Intergenic Spacer–RFLP Analysis and Direct Quantification of Australian *Fusarium oxysporum* f. sp. *vasinfectum* Isolates from Soil and Infected Cotton Tissues

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ABSTRACT

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Fusarium wilt of cotton, caused by *Fusarium oxysporum* f. sp. *vasinfectum*, can have devastating effects on the vascular system of cotton plants and is a major threat to cotton production throughout the world. Accurate characterization and improved detection of these pathogenic isolates is needed for the implementation of a disease prevention program and the development of disease management strategies. Polymerase chain reaction (PCR) amplification of the ribosomal intergenic spacer (IGS) regions combined with digestion with three restriction enzymes (*AluI*, *HaeIII*, *RsaI*) resulted in three unique restriction profiles (IGS–restriction fragment length polymorphism [RFLP] haplotypes) for Australian *F. oxysporum* f. sp. *vasinfectum* isolates, which were capable of distinguishing them from other formae speciales of *F. oxysporum*. Furthermore, a portion of the IGS region flanking the 5' end was sequenced and single nucleotide polymorphisms (SNPs) were revealed. Using these sequence data, two specific real-time PCR-based assays were developed for the absolute quantification of genomic DNA from isolates obtained from soil substrates and infected cotton tissues. Standard curves of real-time PCR-based assays showed a linear relation ($R^2 = 0.993$ to 0.994) between log values of fungal genomic DNA and real-time PCR cycle thresholds. Using these assays, it was possible to detect fungal DNA as low as 5 pg/μl. The detection sensitivity for inoculum added to sterile soils was lower than 10^4 conidia/g soil. In plant samples, the quantified fungal DNA varied from 30 pg to 1 ng/100 ng of total plant genomic DNA.

Additional keywords: *Gossypium hirsutum*, molecular detection

Fusarium oxysporum (5) is well known to cause vascular diseases on a broad range of agricultural and ornamental plant species. In order to characterize and study the wide diversity of *F. oxysporum* isolates infecting a variety of plant hosts, Snyder and Hansen (40) have proposed a classification system that arranges individual pathogenic strains with similar or identical host range into groups called formae speciales. In this manner, *Fusarium* wilt of cotton is caused by *F. oxysporum* f. sp. *vasinfectum* and has been reported as a major threat to cotton production (16). Several races have been characterized

within this forma specialis, such as American races 1, 2, and 6, Egyptian race 3, and Indian race 4 (15). In Australia, *Fusarium* wilt of cotton was first confirmed in 1993, and since then it has spread significantly in the country. It has been reported that the disease is attributable to two pathogenic biotypes, 01111 and 01112 vegetative compatibility groups (VCGs) that are vegetatively incompatible (16). Later, genetic analyses indicated that the isolates were clearly distinct from the American isolates and formed an independent clade (26). Furthermore, Kim et al. (26) reported the occurrence of *F. oxysporum* f. sp. *vasinfectum* races 3, 4, and 8 in California as possible causes of serious economic losses in some cotton fields due to the importation of large quantities of infected Australian cotton seed to California. Davis et al. (15) assumed that Australian biotypes were limited to Australia. In a 1952 survey, isolates of *F. oxysporum* f. sp. *vasinfectum* were not detected in Greece (2) and currently remain unreported in this country.

Nuclear ribosomal DNA (rDNA) is known to be a useful source of inter- and intra-specific polymorphisms in fungal species (23). Ribosomal genes exist in

multiple copies that are arranged in tandem repeats interrupted by noncoding spacer regions. The intergenic spacer (IGS) region is located between the large and small subunit of consecutive cistrons and is reported to be evolving more rapidly than any other region in rDNA repeat units. IGS polymorphisms, based on length or sequence variation, can distinguish between closely related species. Numerous studies have reported the application of an approach involving restriction digestion of polymerase chain reaction (PCR) products to identify polymorphisms in IGS or internal transcribed spacer regions (ITS1 and ITS2) for conducting diversity studies between *Fusarium* species or individual isolates within a *Fusarium* species (3,12,26,27).

Real-time PCR technology can accurately quantify the extent of pathogen biomass and, with multiplex formats, can simultaneously detect different organisms. The applications of this technique are based on the use of fluorogenic probes (hybridization probes or exonuclease probes such as TaqMan) (32) or dyes (SYBR Green dye) (31). In contrast to fluorogenic probes, the SYBR Green dye does not require the design of specific fragments complementary to the target DNA. Because the dye has no sequence specificity, it can be used to detect any double-stranded (ds) DNA PCR product. In its unbound state, SYBR Green emits relatively low fluorescence, but it fluoresces brightly when bound to dsDNA. As the amount of amplification product increases, the amount of fluorescence emitted by the dye increases proportionally (6).

This technology is being used increasingly in plant pathology for the accurate detection and quantification of plant pathogens, even at very low levels of infection (7,9,18,36–38,41–43,45). However, its application for the detection and quantification of fungi from soil samples remains difficult for *Helminthosporium solani* (14), *Colletotrichum coccodes* (13), *Rhizoctonia solani* (28), *F. solani* f. sp. *glycines* (29), and *F. oxysporum* f. sp. *niveum* (46).

