity within a forma specialis (14,38,39) and, in some cases, for distinguishing pathogens from nonpathogens (28).

Knowledge of the genetic diversity of *F. oxysporum* formae speciales may provide an indication of the evolutionary potential of pathogens to overcome management strategies such as host resistance (8,12,55), or provide clues as to how pathogenicity traits are acquired and transferred in populations (9,39). Genetic diversity and evolution of *F. oxysporum* formae speciales can be studied using multi-gene sequence phylogenies; namely, mitochondrial small subunit (mtSSU), translation elongation factor 1- α gene (EF-1 α), and the intergenic spacer (IGS) region of rDNA (5,10,26,46,47). Additionally, information on the presence of mating type (*MAT*) idiomorphs *MAT1-1* and *MAT1-2* in isolates and across phylogenetic clades can be used to formulate hypotheses on the potential of sexual reproduction or other mechanisms that may contribute to the generation of genetic diversity (48). However, it is important to note that, although isolates of *F. oxysporum* contain functional *MAT* genes (63), and DNA fingerprint data for the species is consistent with recombination (60), the sexual stage is unknown (32,39,43).

Limited information is available on the phylogenetic diversity, evolution, VCG status, and *MAT* idiomorphs of *F. oxysporum* f. sp. *cepae* populations worldwide, whereas no information is available for South African isolates. The first genetic studies conducted on *F. oxysporum* f. sp. *cepae* used VCG analyses, which showed that two Japanese isolates belonged to the same VCG (62); namely VCG

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0420 (33,58). Subsequently, 19 *F. oxysporum* f. sp. *cepae* isolates from Colorado (United States) were classified into three VCGs (VCG 0421, 0422, and 0423) and two single-member VCGs (SMVs) (58). In Colorado, VCG 0421 was the predominant VCG, followed by VCG 0423. In contrast, a recent study of *F. oxysporum* f. sp. *cepae* isolates in Turkey identified 48 VCGs, each containing only a single isolate (11). The polyphyletic origin of *F. oxysporum* f. sp. *cepae* in several countries has recently been studied by Galván et al. (25) and Dissanayake et al. (16) using an amplified fragment length polymorphism (AFLP) and rDNA IGS phylogeny, respectively. Dissanayake et al. (16) also showed that only the *MAT1-1* idiomorph was present among 18 *F. oxysporum* f. sp. *cepae* isolates as well as 13 *F. oxysporum* isolates that are pathogenic to Welsh onion in Japan. No races have been identified within *F. oxysporum* f. sp. *cepae* (29), although isolates have been found to differ in virulence on resistant selections (16,25,49).

The correct identification of *F. oxysporum* formae speciales is dependent on the use of an appropriate pathogenicity testing method, except for a few formae speciales where pathogen-specific molecular markers have been developed (39). Unfortunately, for many host systems, including onion, the best pathogenicity testing method is controversial due to the inevitable variation involved in biological systems (3,13,61). Several pathogenicity tests and criteria have been published for *F. oxysporum* f. sp. *cepae* identification but these have not been evaluated comparatively, thus precluding identification of the best method. Some discrepancies in these methods result from the use of either onion seedlings or bulbs for pathogenicity testing, because *F. oxysporum* f. sp. *cepae* can cause both seedling and post-harvest bulb damage. Published pathogenicity tests and criteria for *F. oxysporum* f. sp. *cepae* identification include the evaluation of (i) seedling emergence, damping-off, and stunted growth after inoculation of onion seed (1,16,17); (ii) damping-off and root plate necrosis of onion seedlings planted in inoculated soil (2,40); (iii) the incidence of *F. oxysporum* colonies developing on cross-sections of surface-sterilized onion bulbs 5 days after inoculation (58); and (vi) percentage seedling emergence after pathogen inoculation of soil (25).

The aim of this study was first to determine the pathogenicity of 60 *F. oxysporum* isolates collected from onion bulbs in South Africa using a pathogenicity test that was developed during the course of the study. This test was also used to validate the pathogenicity results of 19 *F. oxysporum* f. sp. *cepae* isolates from Colorado (58). Second, the VCGs of South African *F. oxysporum* f. sp. *cepae* isolates were determined and compared with VCGs in Colorado (58). Finally, the *MAT* idiomorphs and phylogenetic diversity of Colorado and South African *F. oxysporum* f. sp. *cepae* isolates and isolates of *F. oxysporum* not pathogenic to onion from South Africa were investigated.

Materials and Methods

Fungal isolates. Nineteen *F. oxysporum* f. sp. *cepae* isolates previously characterized by Swift et al. (58) were kindly provided by H. F. Schwartz (Department of Bioagricultural Sciences and Pest Management, Colorado State University, Fort Collins). The isolates originated from three regions in Colorado; namely, the northeast (Weld County), southeast (Otero County), and west (Delta, Mesa, and Montrose Counties). The VCG status of each of these isolates has previously been reported (58).

Eight *Fusarium* isolates were used as negative controls in pathogenicity studies. These isolates included two *F. graminearum* Schwabe (teleomorph: *Gibberella zeae* (Schwein.) Petch) isolates (SP 249 and C 381 AU) from wheat plants in Moorreesburg, South Africa, and isolates from other formae speciales of *F. oxysporum* (*F. oxysporum* f. sp. *lini* CBS 259.52, *F. oxysporum* f. sp. *gladioli* CBS 137.97, *F. oxysporum* f. sp. *lycopersici* CBS 412.90 from the Centraalbureau voor Schimmelcultures [Utrecht, The Netherlands], *F. oxysporum* f. sp. *lycopersici* PPRI 5457 from the South African National Collection of Fungi [PPRI, Pretoria, South Africa] and *F. oxysporum* f. sp. *cubense* CAV 005 and CAV 013 from KwaZulu-Natal, South Africa).

***Fusarium* isolations from onion bulbs in South Africa.** Onion bulbs with and without basal rot symptoms were collected from five regions within three provinces in South Africa over a 3-year period (2005 to 2007). The three provinces were Limpopo Province, the Northern Cape Province, and the Western Cape Province, with the three regions in the latter being the Koue Bokkeveld, Boland, and Southern Cape. The collected bulbs represented 27 different onion varieties. After removal of the outer two fleshy bulb scales and 3 to 5 mm of the outer basal plate tissue, small tissue pieces (1 to 3 mm in diameter) were taken from inside the basal plate and placed onto potato dextrose agar (PDA; Biolab, Gauteng, South Africa) supplemented with treptomycin sulfate at 0.04 g/liter of agar (PDA⁺). Plates were incubated at 25 ± 4°C on the laboratory bench and were inspected regularly for fungal growth. All isolates were hyphal tipped, followed by single-spore isolation. All *Fusarium* isolates were identified to the species level as described by Leslie and Summerell (38). Isolates were stored in 15% glycerol at -80°C. All single-spored isolates were deposited in the fungal culture collection of Stellenbosch University, Department of Plant Pathology, Stellenbosch, South Africa.

Genomic DNA extraction. DNA was extracted from *Fusarium* cultures by first growing isolates on PDA for 2 weeks at room temperature. Mycelia were scraped from the plates using a spatula, and DNA was extracted with the Wizard SV Genomic DNA Purification system (Promega Corporation, Madison, WI) according to the manufacturer's instructions.

Identification of *Fusarium* spp. using polymerase chain reaction amplification and sequencing of the EF-1 α region. *Fusarium* isolates were identified to species based on partial sequence of the EF-1 α gene (620 bp, including introns 1 to 3 and exons 2 and 3), and nucleotide BLAST analyses in the FUSARIUM-ID v. 1.0 database (26). The percentage of identities that were used for identifying species complexes and species are indicated in the Results section.

PCR amplification of the EF-1 α gene was conducted using primers EF-1 and EF-2 (47). The PCR reaction consisted of 1 unit of BIOTAQ DNA polymerase (Bioline, London), 1× PCR buffer, 0.2 μ M each primer, 4 mM MgCl₂, bovine serum albumin (BSA) Fraction V (Roche Diagnostics South Africa, Randburg, South Africa) at 1 mg/ml, 200 μ M each dNTP, and 20 to 40 ng of genomic DNA in a final reaction volume of 40 μ l. Amplification was carried out in a 2700 GeneAmp thermocycler (Applied Biosystems, Foster City, CA) using an initial denaturation at 94°C for 5 min; followed by 35 cycles at 94°C for 45 s, 55°C for 45 s, and 72°C for 1 min; with a final extension step at 72°C for 7 min. Amplicons were separated on 1% agarose gels and stained with ethidium bromide.