In order to prevent and monitor the possible spread of the Australian *F. oxysporum* f. sp. *vasinfectum* isolates in Greece and other cotton growing areas, a reliable and rapid characterization and diagnostic method is needed that allows for the detec-

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tion of contaminated plant or soil samples. Therefore, the objectives of this research were to develop: (i) unique IGS-restriction fragment length polymorphism [RFLP] markers that can be used to characterize and differentiate Australian *F. oxysporum* f. sp. *vasinfectum* (VCG01111 and VCG01112) isolates from other cotton root fungal colonizers and especially from other formae speciales of *F. oxysporum* and (ii) reliable real-time PCR assays for the quantification of these isolates from various tissues of cotton cultivars and representative soil types.

MATERIALS AND METHODS

Fungal isolates and growth conditions. Six isolates of *F. oxysporum* f. sp. *vasinfectum* (*FOV*) (three of each VCG) from Australia, seven isolates of *F. oxysporum* formae speciales, four nonpathogenic *F. oxysporum* isolates, two isolates of other *Fusarium* species, and two fungal isolates of *Verticillium dahliae* and *Thielaviopsis basicola* that cause cotton vascular wilt disease and black root rot, respectively, were included in this study (Table 1). Cultures of all isolates were maintained on potato dextrose agar (PDA) at 4°C. For DNA extraction, cultures were grown in 250 ml of potato dextrose broth (PDB) in a rotary shaker at 180 rpm for 4 days at 28°C. After vacuum filtration, the mycelia were lyophilized, ground with sand using a mortar and pestle, and stored at -20°C.

Conidia of pure *FOV* cultures (isolates *FOV 1.2* and *FOV 2.1*) selected for construction of real-time PCR-based standard curves and inoculations of plant samples were produced on potato-carrot medium (13). Cultures were incubated at 22°C under a fluorescent and a black light (16 h day/8 h night) for 10 days. To obtain a conidial suspension, the surface of each culture plate was flooded with 2 ml of sterile distilled water, agitated for 30 s by manually swirling each petri plate, serially diluted, and adjusted using a hemacytometer to the desired concentrations of 5×10^7 , 5×10^6 , 5×10^5 , 5×10^4 , and 5×10^3 conidia/ml (pure *FOV* cultures). For plant inoculations, a dilution of 10^7 conidia/ml was used for drenching young (5 week old) cotton plantlets of cvs. Lacta and Emerald maintained in a growth chamber under optimal conditions (28°C day/26°C night with a 16-h photoperiod at 65% relative humidity).

Soil preparation and inoculation. Soil samples were collected from two cotton cultivation sites in Greece representing two different soil types. The sites were located at Larissa lowlands (the main area of cotton cultivation in Greece), soil type sandy loam (type A), and at Thessaloniki University Farm in Northern Greece, soil type clay loam (type B). Soil mixtures consisting of one part of the two above sieved (3 mm) field soils were thoroughly mixed with one part each of Turface (Profile

Products LLC, IL), peat (4-mm sieved), and perlite as described by Fillion et al. (22). The soil mixtures were autoclaved for 30 min at 121°C three times on three consecutive days before use (sterile soil substrates). Soil filtrates prepared from suspensions of 20 g of field soil diluted in 1 liter of distilled water were added to the sterile soil substrates in order to reintroduce soil microflora. These preparations were used only as negative controls for testing the specificity of primer pairs to detect only the Australian *FOV* isolates in soil samples.

Samples consisting of 2 ml each of *FOV 1.2* and *FOV 2.1* conidia suspensions (10^7 , 10^6 , 10^5 , 10^4 , and 10^3 conidia/ml) were added to 2 g of sterile soil substrates. Controls consisted of duplicate tubes containing sterile or nonsterile soil substrates inoculated with 2 ml of sterile distilled water. All tubes were vortexed for 1 min, freeze-dried for 2 to 3 days, and stored at -80°C prior to soil fungal genomic DNA extraction.

DNA extraction. Genomic fungal and plant DNA was extracted according to DNeasy Plant Mini Kit (Qiagen Inc., Chatsworth, CA). Total soil genomic DNA was isolated with the UltraClean Soil DNA Kit from MoBio Laboratories, Inc. (Solana Beach, CA) according to the manufacturer's protocol without any modification and stored at -20°C. DNA concentrations were determined spectrophotometrically and/or by quantitation on agarose gels stained with ethidium bromide in comparison with molecular marker λ -DNA-*Hind*III (Gibco-BRL, Gaithersburg, MD).

PCR amplification of the IGS regions. The IGS regions of all fungal strains used in this study were amplified by the univer-

sal primers CNL12 and CNS1 (44). Amplifications were performed in 50- μ l reaction mixtures containing 5 μ l of 10 $\times$ PCR buffer; 0.2 μ M Mg²⁺, 0.2 μ M of each primer, 0.2 mM each deoxyribonucleotide triphosphate (dNTPs), 0.5 units of DyNAzyme II DNA polymerase (Finnzymes, Espoo, Finland), and 100 ng fungal DNA. A PTC-200 thermocycler (MJ Research, Waltham, MA) was used with an initial denaturation step of 5 min at 94°C followed by 35 cycles of 1 min at 94°C, 1 min at 55°C, 2 min at 72°C, and a final step of 10 min at 72°C. Amplification products were separated by electrophoresis through 1.5% (wt/vol) agarose gels in 1 $\times$ TBE buffer. Gels were stained with ethidium bromide and photographed on a UV transilluminator.

Restriction digestion of PCR products and phylogenetic analysis of the IGS-RFLP haplotypes. Amplified PCR products were purified by precipitation by adding 0.1 volume of 3 M sodium acetate and 2.5 volumes of absolute ethanol to remove excess dNTPs and primers, and then resuspended in sterile distilled water. Four restriction enzymes (10 U/ μ l) were used to digest amplified DNA: *Alu*I, *Rsa*I, *Hae*III, *Taq*Ia (New England Biolabs, Beverly, MA) according to the manufacturer's instructions. Electrophoresis of the restriction fragments was carried out in 1.5% agarose or 3.5% (wt/vol) metaphor gel (FMC Bio-products, Rockland, ME) in 1 $\times$ TBE buffer. The molecular size of each fragment was estimated precisely using the original UVI-Doc Mw package (St John's Innovation Center, Cambridge, UK) software by comparisons with standard DNA molecular weight markers (Cambrex, Rockland, ME).

Table 1. Fungal species used in this study with their abbreviations, number of isolates, and host/geographic origin

Fungal species	Isolate abbreviation	No. of isolates	Host/collection site
<i>Fusarium oxysporum</i> f. sp. <i>vasinfectum</i> (VGG 01111)	<i>FOV 1.1</i> , <i>FOV 1.2</i> , <i>FOV 1.3</i>	3	Cotton/Australia ^y
<i>F. oxysporum</i> f. sp. <i>vasinfectum</i> (VGG 01112)	<i>FOV 2.1</i> , <i>FOV 2.2</i> , <i>FOV 2.3</i>	3	Cotton/Australia ^y
<i>F. oxysporum</i> f. sp. <i>phaseoli</i> (F123)	<i>FOP</i>	1	Phaseolum/Kastoria ^z
<i>F. oxysporum</i> f. sp. <i>lycopersici</i> (F421)	<i>FOL</i>	1	Tomato/Kiparissia ^z
<i>F. oxysporum</i> f. sp. <i>radicis.cucumericum</i> (F614)	<i>FORC</i>	1	Cucumber/Trifolia ^z
<i>F. oxysporum</i> f. sp. <i>radicis.lycopersici</i> (F623)	<i>FORL</i>	1	Cucumber/Ierapetra ^z
<i>F. oxysporum</i> f. sp. <i>niveum</i> (F42)	<i>FON</i>	1	Watermelon/Agrinio ^z
<i>F. oxysporum</i> f. sp. <i>melonis</i> (F589)	<i>FOM</i>	1	Melon/Alexandroupoli ^z
<i>F. oxysporum</i> f. sp. <i>dianthi</i> (F636)	<i>FOD</i>	1	Carnation/Marathonas ^z
<i>F. oxysporum</i> (F389)	<i>FOX</i>	1	Tomato/Ag. Vlassis ^z
<i>F. oxysporum</i> (F371)	<i>FONP 1</i>	1	Tomato/Ag. Vlassis ^z
<i>F. oxysporum</i> (F388)	<i>FONP 2</i>	1	Tomato/Ag. Vlassis ^z
<i>F. oxysporum</i> (F376)	<i>FONP 3</i>	1	Tomato/Ag. Vlassis ^z
<i>F. proliferatum</i> (F647)	<i>FPRO 1</i>	1	Asparagus/Kavala ^z
<i>F. proliferatum</i> (F643)	<i>FPRO 2</i>	1	Asparagus/Orestida ^z
<i>Thielaviopsis basicola</i> (BPIC1693)	<i>TBAS</i>	1	Tobacco/Locrida ^z
<i>Verticillium dahliae</i> (70V)	<i>VERT</i>	1	Tomato/Scala ^z

^y Isolates were obtained from W. O'Neill (Plant Pathology Building, Department of Primary Industries, Indooroopilly, Queensland, Australia).

^z Isolates were obtained from K. Elena (Benaki Phytopathological Institute of NAGREF, Athens, Greece).

The phylogenetic analysis based on the RFLP patterns was performed using the RESTDIST procedure of PHYLIP version 3.62 software (19). Subsequently, a neighbor-joining tree (NJ) with 1,000 bootstrap replicates was reconstructed from the distance matrix using Neighbor program (20), and the trees were exported into TreeView version 1.6.6 as described by Page (33). Isolate *FOV 1.1* was included as an outgroup sample.