PCR amplification products were purified using the Wizard SV gel and PCR clean-up system (Promega Corporation) following the manufacturer's protocol. PCR products were sequenced in both directions using the ABI PRISM BigDye Terminator Cycle Sequencing Ready reaction kit (version 3.0; Applied Biosystems) according to the manufacturer's specifications. Amplification for sequencing consisted of the following steps: 94°C for 5 min; followed by 30 cycles of 95°C for 10 s, 50°C for 5 s, and 60°C for 4 min; with a final extension step at 60°C for 30 s. Analyses of the resulting fragments were done on an ABI 3130XL Genetic Analyzer (Perkin-Elmer, Norwalk, CT). Geneious Pro 3.5.6 (Biomatters Ltd., Auckland, New Zealand) was used to view the ABI trace files and to obtain consensus double-strand sequences for each isolate.

Vegetative compatibility tests. The VCG status of 27 *F. oxysporum* f. sp. *cepae* isolates (see Results section, Pathogenicity test) from South Africa was determined by using nine reference isolates (Foc 1, 4, 21, 22, 23, 31, 32, 41, and 42) that represent the known *F. oxysporum* f. sp. *cepae* VCGs (VCG 0421, 0422, and 0423) and SMVs (SMV 4 and SMV 5) (58). The VCGs of five *F. oxysporum* isolates nonpathogenic to onion (STE-U 6675, 6682, 6683, 6684, and 6688) were also determined.

VCG determination was done using standard techniques as previously described (3,50). Nitrate nonutilizing (*nir*) mutants were

generated as spontaneous chlorate-resistant sectors on minimal medium containing 4% chlorate (ClO_3^-) (50). The chlorate concentration was altered for all isolates from which *nit* mutants could not be obtained. For all isolates used in vegetative compatibility tests, heterokaryon self compatibility was first determined by pairing *nit1* and *Nit M* mutants of the same isolate with each other. Subsequently, all 27 *F. oxysporum* f. sp. *cepae* isolates from South Africa were paired in all possible combinations. Fourteen isolates that represented all South African *F. oxysporum* f. sp. *cepae* VCGs (see Results; five isolates each representing 0425 and 0426) and SMVs (four isolates) were then tested against nine Colorado *F. oxysporum* f. sp. *cepae* isolates, representing the known *F. oxysporum* f. sp. *cepae* VCGs (VCG 0421, 0422, and 0423) and SMVs (SMV 4 and SMV 5) from Colorado, and five South African *F. oxysporum* isolates nonpathogenic to onion. All pairings were conducted at least twice.

Pathogenicity tests. A preliminary pathogenicity trial evaluated the reliability of various inoculation assays using onion bulbs. In these assays, the inoculation on lower, middle, and upper bulb cross-sections, small basal plate pieces, and whole bulbs were

compared with one another. The most reliable assay was the whole-bulb inoculation method (*unpublished data*). This assay was chosen for pathogenicity testing.

All the *Fusarium* isolates from onion bulbs in South Africa (indicated with STE-U numbers; $n = 63$) and Colorado ($n = 19$) were included in pathogenicity tests (Table 1). 'Coastal Cream' onion bulbs, a Pukekohe-type onion, were used in pathogenicity testing because this cultivar is economically important and also susceptible to *Fusarium* basal rot. Onion bulbs were purchased from a grower in the Western Cape and were stored for a minimum of 13 weeks before use. Single-spored *Fusarium* isolates were grown for 3 weeks on PDA⁺. Spores were harvested from plates using sterile distilled water and adjusted to a final concentration of 5×10^4 conidia/ml. Prior to inoculation of onion bulbs, the dry bulb scales, one of the fleshy inner scales, as well as the roots were removed. The basal plate was wounded using a sterile scalpel (approximately 20 shallow, 5-mm wounds per plate), and inoculated with 1 ml of spore suspension using small droplets (30 to 50 μl). The bulbs were allowed to dry in a laminar flow cabinet before

Table 1. Characteristics (pathogenicity, vegetative compatibility group [VCG], and mating type [MAT] idiomorph) of *Fusarium* isolates collected from different regions in South Africa, and their GenBank accession numbers for three sequenced regions

Isolate ^b	Species name ^c	Regional origin ^d	Year ^e	DS (%) ^f	VCG ^g	MAT	GenBank accession number ^a		
							mtSSU	TEF	IGS
STE-U 6636	<i>Focep</i>	Limpopo, RSA	2005	100	SMV 1	MAT1-1	GU166046	GU165888	GU165967
STE-U 6637	<i>Focep</i>	Limpopo, RSA	2005	83	0426	MAT1-1	GU166047	GU165889	GU165968
STE-U 6638	<i>Focep</i>	Koue Bokkeveld, RSA	2005	97	0426	MAT1-1	GU166048	GU165890	GU165969
STE-U 6639	<i>Focep</i>	Koue Bokkeveld, RSA	2005	83	0425	MAT1-1	GU166049	GU165891	GU165970
STE-U 6640	<i>Focep</i>	Koue Bokkeveld, RSA	2005	100	0425	MAT1-1	GU166050	GU165892	GU165971
STE-U 6641	<i>Focep</i>	Koue Bokkeveld, RSA	2005	100	0425	MAT1-1	GU166051	GU165893	GU165972
STE-U 6642	<i>Focep</i>	Koue Bokkeveld, RSA	2005	90	0425	MAT1-1	GU166052	GU165894	GU165973
STE-U 6643	<i>Focep</i>	Koue Bokkeveld, RSA	2005	100	0425	MAT1-1	GU166053	GU165895	GU165974
STE-U 6644	<i>Focep</i>	Koue Bokkeveld, RSA	2005	100	0425	MAT1-1	GU166054	GU165896	GU165975
STE-U 6645	<i>Focep</i>	Koue Bokkeveld, RSA	2005	100	SMV2	MAT1-1	GU166055	GU165897	GU165976
STE-U 6646	<i>Focep</i>	Koue Bokkeveld, RSA	2005	99	0425	MAT1-1	GU166056	GU165898	GU165977
STE-U 6647	<i>Focep</i>	Koue Bokkeveld, RSA	2005	83	0425	MAT1-1	GU166057	GU165899	GU165978
STE-U 6648	<i>Focep</i>	Koue Bokkeveld, RSA	2005	91	0425	MAT1-1	GU166058	GU165900	GU165979
STE-U 6649	<i>Focep</i>	Koue Bokkeveld, RSA	2005	100	0425	MAT1-1	GU166059	GU165901	GU165980
STE-U 6650	<i>Focep</i>	Koue Bokkeveld, RSA	2005	100	0425	MAT1-1	GU166060	GU165902	GU165981
STE-U 6651	<i>Focep</i>	Koue Bokkeveld, RSA	2005	100	0425	MAT1-1	GU166061	GU165903	GU165982
STE-U 6652	<i>Focep</i>	Koue Bokkeveld, RSA	2005	100	0425	MAT1-1	GU166062	GU165904	GU165983
STE-U 6653	<i>Focep</i>	Koue Bokkeveld, RSA	2005	92	0425	MAT1-1	GU166063	GU165905	GU165984
STE-U 6654	<i>Focep</i>	Koue Bokkeveld, RSA	2005	100	0425	MAT1-1	GU166064	GU165906	GU165985
STE-U 6655	<i>Focep</i>	Koue Bokkeveld, RSA	2005	77	HIS	MAT1-1	GU166065	GU165907	GU165986
STE-U 6656	<i>Focep</i>	Koue Bokkeveld, RSA	2005	83	0425	MAT1-1	GU166066	GU165908	GU165987
STE-U 6657	<i>Focep</i>	Koue Bokkeveld, RSA	2005	100	0425	MAT1-1	GU166067	GU165909	GU165988
STE-U 6658	<i>Focep</i>	Koue Bokkeveld, RSA	2005	100	SMV3	MAT1-1	GU166068	GU165910	GU165989
STE-U 6659	<i>Focep</i>	Koue Bokkeveld, RSA	2005	50	0423	MAT1-1	GU166069	GU165911	GU165990
STE-U 6660	<i>Focep</i>	Koue Bokkeveld, RSA	2006	100	0425	MAT1-1	GU166070	GU165912	GU165991
STE-U 6661	<i>Focep</i>	Koue Bokkeveld, RSA	2006	99	0425	MAT1-1	GU166071	GU165913	GU165992
STE-U 6662	<i>Focep</i>	Boland, RSA	2006	97	0425	MAT1-1	GU166072	GU165914	GU165993
Foc 1 ^h	<i>Focep</i>	Eastern CO, USA	1992	91	0421	MAT1-1&MAT1-2	GU166073	GU165915	GU165994
Foc 4 ^h	<i>Focep</i>	Southern CO, USA	1988	100	0421	MAT1-1&MAT1-2	GU166074	GU165916	GU165995
Foc 8	<i>Focep</i>	Western CO, USA	1993	100	0421	MAT1-1&MAT1-2	GU166075	GU165917	GU165996
Foc 25	<i>Focep</i>	Western CO, USA	1999	100	0421	MAT1-1&MAT1-2	GU166076	GU165918	GU165997
Foc 27	<i>Focep</i>	Western CO, USA	1999	79	0421	MAT1-1&MAT1-2	GU166077	GU165919	GU165998