Sequencing of IGS fragments and design of *FOV*-specific primers. PCR amplification products of all fungal IGS regions were purified using the NucleoSpin Extract Kit (Macherey Nagel, Duren, Germany) in order to remove secondary metabolites prior to sequencing. The 5' end of each fragment was sequenced using the reverse primer CNS1. Sequencing was carried out by the DNA facility in the Laboratory of Institute of Molecular Biology and Biotechnology (I.M.B.B., Crete, Greece) in a Long Read IR2 4200 DNA sequencer (Li-Cor, Lincoln, NE). Gene sequence data are available in the GenBank database under accession numbers AY997771 through AY997789 and AY997790.

Two sets of Australian *FOV*-specific primer pairs, *FOV-1/FOV-2* and *FOV-3/FOV-4*, were commercially synthesized based on the previously described IGS sequence. The *FOV-1* (5'-TGTAGG GGTGTGGGTTTTTTC-3') and *FOV-2* (5'-CCAACACACAACCGCACACGA-3') primers, which flank a 125-bp DNA fragment located near the 5' end of the IGS rRNA gene, were used to amplify all three isolates of VCG01111 and one isolate (*FOV 2.3*) of VCG01112. The *FOV-3* (5'-AGCAGTAGAGTAGCCTTGTG-3') and *FOV-4* (5'-TTCCACCAAGTCTAAGTC GC-3') primers were designed to generate a 165-bp DNA product from the 5' end of isolates *FOV 2.1* and *FOV 2.2* (VCG01112). The specificity of each primer pair was confirmed by absence of significant homology with other known DNA sequences using the Basic Local Alignment Search Tool (BLAST) of the GenBank database. To evaluate the specificity of these Australian *FOV*-specific primer pairs, we extended our work to two other isolates of *FOV* races: race 3 (Egyptian) and race 4 (Indian) obtained from C. Steinberg (INRA, Dijon, France).

Standard curves and real-time PCR amplification. Standard fluorescent ampli-

fication curves representing an exponential growth of PCR products based on cycle thresholds (C_T) for 10-fold dilution series of pure fungal cultures genomic DNA (5×10^1 , 5×10^0 , 5×10^{-1} , 5×10^{-2} , and 5×10^{-3} ng/ μ l) were constructed for the two representative Australian *FOV* isolates (*FOV 1.2* and *FOV 2.1*) detected by the corresponding specific primer pairs. C_T values were calculated to indicate significant fluorescence signals rising above background during the early cycles of the exponent amplification phase of the real-time PCR process.

Quantitative PCR amplifications were carried out in a total volume of 25 μ l using an Opticon 2 (MJ Research) real-time PCR system. PCR was performed in 1 $\times$ PCR buffer, 0.2 mM dNTPs, 0.4 pmol for each *FOV* primer, 0.2 $\times$ SYBR Green (Sigma-Aldrich, Dorset, UK), and 1 U of the DYNazyme II DNA polymerase (Finnzymes). Different concentrations of *FOV* conidia were added to sterile soil. DNA was extracted from these amended soil samples, and 4 μ l of the undiluted DNA extract was amplified as described above to examine the relationship between the number of conidia in a soil sample and real-time PCR results. In separate reactions, 4 μ l (100 ng) of DNA extracted from plant tissues were used for real-time PCR. Template control reactions contained 4 μ l of sterile water replacing the DNA solution. The thermal cycling conditions for *FOV 1.2* isolate (VCG01111) and *FOV 2.1* isolate (VCG01112) DNA template amplification were: incubation at 94°C for 5 min, followed by 35 cycles of 1 min at 94°C, 30 s at 61°C and 63°C, respectively, and 1 min at 72°C, and a final extension step of 5 min at 72°C. For identification of the PCR products, a melting curve was performed from 65 to 95°C by reading every 0.2°C with a 10-s hold between each temperature increment. All reactions were performed in triplicate.

Post-PCR statistical analysis. Statistical analyses were performed using the Correlation and General Linear Model procedure of the SPSS (version.12) statistical software (SPSS Institute, 1998). The relation between C_T and fungal DNA concentration was analyzed by correlation analyses. The effect of conidia concentration on fungal genomic DNA quantification was analyzed by multifunctional ANOVA (three-way ANOVA) in which we examined the main level effect of each factor, as well as two-way and three-way interactions be-

tween factors. For each factor, we made all possible by two comparisons (Post hoc tests) between all levels using the Sidak correction test (24). Significant differences were detected at $P < 0.05$.

RESULTS

IGS-RFLP analysis. A PCR procedure using the universal primers CNL12 and CNS1 was conducted to amplify the IGS region of all isolates, and a single fragment was generated from each isolate (Fig. 1). The average size of the amplified fragments was approximately 2.6 kb. A considerable decrease in length of amplified fragments was revealed with *FOP* and *VERT* isolates (Fig. 1). The observed polymorphism for sizes of fungal IGS regions of even similar formae speciales is well documented (20). In our experiments, the lengths of the IGS regions were also estimated by a summation of individual fragment sizes generated by RFLP analysis. However, this estimation was different for each enzyme used and did not always match the sizes of the nondigested regions even when Metaphor high-resolution agarose was used (Fig. 2).

Digestion of the IGS regions with the four restriction enzymes generated 53 (16 *AluI*-IGS, 13 *TaqI*-IGS, 12 *RsaI*-IGS, and 12 *HaeIII*-IGS) different restriction patterns (Table 2). Three *AluI* restriction profiles were identified that were unique to Australian *FOV* isolates (Table 2). A single *RsaI* and two *HaeIII* restriction profiles were identified that were unique to Australian *FOV* isolates. No restriction profiles unique to *FOV* isolates were identified from digestions of IGS regions with *TaqI*. In total, 18 IGS haplotypes were identified based on restriction analysis, of which three were haplotypes unique to *FOV* isolates (Table 2).

Phylogenetic analysis of the data generated a consensus tree in which several robust clusters could be observed (Fig. 3). The Australian isolates formed clusters that appear to be independent of each other. Specifically, two isolates of VCG01112 (*FOV 2.1/FOV 2.2*), two isolates of VCG01111 (*FOV 1.2/FOV 1.3*), two isolates of *FPRO*, and *FONP 1* and *FONP 3* form distinct clusters supported by bootstrap values higher than 90%. The other two remaining *FOV* isolates were not grouped in the same clusters and showed little genetic similarity to their corresponding VCGs. All isolates were classified into

Fig. 1. Polymerase chain reaction (PCR) amplification products corresponding to the intergenic spacer (IGS) regions of the 21 fungal isolates used in the present study using primers CNL12/CNS1 visualized on EtBr-stained agarose gel (1.5%). Lanes: 1, molecular marker λ -DNA-*HindIII*/ ϕ X-*HaeIII*; 2–4, three different isolates of *FOV* VCG01111; 5–7, three different isolates of *FOV* VCG01112; 8, *FOP*; 9, *FOL*; 10, *FORC*; 11, *FORL*; 12, *FON*; 13, *FOM*; 14, *FOX*; 15, *FOD*; 16, *FONP 1*; 17, *FONP 2*; 18, *FONP 3*; 19, *FPRO 1*; 20, *FPRO 2*; 21, *TBAS*; 22, *VERT*; 23, H_2O .

five major lineages based on this analysis, where *FOV* isolates were observed in the separate lineages I, V, and II (Table 3).

Sequence data, design, and specificity of *FOV*-specific primers. IGS amplicons also were used as templates for direct sequencing of a portion of the IGS region flanking the 5' end. A fragment ranging in size from 442 to 451 bp was obtained for all isolates with the exception of *FOM*, for which a fragment of 378 bp was identified. A unique sequence was identified for each isolate except for *FORL* and *FORC*, for

which identical sequences were determined. Single nucleotide polymorphisms (SNPs) unique for isolates of *FOV* were identified by nucleotide alignments of the sequenced portions of IGS regions of all fungal isolates used (data not shown). Two sets of *FOV*-specific primer pairs (*FOV*-1/*FOV*-2 and *FOV*-3/*FOV*-4) were subsequently designed in order to exploit SNPs in the 3' region that differentiate Australian *FOV* isolates from other isolates. Comparisons of each primer sequence with nucleotide sequences of other plant-pathogenic

fungi and bacteria deposited in GenBank databases revealed no significant levels of similarity. The specificity of these primers for *FOV* isolates was tested, and a specific product (125 bp) was reliably detected after conventional or real-time PCR using primers *FOV*-1/*FOV*-2. Similarly, primer pairs *FOV*-3/*FOV*-4 amplified a unique DNA fragment of approximately 165 bp (Fig. 4A and B).

However, fragments of the same size as those generated from *FOV* isolates were also unexpectedly amplified from an iso-

Fig. 2. Restriction fragments generated by restriction fragment length polymorphism (RFLP) analysis on polymerase chain reaction (PCR)-amplified intergenic spacer (IGS) regions of the 21 fungal isolates used in the present study visualized on EtBr-stained agarose (1.5%, A and B) or metaphor (3.5%, C and D) gel after digestion with the restriction endonucleases *AluI* (A), *RsaI* (B), *HaeIII* (C), *TaqIa* (D). **A and B.** Lanes: 1, molecular markers λ -DNA-*HindIII*/X-*HaeIII*; 2,6,7, three different isolates of *FOV* VCG01111; 3–5, three different isolates of *FOV* VCG01112; 8, *FOP*; 9, *FOL*; 10, *FORC*; 11, *FORL*; 12, *FON*; 13, *FOM*; 14, *FOX*; 15, *FOD*; 16, *FONP 1*; 17, *FONP 2*; 18, *FONP 3*; 19, *FPRO 1*; 20, *FPRO 2*; 21, *TBAS*; 22, *VERT*; 23, H_2O . **C and D.** Lanes: 1,10,19,26, molecular markers 20 bp ext. ladder; 2,6,7, three different isolates of *FOV* VCG01111; 3–5, three different isolates of *FOV* VCG01112; 8, *FOP*; 9, *FOL*; 11, *FORC*; 12, *FORL*; 13, *FON*; 14, *FOM*; 15, *FOX*; 16, *FOD*; 17, *FONP 1*; 18, *FONP 2*; 20, *FONP 3*; 21, *FPRO 1*; 22, *FPRO 2*; 23, *TBAS*; 24, *VERT*; 25, H_2O .