(continued on next page)

^a mtSSU = mitochondrial small subunit, TEF = translation elongation factor, and IGS = intergenic spacer; n.d. = not determined.

^b Isolates collected from onion in South Africa were deposited in the fungal culture collection of Stellenbosch University, Department of Plant Pathology.

^c *Focep* = *Fusarium oxysporum* f. sp. *cepae*.

^d RSA = Republic of South Africa, CO = Colorado, and USA = United States.

^e Year of collection.

^f For disease severity (DS) determination, the basal plates of four onion bulbs were inoculated with *Fusarium* spp. in two repeated experiments. An isolate was designated as being pathogenic toward onion and, thus, as *F. oxysporum* f. sp. *cepae*, if the isolate caused 20% or more necrosis of the basal plate as an average over the eight onion bulbs.

^g The VCG code consists of a four-digit number, with the first three digits corresponding to forma specialis and the fourth digit to designate VCGs within the forma specialis. For *F. oxysporum* isolates that did not anastomose with any other isolate, a single-member VCG (SMV) number was assigned.

^h Nine representative *F. oxysporum* f. sp. *cepae* isolates from Colorado were used in VCG determination of the *F. oxysporum* f. sp. *cepae* isolates from South Africa.

ⁱ Isolate Foc 22 was formerly numbered as VCG 0421 (58). VCG and phylogenetic analysis in the current study placed this isolate into a new Colorado VCG; namely, VCG 0424.

^j Isolate Foc 23 was formerly identified as an SMV with no assigned VCG coding (58). VCG and phylogenetic analysis in the current study placed this isolate into a new Colorado VCG; namely, VCG 0424.

being transferred to brown paper bags. These bags were placed in moisture chambers (clear Perspex box of 60 by 30 by 60 cm, lined with a sheet of chromatography paper, the base of which rested in a tray containing deionized water) on bench tops in a temperature-regulated room for a 6-week period. Pathogenicity testing of each isolate was conducted using four onion bulbs and was repeated at least once. For each isolate, four inoculated onion bulbs were placed in separate paper bags, and each bag was placed in a different moisture chamber. The moisture chambers were kept closed for the first 48 h in order to create a high-humidity environment ($\geq 93\%$), whereafter two small openings (approximately 2 cm in diameter) at the top of the chamber was opened to reduce the relative humidity in the chamber (50 to 60%). The temperatures in the moisture chambers were 24 to 26°C.

After 6 weeks of incubation, 5 mm of the outer basal plate was cut off, and the percent necrosis was evaluated on the cut basal plate section. The virulence of isolates was designated based on the percent necrosis present on the basal plate section of four inoculated bulbs in two independent experiments. The virulence classes

included highly virulent ($>70\%$), moderately virulent (20 to 70%), and nonpathogenic ($<10\%$). For the purpose of this study, isolates that caused 0 to 10% necrosis were designated nonpathogenic because a low percent necrosis developed inconsistently where wounds were created for the purpose of inoculation (Table 1). The pathogen was reisolated from the basal plates onto PDA⁺ and identified as *F. oxysporum* using conidial morphology, thus fulfilling Koch's postulate for each isolate.

Negative controls for the experiments consisted of two *F. graminearum* isolates, four formae speciales of *F. oxysporum* (two *F. oxysporum* f. sp. *cubense* isolates, two *F. oxysporum* f. sp. *lycopersici* isolates, and one isolate each of *F. oxysporum* f. sp. *gladioli* and *F. oxysporum* f. sp. *lini*), a nonpathogenic *F. oxysporum* isolate from onion (identified in the preliminary pathogenicity experiments), and a water control. The positive control consisted of a highly virulent *F. oxysporum* f. sp. *cepae* isolate, also identified in the preliminary pathogenicity experiment.

PCR amplification and sequencing of the mtSSU and IGS regions. Multilocus gene sequences were determined for all *F. oxy-*

Table 1. (continued from preceding page)