Table 2. Distribution of lineage groups and intergenic spacer (IGS) haplotypes based on IGS–restriction fragment length polymorphism (RFLP) patterns of the 21 fungal isolates used in the present study

Isolates	Lineage	IGS haplotypes	IGS-RFLP pattern			
			<i>AluI</i>	<i>RsaI</i>	<i>HaeIII</i>	<i>TaqIa</i>
<i>Fusarium oxysporum</i> f. sp. <i>vasinfectum</i> <i>FOV 1.1</i> (VGG01111) / <i>FOV 2.3</i> (VGG01112)	I	1	A	A	A	A
<i>F. oxysporum</i> f. sp. <i>vasinfectum</i> <i>FOV 1.2</i> (VGG01111) / <i>FOV 1.3</i> (VGG01111)	V	2	B	A	A	A
<i>F. oxysporum</i> f. sp. <i>vasinfectum</i> <i>FOV 2.1</i> (VGG01112) / <i>FOV 2.2</i> (VGG01112)	II	3	C	B	B	A
<i>F. oxysporum</i> f. sp. <i>phaseoli</i> (<i>FOP</i>)	IV	4	D	C	C	B
<i>F. oxysporum</i> f. sp. <i>lycopersici</i> (<i>FOL</i>)	I	5	F	E	E	A
<i>F. oxysporum</i> f. sp. <i>radicis.cucumericum</i> (<i>FORC</i>)	III	6	I	B	F	F
<i>F. oxysporum</i> f. sp. <i>radicis.lycopersici</i> (<i>FORL</i>)	III	7	J	B	F	G
<i>F. oxysporum</i> f. sp. <i>niveum</i> (<i>FON</i>)	II	8	K	G	G	H
<i>F. oxysporum</i> f. sp. <i>melonis</i> (<i>FOM</i>)	III	9	L	H	H	I
<i>F. oxysporum</i> f. sp. <i>dianthi</i> (<i>FOD</i>)	II	10	P	L	K	L
<i>F. oxysporum</i> (<i>FOX</i>)	II	11	M	I	E	A
<i>F. oxysporum</i> (<i>FONP 1</i>)	III	12	G	F	E	D
<i>F. oxysporum</i> (<i>FONP 2</i>)	III	13	H	F	E	D
<i>F. oxysporum</i> (<i>FONP 3</i>)	III	14	G	F	E	E
<i>F. proliferatum</i> (<i>FPRO 1</i>)	II	15	N	J	I	J
<i>F. proliferatum</i> (<i>FPRO 2</i>)	II	16	N	J	L	M
<i>Thielaviopsis basicola</i> (<i>TBAS</i>)	III	17	E	D	D	C
<i>Verticillium dahliae</i> (<i>VERT</i>)	IV	18	O	K	J	K

late of *F. oxysporum* f. sp. *dianthi* (*FOD*) in both PCR reactions. The additional fragments may be due to sequence variation among the thousands of copies of IGS regions within a fungal species (44) such as *FOD*, in this case. To solve this problem, we developed two different approaches. First, restriction enzyme sites were identified based on obtained nucleotide data of the sequenced portions of IGS regions that could produce different digestion fragment patterns of the PCR amplicons. *PleI* and *TaqIa* revealed a different digestion pattern between Australian *FOV* isolates and *FOD* isolate in both cases (Fig. 4C and D). Second, possible differentiations of melting temperature (*T_m*) curves in real-time PCR

products were examined using each *FOV*-specific primer pair. In both cases, the results indicated that the *T_m* values differed in cases of *FOV* and *FOD* isolates from 0.2°C to 0.4°C (for *FOV*-1/*FOV*-2 primers) and from 0.2 to 0.6°C (for *FOV*-3/*FOV*-4 primers) (Fig. 4E and F).

The specificities of the two Australian *FOV*-specific primer pairs were also evaluated using two other *FOV* races including race 3 (Egyptian) and race 4 (Indian) using real-time PCR assays. The results of amplification products in both cases clearly showed that these primer pairs were specific only for Australian *FOV* isolates (Fig. 5).

Real-time quantification of *FOV* DNA extracted from soil samples and cotton

tissues. Different concentrations of 10-fold serially diluted *FOV* genomic DNA ranging from 5×10^1 to 5×10^{-3} ng/μl of *FOV* 1.2 and *FOV* 2.1 showed single amplicons with the expected sizes of 125 and 165 bp, respectively, as determined by either melting curve analysis or agarose gel electrophoresis. The use of standard curves based on known concentrations of DNA makes it theoretically possible to quantify DNA from any source. In this study, standard curves were constructed using the above-mentioned concentrations in which we plotted the samples from *FOV* 1.2 and *FOV* 2.1 infested soils or infected cotton tissues, respectively. Quantification showed linear relations ($R^2 = 0.994$ and

Fig. 3. Neighbor-joining tree illustrating phylogenetic relationships within the 21 fungal isolates used in the present study based on intergenic spacer-restriction fragment length polymorphism (IGS-RFLP) data. Horizontal branch lengths correspond to evolutionary distances assigned by PHYLIP program. Bootstrap values greater than 50% (based on 1,000 resamples) are indicated next to the branches.