Isolate ^b	Species name ^c	Regional origin ^d	Year ^e	DS (%) ^f	VCG ^g	MAT	GenBank accession number ^a		
							mtSSU	TEF	IGS
Foc 28	<i>Focep</i>	Western CO, USA	1999	84	0421	MAT1-1&MAT1-2	GU166078	GU165920	GU165999
Foc 22 ^{h,i}	<i>Focep</i>	Western CO, USA	1999	100	0424	MAT1-1	GU166079	GU165921	GU166000
Foc 201 A	<i>Focep</i>	Eastern CO, USA	1998	100	0421	MAT1-1&MAT1-2	GU166080	GU165922	GU166001
Foc 201 D	<i>Focep</i>	Eastern CO, USA	1998	100	0421	MAT1-1&MAT1-2	GU166081	GU165923	GU166002
Foc 204 A	<i>Focep</i>	Eastern CO, USA	1998	100	0421	MAT1-1&MAT1-2	GU166082	GU165924	GU166003
Foc 31 ^h	<i>Focep</i>	Western CO, USA	1999	80	0422	MAT1-1	GU166083	GU165925	GU166004
Foc 32 ^h	<i>Focep</i>	Western CO, USA	1999	20	0422	MAT1-1	GU166084	GU165926	GU166005
Foc 41 ^h	<i>Focep</i>	Western CO, USA	1999	100	0423	MAT1-1	GU166085	GU165927	GU166006
Foc 42 ^h	<i>Focep</i>	Western CO, USA	1999	50	0423	MAT1-1	GU166086	GU165928	GU166007
Foc 48	<i>Focep</i>	Western CO, USA	1999	50	0423	MAT1-1	GU166087	GU165929	GU166008
Foc 50	<i>Focep</i>	Western CO, USA	1999	30	0423	MAT1-1	GU166088	GU165930	GU166009
Foc 47	<i>Focep</i>	Western CO, USA	1999	46	0423	MAT1-1	GU166089	GU165931	GU166010
Foc 21 ^h	<i>Focep</i>	Western CO, USA	1999	100	SMV4	MAT1-1&MAT1-2	GU166090	GU165932	GU166011
Foc 23 ^{h,j}	<i>Focep</i>	Western CO, USA	1999	75	0424	MAT1-1	GU166091	GU165933	GU166012
STE-U 6663	<i>F. oxysporum</i>	Southern Cape, RSA	2005	1	n.d.	MAT1-1&MAT1-2	GU166092	GU165934	GU166013
STE-U 6664	<i>F. oxysporum</i>	Boland, RSA	2005	4	n.d.	MAT1-1	GU166093	GU165935	GU166014
STE-U 6665	<i>F. oxysporum</i>	Boland, RSA	2005	1	n.d.	MAT1-1	GU166094	GU165936	GU166015
STE-U 6666	<i>F. oxysporum</i>	Boland, RSA	2005	0	n.d.	MAT1-1	GU166095	GU165937	GU166016
STE-U 6667	<i>F. oxysporum</i>	Limpopo, RSA	2005	7	n.d.	MAT1-1&MAT1-2	GU166096	GU165938	GU166017
STE-U 6668	<i>F. oxysporum</i>	Limpopo, RSA	2005	0	n.d.	MAT1-1&MAT1-2	GU166097	GU165939	GU166018
STE-U 6669	<i>F. oxysporum</i>	Limpopo, RSA	2005	0	n.d.	MAT1-2	GU166098	GU165940	GU166019
STE-U 6671	<i>F. oxysporum</i>	Limpopo, RSA	2005	3	n.d.	MAT1-1	GU166099	GU165941	GU166020
STE-U 6672	<i>F. oxysporum</i>	Limpopo, RSA	2005	0	n.d.	MAT1-1&MAT1-2	GU166100	GU165942	GU166021
STE-U 6673	<i>F. oxysporum</i>	Limpopo, RSA	2005	8	n.d.	MAT1-1	GU166101	GU165943	GU166022
STE-U 6674	<i>F. oxysporum</i>	Limpopo, RSA	2005	0	n.d.	MAT1-1&MAT1-2	GU166102	GU165944	GU166023
STE-U 6675	<i>F. oxysporum</i>	Limpopo, RSA	2005	1	SMV5	MAT1-1	GU166103	GU165945	GU166024
STE-U 6676	<i>F. oxysporum</i>	Limpopo, RSA	2005	3	n.d.	MAT1-1&MAT1-2	GU166104	GU165946	GU166025
STE-U 6677	<i>F. oxysporum</i>	Northern Cape, RSA	2005	0	n.d.	MAT1-1&MAT1-2	GU166105	GU165947	GU166026
STE-U 6678	<i>F. oxysporum</i>	Northern Cape, RSA	2005	0	n.d.	MAT1-1&MAT1-2	GU166106	GU165948	GU166027
STE-U 6679	<i>F. oxysporum</i>	Northern Cape, RSA	2005	0	n.d.	MAT1-1	GU166107	GU165949	GU166028
STE-U 6680	<i>F. oxysporum</i>	Northern Cape, RSA	2005	0	n.d.	MAT1-1	GU166108	GU165950	GU166029
STE-U 6681	<i>F. oxysporum</i>	Koue Bokkeveld, RSA	2005	9	n.d.	MAT1-1&MAT1-2	GU166109	GU165951	GU166030
STE-U 6682	<i>F. oxysporum</i>	Koue Bokkeveld, RSA	2005	0	SMV6	MAT1-1&MAT1-2	GU166110	GU165952	GU166031
STE-U 6683	<i>F. oxysporum</i>	Koue Bokkeveld, RSA	2005	1	SMV7	MAT1-1&MAT1-2	GU166111	GU165953	GU166032
STE-U 6684	<i>F. oxysporum</i>	Koue Bokkeveld, RSA	2005	8	SMV8	MAT1-1	GU166112	GU165954	GU166033
STE-U 6685	<i>F. oxysporum</i>	Koue Bokkeveld, RSA	2005	0	n.d.	MAT1-1&MAT1-2	GU166113	GU165955	GU166034
STE-U 6686	<i>F. oxysporum</i>	Koue Bokkeveld, RSA	2005	8	n.d.	MAT1-1	GU166114	GU165956	GU166035
STE-U 6687	<i>F. oxysporum</i>	Koue Bokkeveld, RSA	2006	1	n.d.	MAT1-1	GU166115	GU165957	GU166036
STE-U 6688	<i>F. oxysporum</i>	Koue Bokkeveld, RSA	2006	0	SMV9	MAT1-1&MAT1-2	GU166116	GU165958	GU166037
STE-U 6689	<i>F. oxysporum</i>	Koue Bokkeveld, RSA	2007	0	n.d.	MAT1-1&MAT1-2	GU166117	GU165959	GU166038
STE-U 6690	<i>F. oxysporum</i>	Koue Bokkeveld, RSA	2007	0	n.d.	MAT1-1&MAT1-2	GU166118	GU165960	GU166039
STE-U 6691	<i>F. oxysporum</i>	Koue Bokkeveld, RSA	2007	2	n.d.	MAT1-1&MAT1-2	GU166119	GU165961	GU166040
STE-U 6692	<i>F. oxysporum</i>	Koue Bokkeveld, RSA	2007	0	n.d.	MAT1-1&MAT1-2	GU166120	GU165962	GU166041
STE-U 6693	<i>F. oxysporum</i>	Koue Bokkeveld, RSA	2007	0	n.d.	MAT1-1&MAT1-2	GU166121	GU165963	GU166042
STE-U 6694	<i>F. oxysporum</i>	Koue Bokkeveld, RSA	2007	3	n.d.	MAT1-1&MAT1-2	GU166122	GU165964	GU166043
STE-U 6695	<i>F. oxysporum</i>	Koue Bokkeveld, RSA	2007	0	n.d.	MAT1-1	GU166123	GU165965	GU166044
STE-U 6696	<i>F. oxysporum</i>	Koue Bokkeveld, RSA	2007	0	n.d.	MAT1-1&MAT1-2	GU166124	GU165966	GU166045
STE-U 6947	<i>F. proliferatum</i>	Limpopo, RSA	2005	44	n.d.	n.d.	n.d.	n.d.	n.d.
STE-U 6948	<i>F. solani</i> compl	Southern Cape, RSA	2005	0	n.d.	n.d.	n.d.	n.d.	n.d.
STE-U 6949	<i>F. proliferatum</i>	Southern Cape, RSA	2005	16	n.d.	n.d.	n.d.	n.d.	n.d.

sporum isolates from onion bulbs in South Africa (pathogenic and putative nonpathogenic to onion isolates) as well as *F. oxysporum* f. sp. *cepae* isolates from Colorado (58).

The mtSSU rDNA region was amplified using primers MS1-1 (5'-GTGTTCCAGTATGGGGAGC) and MS2-1 (5'-CTTCAC TACTGGTGTGACAAACG), which were designed using the Primer3 program (<http://fokker.wi.mit.edu/primer3/input.htm>) and yielded an amplicon size of approximately 650 bp. The PCR reaction mixture was the same as for the EF-1 α region, except that 3 mM MgCl₂ was used. The amplification conditions and sequencing were also conducted as described for the EF-1 α region.

A partial sequence of the IGS (approximately 1.6 kb) was amplified using primers PNfO and PN22 (21). The PCR reaction mixture contained 1.5 units of BIOTAQ (Bioline), 1 $\times$ PCR buffer, 0.2 μ M each primer, 2.8 mM MgCl₂, BSA Fraction V at 0.8 mg/ml, 250

μ M each dNTP, and 20 to 40 ng of genomic DNA in a total volume of 25 μ l. The amplification conditions were similar to that of the mtSSU and EF-1 α regions, except that an extension time of 2 min was used. Sequencing reactions were conducted using primers PNfO, PN22, and IGS2 (internal reverse primer; 23), as described for the EF-1 α region. All sequences were deposited in NCBI GenBank (<http://www.ncbi.nlm.nih.gov/>; Table 1).

Phylogenetic analyses. Automated sequence alignments were done using MAFFT version 5.85 (<http://mafft.cbrc.jp/alignment/server/index.html>), followed by manual adjustments of the alignments in Sequence Alignment Editor v2.0 (51). Reference sequences of other *F. oxysporum* formae speciales (10,42,47) were also included in the alignments. In the combined EF-1 α and mtSSU phylogeny, the only other *F. oxysporum* f. sp. *cepae* isolate that could be included was one isolate from Germany (NRRL

Fig. 1. Phylogeny of *Fusarium oxysporum* (*F.o.*) isolates based on the combined analysis of translation elongation factor and mitochondrial small subunit sequences. The tree presents 1 of 100 equally parsimonious trees of a heuristic search. Numbers within the tree represent the bootstrap values (1,000 replicates) followed by posterior probability values in brackets. Only bootstrap values above 60% are shown. Length = 219, CI = 0.863, RI = 0.977, and RC = 0.844. South African (RSA) *F. oxysporum* f. sp. *cepae* (*F.o.cepae*) and *F.o.* sequences have "STE-U" codes, and Colorado isolates have "Foc" codes. Each of the specific *F.o.cepae* vegetative compatibility groups (VCGs) are indicated by a specific color. Single-member VCGs (SMVs) and heterokaryon self-incompatible (HSI) isolates are shown in bold. The place of origin of isolates is shown to the right of the phylogram, as well as their mating type (MAT) idiomorph. NRRL 25184 and NRRL 28387 (10) were used as outgroups to root the phylogram.