Table 3. Quantification of *Fusarium oxysporum* f. sp. *vasinfectum* (*FOV* 1.2 and *FOV* 2.1) genomic DNA in sterile infested soil samples by real-time polymerase chain reaction

Number of conidia	DNA amount (ng) ^w					
	<i>FOV</i> 1.2		<i>FOV</i> 2.1		<i>FOV</i> 1.2	<i>FOV</i> 2.1
	Soil type A ^x	Soil type B ^x	Soil type A	Soil type B	Mean of soils	
10 ⁴	0.01 ± 0.001	0.02 ± 0.001	ND ^y	ND	0.01 ± 0.002 a ^z	...
10 ⁵	0.28 ± 0.050	0.19 ± 0.030	0.27 ± 0.010	ND	0.24 ± 0.030 a	0.13 ± 0.060 a
10 ⁶	5.03 ± 0.100	6.93 ± 0.170	6.89 ± 0.440	7.87 ± 1.410	5.98 ± 0.620 a	7.38 ± 0.690 a
10 ⁷	94.29 ± 3.330	61.39 ± 1.150	64.80 ± 7.280	60.83 ± 6.690	77.84 ± 7.520 b	62.8 ± 4.510 b
Mean conidia	24.90 ± 12.120 a ^z	17.13 ± 7.750 b	17.99 ± 8.340 a	17.17 ± 7.800 a		

^w Values are the mean of three replicates ± standard error of mean.

^x Composition of soil type A is 66% sand, 22% loam, 12% clay, and of soil type B is 23% sand, 47% loam, 30% clay.

^y ND: not detected.

^z Within rows and columns, values followed by a different letter are significantly different for each *FOV* isolate using Sidac's studentized range test ($P < 0.05$).

Fig. 4. A and B, Polymerase chain reactions (PCR) performed with the two primer pairs specific for *Fusarium oxysporum* f. sp. *vasinfectum* isolates (FOV-1/FOV-2 and FOV-3/FOV-4, respectively). Lane 1, 100-bp DNA ladder markers; **A**, lanes: 2–5, amplified products of *FOV* isolates (*FOV* 1.1, *FOV* 1.2, *FOV* 1.3, *FOV* 2.3); 12, *FOD* isolate; 6–11 and 13–20, amplified products of the other tested isolates; **B**, lanes: 2 and 3, amplified products of *FOV* isolates (*FOV* 2.1, *FOV* 2.2); 9, *FOD* isolate; 4–8 and 10–18, amplified products of the other tested isolates. **C and D**, Restriction digests of the previous amplicons with *PleI* (**C**) and *TaqIa* (**D**): lanes: 1, 100-bp DNA ladder markers; 2,4,6,8,13,15, amplified products of *FOV* isolates (*FOV* 1.1, *FOV* 1.2, *FOV* 1.3, *FOV* 2.3, *FOV* 2.1, *FOV* 2.2); 10,17, amplified products of *FOD* isolate; 3,5,7,9,14,16, restriction products of *FOV* amplicons (*FOV* 1.1, *FOV* 1.2, *FOV* 1.3, *FOV* 2.3, *FOV* 2.1, *FOV* 2.2); 11,18, restriction products of *FOD* amplicons; 12,19, H_2O . **E and F**, Melting curve profiles of real-time PCR-based assays using the *FOV*-specific primer pairs depicting the T_m difference in each case between *FOV* and *FOD* isolates.

Fig. 5. A and B, Amplified products of real-time polymerase chain reactions (PCR) performed with the two primer pairs specific for *Fusarium oxysporum* f. sp. *vasinfectum* isolates (FOV-1/FOV-2 and FOV-3/FOV-4, respectively). **A**, Lanes: 1–2, two representative *FOV* isolates (*FOV* 1.2, *FOV* 2.3); 3–4, *FOV* (race 3 and 4) isolates; 5, H_2O ; **B**, lanes 1–2, two representative *FOV* isolates (*FOV* 2.1, *FOV* 2.2); 3–4, *FOV* (race 3 and 4) isolates; 5, H_2O . **C and D**, Corresponding amplification plots (kinetics of fluorescence signal) of *FOV* 1.2 (curve 1) and *FOV* 2.3 (curve 2), (**C**) *FOV* 2.1 (curve 1) and *FOV* 2.2 (curve 2), (**D**) fungal DNA after using the two specific primer pairs FOV-1/FOV-2 (**C**) and FOV-3/FOV-4 (**D**). No fluorescence signal for *FOV* isolates race 3 and 4 was present in any real-time PCR reaction, indicative of the accuracy of the specific detection assays.