22548) (42), because GenBank did not contain any other *F. oxysporum* f. sp. *cepae* isolates that had both EF-1 α and mtSSU sequence data. The sequences (NRRL 25184 and NRRL 28387) that were used as outgroups in the EF-1 α and mtSSU trees were the same as those used by Baayen et al. (10) and O'Donnell et al. (47). Sequences used as outgroups in the IGS phylogeny were *F. subglutinans* (AY 249387.1) (54) and *F. avenaceum* (FJ 154746) (45).

The relatedness of *F. oxysporum* f. sp. *cepae* isolates from Colorado and South Africa to *F. oxysporum* f. sp. *cepae* isolates from other regions of the world was investigated by constructing an expanded EF-1 α phylogeny. This phylogeny included the GenBank *F. oxysporum* f. sp. *cepae* EF-1 α sequences from the study of Galván et al. (25) and isolate NRRL 22538 from Mbofung et al. (42). Although Dissanayake et al. (16) submitted IGS sequences of 18 *F. oxysporum* f. sp. *cepae* isolates to GenBank, these sequences could

not be included in the IGS phylogeny of the current study because these were partial sequences of only 660 bp.

Maximum parsimony analysis was conducted on the alignments using PAUP v.4.0b10 (59). The heuristic search option with 10 random taxon additions was used, and tree bisection and reconstruction (TBR) was used as the branch swapping algorithm. All characters were unordered and of equal weight, and gaps were treated as missing data. Bootstrap analysis of 1,000 heuristic search replicates was performed to estimate the reliability of inferred phylogenies. The conditions used for the bootstrap were the same as that used for the maximum parsimony analysis. A partition homogeneity test was conducted in PAUP to test the pairwise congruence between the three gene area datasets (EF-1 α , mtSSU, and IGS).

Bayesian analysis was conducted using MrBayes v. 3.1.2. (53). The program MrModeltest version 3.6 was used for selecting the

Fig. 2. Phylogeny of *Fusarium oxysporum* (F.o.) isolates based on translation elongation factor sequences. The tree presents 1 of 100 equally parsimonious trees of a heuristic search. Numbers within the tree represent the bootstrap values (1,000 replicates) followed by posterior probability values in brackets. Only bootstrap values above 60% are shown. Length = 152, CI = 0.868, RI = 0.978, and RC = 0.849. South African (RSA) *F. oxysporum* f. sp. *cepae* (F.o. *cepae*) and F.o. sequences have STE-U codes. United States (USA) Colorado F.o. *cepae* sequences are followed by a circle (●) and those from the study of Galván et al. (25) are followed by an asterisk (*). Each of the specific F.o. *cepae* vegetative compatibility groups (VCGs) are color coded. Sequences of F.o. *cepae* single-member VCGs (SMVs) and heterokaryon self-incompatible (HSI) isolates are in bold. The place of origin of isolates is shown to the right of the phylogram. NRRL 25184 and NRRL 28387 (10) were used as outgroups to root the phylogram.

optimal model of sequence evolution for each dataset alignment. The likelihood and prior settings were specified in MrBayes according to the models found with MrModeltest for each partition. Metropolis-coupled Monte Carlo Markov chains were initiated from a random tree and run for two million generations, keeping 1 of every 100th generation. Convergence among chains was monitored by examining plots of log-likelihood values and observing when the values of the four chains reached a plateau. The average deviation of split frequencies was 0.0101 for the combined EF-1 α and mtSSU dataset, 0.0123 for the EF-1 α and Galván et al. (25) dataset, and 0.0201 for the IGS dataset. The potential scale-reduction factors were one for each of the parameters. The first 14,000 generations (burn-in) were discarded for the combined EF-1 α and

mtSSU sequences, 14,200 generations for the EF-1 α and Galván et al. (25) sequences, and 33,000 generations for the IGS sequences. The remaining samples were used to calculate the 50% majority-rule tree and the posterior probability for the individual branches.

Amplification of *MAT* idiomorphs. The presence of *MAT* idiomorphs (*MAT1-1* and *MAT1-2*) in each of the *F. oxysporum* isolates from onion was determined using several different primer pairs. Amplification of the *MAT1-1* idiomorph was conducted using primer pairs FOM111 and FOM112 (48), Falpha1 and Falpha2 (6), and FOM111 and Falpha2. Amplification of the *MAT1-2* idiomorph was conducted using two primer pair combinations consisting of FOM211 and FOM212 (48) and FOM211 (48) and GFMH2 (34). Attempts to amplify the *MAT1-2* idiomorph using

Fig. 3. Phylogeny of *Fusarium oxysporum* (*F.o.*) isolates based on intergenic spacer (IGS) region sequences. The tree presents 1 of 100 equally parsimonious trees of a heuristic search. Numbers within the tree represent the bootstrap values (1,000 replicates) followed by posterior probability values in brackets. Only bootstrap values above 60% are shown. Length = 1242, CI = 0.781, RI = 0.913, and RC = 0.713. South African (RSA) *F. oxysporum* f. sp. cepae (*F.o. cepae*) and *F.o.* sequences have STE-U codes. United States (USA) Colorado *F.o. cepae* sequences are followed by a circle (●). Each of the specific *F.o. cepae* vegetative compatibility groups (VCGs) are color coded. Sequences of *F.o. cepae* single-member VCGs (SMVs) and heterokaryon self-incompatible (HSI) isolates are in bold. The place of origin of isolates is shown to the right of the phylogram, followed by the mating type (*MAT*) idiomorph identified within each isolate. Accession numbers AY249387.1 (*F. subglutinans*) (54) and FJ154746 (*F. avenaceum*) (45) were used as outgroups to root the phylogram.

primer pairs FHM11 and FHM12 (6,7) and GFMH1/GFMH2 (34), with several different PCR reaction and amplification conditions, were unsuccessful.

Amplification with primer pairs FOM111/FOM112 and FOM211/212 was conducted in a total reaction volume of 25 µl containing 1× PCR buffer (Immolaase; Bioline), 0.25 mM dNTPs, 0.35 µM each primer, 50 ng of genomic DNA, 1 U of Immolaase Taq (Bioline), and 3 mM MgCl₂. The amplification reaction of primer pair Falpha1/Falpa2 was similar, except that 1.5 mM MgCl₂ and 0.3 µM each primer was used. Amplification with primer pairs FOM111/Falpa2 and FOM211/GFMH2 was similar to that of Falpha1/Falpa2, except that Supertherm Gold (JMR, Holdings, Kent, UK) DNA polymerase and 3 mM MgCl₂ were used.

PCR amplification conditions for all primers consisted of 1 cycle of denaturing at 95°C for 8 min; 36 cycles of 30 s at 94°C, 30 s at 50°C (Falpha1/Falpa2), 54°C (FOM111/FOM112 and FOM211/212), 57°C (FOM211/GFMH2), or 54°C (FOM111/Falpa2), and 40 s at 72°C; and a final extension step of 6 min at 72°C. PCR amplicons were separated on 1.5% agarose gels. The identity of the *MAT* idiomorph PCR products (obtained with primer pairs FOM211/212, FOM211/GFMH2, and FOM111/Falpa2) was confirmed by sequencing the PCR products from a subset of 31 isolates. These isolates represented different VCGs and phylogenetic clades.

Because the *MAT* idiomorph genotyping revealed the presence of both idiomorphs within the same isolate for VCGs such as VCG 0421 (see Results), a selection of the isolates within this VCG were single spored for a second time to confirm that isolates did not consist of mixed cultures.

Results

***Fusarium* isolations from onion bulbs and identification of *F. oxysporum*.** In total, 63 *Fusarium* isolates were obtained from onion bulbs. Most of the isolates (62.5%) were collected from the Koue Bokkeveld (Table 1). Morphological identification revealed three species; namely, 60 isolates of *F. oxysporum*, 2 isolates of *F. proliferatum*, and 1 isolate of the *F. solani* species complex.

The morphological species identifications were supported by sequence comparison of the EF-1α region using BLAST in the FUSARIUM-ID v.1.0 database (26). Three *Fusarium* spp. were identified among the 63 selected isolates, of which *F. oxysporum* was predominant (93.8 to 99.8% sequence similarity to *F. oxysporum*). The two other *Fusarium* spp. were all obtained from areas outside the Koue Bokkeveld and included one *F. solani* species complex isolate and two *F. proliferatum* isolates (97 and 99% sequence similarity to *F. solani* and *F. proliferatum*, respectively). *F. proliferatum* isolates are known to vary in EF-1α alleles by as much as 1.5% (26). Both *F. solani* and *F. oxysporum* are species complexes without a well-defined internal structure (10,26). All 40 isolates from the Koue Bokkeveld were identified as *F. oxysporum* (Table 1).

Vegetative compatibility tests. VCG assignments were based on pairings between Nit M and *nit1* mutants, except for pairings involving either isolate STE-U 6650 or 6659, for which Nit M mutants could not be obtained. In these cases, *nit3* and *nit1* mutants were paired. Only one *F. oxysporum* f. sp. *cepa*e isolate (STE-U 6655) was heterokaryon self-incompatible (HSI).

The VCG or SMV status of all 19 Colorado isolates was confirmed, except for isolates Foc 22 and Foc 23. Contrary to the findings of Swift et al. (58), isolates Foc 22 (previously VCG 0421) and Foc 23 (previously SMV 5) formed a stable heterokaryon with each other and, thus, now represent a new VCG; namely, VCG 0424. The VCG code consists of a four-digit number, with the first three digits corresponding to forma specialis and the fourth digit designating the VCGs within the forma specialis. The new VCG was allocated because Foc 22 did not form a stable heterokaryon with any of the other VCG 0421 isolates. Colorado isolate Foc 21 did not form a stable heterokaryon with any other isolate, thus retaining its status as an SMV, and was renamed SMV 4 for the

purpose of the current study. One of the pathogenic isolates from the Koue Bokkeveld in South Africa (STE-U 6659) was vegetatively compatible with the two VCG 0423 representative isolates Foc 41 and Foc 42 from Colorado and, thus, could be assigned to this VCG (Table 1).

Two new VCGs were identified among the remaining 26 South African *F. oxysporum* f. sp. *cepa*e isolates. These VCGs, excluding the SMVs, were given numbers according to the VCG numbering system of Katan (33), which followed on the VGC numbers published by Swift et al. (58). VCG 0425 represented the bulk (74%) of the *F. oxysporum* f. sp. *cepa*e isolates from South Africa, including 19 isolates from the Koue Bokkeveld and 1 isolate (STE-U 6662) from the Boland. VCG 0426 included two isolates, one each from Limpopo (STE-U 6637) and the Koue Bokkeveld (STE-U 6638). The assignment of VCG numbers 0424 to 0426 to *F. oxysporum* f. sp. *cepa*e isolates was acknowledged by T. Gordon (Department of Plant Pathology, University of California). Three of the South African *F. oxysporum* f. sp. *cepa*e VCGs consisted of single-member isolates (STE-U 6636, 6645, and 6658) obtained from the Limpopo (STE-U 6636) and Koue Bokkeveld (STE-U 6645 and 6658), and will hereafter be referred to as SMV 1, SMV 2, and SMV 3, respectively (Table 1).

Only five nonpathogenic *F. oxysporum* isolates (STE-U 6675, 6682, 6683, 6684, and 6688) from onion in South Africa were subjected to VCG analysis. Each of these represented SMV isolates (Table 1) and were not vegetatively compatible with any of the *F. oxysporum* f. sp. *cepa*e VCGs or SMVs from South Africa or Colorado.

Pathogenicity tests. In all, 82 isolates were tested for pathogenicity, including all South African *Fusarium* isolates from onion (63) and 19 *F. oxysporum* f. sp. *cepa*e isolates from Colorado (58). There appeared to be an association between virulence and VCG, because all the isolates in VCG 0421, 0424, 0425, 0426, and SMV 4 and SMV 1 to 3 were highly virulent (Table 1). In contrast, VCG 0422 and VCG 0424 contained moderately and highly virulent isolates. In VCG 0422, isolate Foc 32 was moderately virulent, whereas Foc 31 was highly virulent. In VCG 0423, isolate Foc 41 was highly virulent, and moderately virulent isolates included Foc 42, Foc 47, Foc 48, Foc 50, and STE-U 6659.

In South Africa, most of the highly virulent isolates were from the Koue Bokkeveld. Isolates from the Northern and Southern Cape were all nonpathogenic. Most isolates from Limpopo and Boland were also nonpathogenic but three highly virulent isolates were identified in these regions (i.e., STE-U 6636 and 6637 in Limpopo and isolate STE-U 6662 in the Boland; Table 1).

The other *Fusarium* spp. (*F. solani* species complex and *F. proliferatum*) obtained from onion bulbs caused 0 to 16% basal plate necrosis, except for one *F. proliferatum* isolate that caused 44% necrosis of the cut basal plate section (Table 1). However, the symptoms caused by the latter *F. proliferatum* isolate, unlike those caused by *F. oxysporum* f. sp. *cepa*e isolates, included a light-brown bulb necrosis of the outer dry and fleshy scales, progressing downward and inward from the top half of the bulbs.

Negative control bulbs remained asymptomatic until the end of each assay. Some negative control bulbs developed limited internal necroses (approximately 2%) but this was not observed in independent experiments or in more than one of the inoculated bulbs. In all experiments, the positive control, consisting of a highly virulent *F. oxysporum* f. sp. *cepa*e isolate, caused 80 to 100% necrosis in all four inoculated bulbs.

Phylogenetic analyses. The first phylogeny that was constructed consisted of a combined EF-1α and mtSSU phylogeny, with partition homogeneity testing showing that these data sets were congruent ($P = 0.315$). The phylogeny contained four main clades (clades 1 to 4) and one large subclade (4a), all with bootstrap support $\geq 79\%$ and posterior probability ≥ 0.96 (Fig. 1). Our clade 2 corresponded with the basal clade 1 of O'Donnell et al. (47). Clade 1 (100% bootstrap support, 1.00 posterior probability) formed the basal clade and included all isolates associated with VCGs 0422, 0423, and 0424 (mostly Colorado *F. oxysporum* f. sp. *cepa*e iso-

lates), as well as two nonpathogenic isolates from South Africa (STE-U 6675 and 6685) and the one HSI isolate (STE-U 6655). Clade 2 (100% bootstrap support, 1.00 posterior probability) contained only two isolates from Limpopo (South Africa): an *F. oxysporum* f. sp. *cepa*e isolate (SMV 1) and a nonpathogenic isolate (STE-U 6674). Clade 3 (94% bootstrap support, 0.98 posterior probability) did not contain any onion *F. oxysporum* isolates but included the three *F. oxysporum* f. sp. *cubense* (NRRL 26029, 26024, and 25603) and two *F. oxysporum* f. sp. *lactucae* (F 9501 and FK 09701) isolates that were shown by O'Donnell et al. (47) and Mbofung et al. (42) to represent the most ancestral lineage within their *F. oxysporum* phylogenies. Clade 4 (79% bootstrap support, 0.96 posterior probability) contained a large, well-supported subclade 4a (98% bootstrap support, 1.00 posterior probability). Most of the *F. oxysporum* f. sp. *cepa*e isolates grouped in subclade 4a. Within subclade 4a, STE-U 6639 showed sequence variation that was attributed to an indel of 15 bp in the EF-1 α region. Subclade 4a consisted of *F. oxysporum* f. sp. *cepa*e isolates from three VCGs (including the two main VCGs 0421 and 0425), three *F. oxysporum* f. sp. *cepa*e SMVs (SMV 2, 3, and 4), two nonpathogenic *F. oxysporum* isolates (STE-U 6687 and 6695) from the Koue Bokkeveld region in South Africa, and one (STE-U 6638) of the two VCG 0426 isolates. The other isolate of VCG 0426 (STE-U 6637) from the Limpopo clustered outside of subclade 4a but still within clade 4. The other *F. oxysporum* isolates that also clustered in clade 4 but outside of subclade 4a mainly consisted of nonpathogenic *F. oxysporum* isolates from different regions in South Africa, other *F. oxysporum* formae speciales isolates, and an *F. oxysporum* f. sp. *cepa*e isolate from Germany (NRRL22548).

The relatedness of the South African and Colorado *F. oxysporum* f. sp. *cepa*e isolates to the *F. oxysporum* f. sp. *cepa*e isolates from the study of Galván et al. (25) was investigated using an expanded EF-1 α phylogeny, because mtSSU sequences were not available for these isolates. The expanded EF-1 α phylogeny included nine *F. oxysporum* f. sp. *cepa*e sequences from Galván et al. (25) that were collected from six countries (Fig. 2). The topology and clade distribution of the EF-1 α phylogeny were comparable with the combined EF-1 α and mtSSU phylogeny, except that isolate NRRL28371 grouped basal to clade 4 and was designated as a fifth clade. In the expanded EF-1 α phylogeny, all nine Galván et al. (25) sequences grouped within clade 4 (81% bootstrap support, 1.00 posterior probability), with only two of the sequences, DSM 62306 from California and EZA from Australia, clustering within subclade 4a (99% bootstrap support, 1.00 posterior probability). None of the Galván et al. (25) *F. oxysporum* f. sp. *cepa*e sequences clustered within the basal clade 1 containing *F. oxysporum* f. sp. *cepa*e isolates from Colorado and South Africa.

Partition homogeneity tests showed low but significant differences between the IGS and EF-1 α or mtSSU datasets (P -values < 0.05). Therefore, we conservatively chose not to combine the IGS dataset with the EF-1 α or mtSSU data sets. The IGS phylogeny had a CI of 0.781, whereas the combined EF-1 α -mtSSU phylogeny had a CI of 0.836. Parsimony and Bayesian analysis of the IGS data yielded two clades with high bootstrap support and posterior probability values (100% and 1.00 for clade 1; 79% and 1.00 for clade 2; Fig. 3). When compared with the combined mtSSU and EF-1 α phylogeny, the IGS phylogeny only supported the same clade 1 containing *F. oxysporum* f. sp. *cepa*e VCGs 0422, 0423, and 0424 and the HSI isolates. The one exception was the divergent placement of the EF-1 α -mtSSU-clade 1 isolate, Foc 50, from Colorado (Mesa) that clustered into clade 2 in the IGS phylogeny among the bulk of the nonpathogenic and *F. oxysporum* f. sp. *cepa*e EF-1 α -mtSSU-clade 4a isolates. This result was confirmed by repeating the sequencing of the three gene areas for Foc 50 from newly isolated DNA. The nonpathogenic isolate STE-U 6675 also did not cluster in clade 1 but grouped basal in clade 2. Even though STE-U 6675 had high support in clustering with the other clade 1 isolates, the long branch lengths in the EF-1 α -mtSSU phylogeny indicated it to also be different. Within the IGS phylogeny, clade 2

contained all the *F. oxysporum* f. sp. *cepa*e VCG 0421, 0425, and 0426 isolates, as well as most of the nonpathogenic and all of the Mbofung et al. (42) reference isolates. Clade 2 also contained the four *F. oxysporum* f. sp. *cepa*e SMV 1, 2, 3, and 4 isolates. Within the combined EF-1 α and mtSSU phylogeny, SMV 2, 3, and 4 grouped within clade 4 but SMV 1 clustered into a separate clade (clade 2). The two *F. oxysporum* f. sp. *lactucae* isolates, F 9501 and FK 09701, that represented the most basal isolate clade in the study of Mbofung et al. (42), grouped basal within clade 2.

Amplification of MAT idiomorphs. The two *MAT1*-2 primer pairs were equally effective in amplifying the *MAT1*-2 idiomorph whereas, for amplification of the *MAT1*-1 idiomorph, primer pair FOM111/F α 2 was most successful (Table 1). However, for some isolates, other amplicons in addition to the 390-bp amplicon were also amplified. Amplification of these isolates with the other *MAT1*-1 primer pairs yielded only the one expected amplicon size. Primer pair FOM111/FOM112 only yielded the expected 1-kb PCR product in 41% of the isolates that were identified as *MAT1*-1 by primer pair FOM111/F α 2. Similarly, primer pair F α 1/F α 2 only yielded the expected 380-bp product in 60% of the known *MAT1*-1 isolates. Attempts to improve the amplification efficiency of the latter two primer pairs by lowering the stringency of reaction (increased magnesium chloride concentration) and amplification (lower annealing temperatures) conditions did not result in the amplification of the *MAT1*-1 idiomorph from more isolates. For amplification of the *MAT1*-2 idiomorph, the FOM211/FOM212 and FOM211/GMH2 primer pairs amplified an approximately 700- and 600-bp *MAT1*-2 PCR product, respectively, from the same set of isolates.

All *F. oxysporum* isolates from onion had the *MAT1*-1 idiomorph, except for STE-U 6669. In some cases, the *MAT1*-1 and *MAT1*-2 idiomorphs were identified within the same isolate (Table 1; Fig. 3). The consensus sequences (387 bp) obtained from 31 randomly selected isolates with primer pair FOM111 and F α 2 had 98 to 100% similarity to *F. oxysporum* *MAT1*-1 GenBank sequences (AY52742 and AY527425; 48). The consensus sequences (523 bp) amplified from 18 isolates with primer pair FOM211/GFMH2 or FOM211/FOM212 had 99% similarity to the *MAT1*-2 *F. oxysporum* f. sp. *lycopersici* GenBank accession AB011378 (6).

Discussion

F. oxysporum is the main *Fusarium* sp. associated with asymptomatic and decayed onion bulbs in South Africa. Most of the *F. oxysporum* isolates from this study were from the Koue Bokkeveld, because this region has a long history of concentrated onion production through the warm summer months when *F. oxysporum* f. sp. *cepa*e is most active (M. J. Southwood, unpublished data). As expected, isolates that are nonpathogenic to onion were also obtained from this region. Only two *F. proliferatum* isolates were obtained. Because Koch's postulate was fulfilled for one of the *F. proliferatum* isolates, this is the first report of this species causing bulb rot in South Africa.

Our test for pathogenicity toward onion used a high inoculum concentration and favorable environmental conditions for disease development. It is, therefore, unlikely that pathogenic isolates would have been identified as nonpathogenic. However, none of the negative controls were able to cause disease under these conditions, suggesting that the test was not overly aggressive. Our results confirmed pathogenicity of *F. oxysporum* f. sp. *cepa*e isolates from Colorado (58), and revealed pathogenic ($n = 27$) and nonpathogenic ($n = 33$) South African isolates. Differences were detected in the virulence of *F. oxysporum* f. sp. *cepa*e isolates, with isolates subjectively being classified as moderately or highly virulent based on percent basal plate necrosis. Future studies should investigate whether similar virulence designations would be observed for these isolates if different inoculation methods, such as artificial inoculation of seedlings, were used. *F. oxysporum* f. sp. *cepa*e also causes seedling damping-off of onion and, therefore, some authors have used seedling emergence, damping-off, and

stunted growth of seedlings as parameters for identifying *F. oxysporum* f. sp. *cepae* isolates (1,2,16,17,40). A thorough comparative study on the pathogenicity of several different *F. oxysporum* f. sp. *cepae* isolates toward mature onion bulbs and seedlings has not yet been published.

VCG analyses of South African and Colorado *F. oxysporum* f. sp. *cepae* isolates resulted in the identification of three new VCGs (0424 to 0426), in addition to the previously reported four VCGs (0420 to 0423) (58,62). Furthermore, four SMVs, three from South Africa (SMV 1 to 3) and one from Colorado (SMV 4), are also now known. An SMV cannot receive official VCG status until another isolate is identified that can form a stable heterokaryon with the existing SMV isolate. Only one South African *F. oxysporum* f. sp. *cepae* isolate belonged to a VCG (VCG 0423) previously identified in Colorado. Similarly, Bayraktar et al. (11) found that VCG 0423 was the only Colorado VCG with which one of their *F. oxysporum* f. sp. *cepae* isolates from Turkey could form a stable heterokaryon. In each region, one VCG dominated, namely VCG 0421 (47% of isolates) in Colorado and VCG 0425 (74% of isolates) in South Africa, which could be due to a founder effect. In contrast, no single *F. oxysporum* f. sp. *cepae* VCG was found to dominate populations in Turkey (11). The relatively large number of *F. oxysporum* f. sp. *cepae* VCGs and SMVs is in contrast to the few VCGs identified in other bulb and corm rot formae speciales. *F. oxysporum* f. sp. *tulipae* and *F. oxysporum* f. sp. *lilii* each contains only one VCG, whereas *F. oxysporum* f. sp. *gladioli* contains two VCGs (9,52).

The combined EF-1 α and mtSSU phylogeny was helpful for identifying clonal relationships within some VCGs and, further, showed that isolates belonging to different VCGs can also be clonally related. The phylogeny showed the clonal relationship between Colorado *F. oxysporum* f. sp. *cepae* isolate Foc 22 (previously identified as VCG 0421 by Swift et al. [58]) and isolate Foc 23 (previously SMV 5) (Fig. 1) which, along with their somatic compatibility, resulted in the two isolates being classified into a new VCG, VCG 0424. Bayraktar et al. (11) recently also showed, using restriction fragment length polymorphism analysis of the IGS region, that Foc 22 and Foc 23 are genetically similar and distinct from VCG 0421. On the other hand, although *F. oxysporum* f. sp. *cepae* VCGs 0421, 0425, and 0426 and SMV 2, 3, and 4 isolates were vegetatively incompatible, they were all genetically similar based on the phylogenies (Fig. 1). Similarly, in other *F. oxysporum* formae speciales, mtSSU or EF-1 α analyses have also shown that different VCGs can be clonally related and that VCG is not an accurate reflection of genetic diversity (10,35,47). This is due to the fact that, for an isolate to be vegetatively compatible, all alleles at all vic loci must be identical (14).

In our study, there seemed to be a relationship between virulence and phylogenetic clade, which is important to consider in selecting isolates for resistance screening of cultivars or lines against Fusarium basal rot. For example, as new isolates are genotyped and identified as belonging to new phylogenetic clades, these isolates should be investigated for virulence and included in resistance screenings. Dissanayake et al. (16) made a similar observation on Welsh onion, where a correlation between IGS genotype and pathogenicity was found.

The phylogenies from our study confirmed the previously identified polyphyletic origin of *F. oxysporum* f. sp. *cepae* (16,25) and revealed a regional distribution of *F. oxysporum* f. sp. *cepae* clades. *F. oxysporum* f. sp. *cepae* clade 4 has a worldwide distribution, because it included most isolates from South Africa and Colorado, as well as isolates from other U.S. states, Australia, Germany, the Netherlands, and Turkey (Fig. 2). In contrast, *F. oxysporum* f. sp. *cepae* clade 2 contained only one South African *F. oxysporum* f. sp. *cepae* isolate, and clade 1 contained only a few isolates from Colorado and one HSI isolate from South Africa. Clade 1 grouped basal (EF-1 α and mtSSU phylogeny) to all the other *F. oxysporum* f. sp. *cepae* clades and to the most basal *F. oxysporum* clades from other studies (10,42,47). Interestingly, *F. oxysporum* f. sp. *cepae* isolates within clade 1 also contained the largest variation in virulence (20

to 100% necrosis), with most isolates being moderately virulent. This variance in isolate virulence may be due to the virulence of the isolates declining during storage, or due to genetic differences in virulence of the isolates on 'Coastal Cream'.

In some formae speciales, the IGS region has been useful for resolving VCGs (4,22,41) and lineages (23), differentiating pathogenic isolates from nonpathogens (42), and distinguishing pathogenic isolates with different virulence characteristics (16). This was not true for our collection of *F. oxysporum* f. sp. *cepae* isolates, because pathogens and nonpathogens grouped in the same clade and VCGs could not be differentiated. Interestingly, the IGS phylogeny did reveal the divergent placement of two isolates, one of the Colorado VCG 0423 isolates (Foc 50) and a nonpathogenic *F. oxysporum* isolate (STE-U 6673). For example, isolate Foc50 was placed in clade 1 in the EF-1 α -mtSSU phylogeny but grouped with EF-1 α -mtSSU clade 4a isolates in the IGS phylogeny. One possible explanation could be genetic recombination or genetic exchange between isolate Foc 50 and an isolate within EF-1 α -mtSSU-clade 4a. If this was through parasexual recombination, the vegetative incompatibility of isolates would have to be overcome. Alternatively, this discrepancy could also be explained by the fact that divergent IGS sequences may reside within a single isolate.

This is the first study to report the presence of two different *MAT* idiomorphs within the same *F. oxysporum* isolate, although this species complex is generally considered to be heterothallic (6). It is important to note that the identification of the specific *MAT1-1* and *MAT1-2* idiomorphs in the current collection of isolates was dependent on the use of the correct primer pair combinations. All the *F. oxysporum* f. sp. *cepae* isolates and most of the nonpathogenic *F. oxysporum* isolates contained the *MAT1-1* idiomorph, whereas the *MAT1-2* idiomorph was only identified in one nonpathogenic isolate, STE-U 6669 from Limpopo. In general, for a specific VCG, all the isolates within the VCG contained both idiomorphs; for example, VCG 0421. The distribution of *F. oxysporum* isolates that contained either the *MAT1-1* or both *MAT1-1* and *MAT1-2* idiomorphs was not specific to any of the phylogenetic clades in the EF-1 α -mtSSU or IGS phylogenies. This may suggest the historic exchange of genetic material among *F. oxysporum* f. sp. *cepae* isolates.

The mechanisms by which exchange of genetic material and recombination could take place between *F. oxysporum* isolates may involve sexual reproduction, parasexuality, horizontal gene transfer, or mutation and transposition. The role of sexual reproduction in *F. oxysporum* is uncertain, because no teleomorph has been described (10,36). Therefore, this species complex is considered to be asexual, although sexual reproduction may have occurred in the early evolution of this species complex in ancient lineages (23,48,60).

Our study has provided new information regarding the biology and phylogenetic diversity of *F. oxysporum* f. sp. *cepae* and *F. oxysporum* isolates associated with onion. Three new *F. oxysporum* f. sp. *cepae* VCGs and three new SMVs were identified among *F. oxysporum* f. sp. *cepae* isolates from South Africa and Colorado. In each of these regions, one VCG dominated; namely, VCG 0421 in Colorado and VCG 0425 in South Africa. The virulence of *F. oxysporum* f. sp. *cepae* isolates differed, with some VCGs mainly containing moderately virulent isolates (VCG0423 and 0422), whereas the dominant VCG 0421 and 0425 isolates were all highly virulent. Multilocus phylogenies supported the putative clonal nature of the main *F. oxysporum* f. sp. *cepae* VCGs (0421 and 0425) and confirmed the polyphyletic origin of *F. oxysporum* f. sp. *cepae*. The information obtained from our molecular data has recently been used to develop markers for the rapid identification of the two main *F. oxysporum* f. sp. *cepae* VCGs (0421 and 0425; 56), and will be valuable for selecting genetically diverse isolates for use in breeding programs. Possible genetic exchange between isolates via parasexuality or sexual reproduction was supported by the (i) divergent placement of two isolates in the IGS and EF-1 α -mtSSU phylogenies, (ii) presence of both *MAT1-1* and *MAT1-2* idiomorphs within the same isolate and across phylogenetic clades,

and (iii) close genetic relationship between some nonpathogenic and *F. oxysporum* f. sp. *cepae* isolates.

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