0.993, respectively) between log values of fungal genomic DNA and real-time PCR C_T s over the range of DNA concentrations examined (Fig. 6). No PCR products were obtained from any negative controls (data not shown).

The amount of *FOV 1.2* or *FOV 2.1* genomic DNA detected in both soils significantly increased ($P < 0.0001$) by increasing the amounts of conidia to $10^7/g$ (Table 3). No significant differences between the amounts of *FOV 2.1* genomic DNA ex-

tracted from soil substrates were found ($P = 0.668$). This was not the case with *FOV 1.2*, for which the amount of genomic DNA extracted from soil B was significantly reduced ($P < 0.01$) compared to DNA from soil A (Table 3). Finally, there was a significant difference ($P = 0.001$) between the detected amounts of *FOV 1.2* and *FOV 2.1* genomic DNA in soil A but not in soil B ($P = 0.982$).

Table 4 shows that the amounts of *FOV 1.2* and *FOV 2.1* genomic DNA detected in

tissues of the two cotton cultivars significantly increased ($P = 0.045$ and $P < 0.001$, respectively) from the resistant cv. Emerald to the susceptible cv. Lacta. Analyzing separately the results obtained in roots or hypocotyls, significant differences between the amounts of the *FOV 2.1* genomic DNA were found for both cultivars ($P = 0.007$). This was quite different for the *FOV 1.2* isolate in comparisons between mean differences for root and hypocotyl tissues of the resistant cv. Emerald,

Fig. 6. **A and B,** Standard curves of real-time polymerase chain reaction (PCR)-based assays used for the absolute quantification of *FOV 1.2* (**A**) and *FOV 2.1* (**B**) target DNA isolated from two different soil types (A and B) and from different tissues (roots and hypocotyls) of infected cotton plants (cvs. Lacta [susceptible] and Emerald [resistant]). **C and D,** Amplification plots (kinetics of fluorescence signal) of *FOV 1.2* (**C**) and *FOV 2.1* (**D**) intergenic spacer (IGS) DNA from 10-fold diluted pure genomic DNA after using the two specific primer pairs FOV-1/FOV-2 (**C**) and FOV-3/FOV-4 (**D**). **E and F,** Corresponding melting curve profiles where the negative derivative of fluorescence with respect to temperature is plotted as dF/dT versus temperature to obtain a graphical representation of the melting peaks.

where no significant differences were determined ($P = 0.972$). A significant difference in the amount of genomic DNA of *FOV 2.1* extracted from hypocotyls was found between the two cultivars ($P < 0.001$). Finally, significantly increased differences ($P = 0.009$ and $P < 0.001$, respectively) occurred between roots and hypocotyls when "Lacta" plants were infected with both *FOV* isolates (Table 4).

DISCUSSION

The rapid and reliable detection of pathogens is important in developing strategies for disease management in plant nurseries (30). Our objectives were to characterize Australian *FOV* isolates in order to identify unique IGS-RFLP haplotypes and to develop a real-time PCR-based assay to reliably detect and quantify these isolates.

Our results indicate that IGS-RFLP analysis is a useful tool in discriminating *FOV* isolates from all other fungal isolates used in this study. The occurrence of restriction site polymorphisms was confirmed by the identification of unique restriction profiles using the restriction enzymes *AluI*, *RsaI*, and *HaeIII*. The use of high-resolution metaphor gels allowed the identification of small molecular weight fragments by increasing the number of resolved polymorphic bands. Different size polymorphisms occurred even in the amplified fungal IGS products. Recently, Kim et al. (26) identified nine haplotypes based on restriction digests of the IGS region by testing 30 isolates of *FOV* from different worldwide geographic origins. The Australian isolates comprised an independent lineage, indicating the existence of a polyphyletic evolutionary process. This is consistent with findings of Skovgaard et al. (39), who found four lineages within eight races of the pathogen. Size polymorphism in the IGS regions of *F. oxysporum* formae speciales has also been observed by Kim et al. (25). Our findings also indicate a strong diversity occurring within these similar isolates.

We were able to identify six unique restriction patterns for Australian *FOV* isolates (3 *AluI*-IGS, 2 *HaeIII*-IGS, and 1 *RsaI*-IGS) that enabled us to develop specific RFLP markers for these isolates. This IGS-RFLP approach has also been re-

ported in several other studies to assess the genetic diversity of isolates within *Fusarium oxysporum* formae speciales such as *phaseoli* (1), *melonis* (4), and *lilii* (8). In these studies, isolates belonging to the same VCG exhibited the same IGS haplotype, which agrees with the hypothesis that VCGs represent clonal lineages that rarely, if ever, reproduce sexually. A similar conclusion has been reported in a study that assessed the genetic relationships among 52 isolates of *FOV* of worldwide origin, with the exception of Australian strains, based on rDNA RFLP analysis (21).

Sequence polymorphism in the IGS region is suitable for the development of PCR-based diagnostic protocols of higher sensitivity and specificity (9,10,34,35). The specificity of such a protocol for the detection and absolute quantification of Australian *FOV* isolates was confirmed by testing them against other *FOV* isolates from Egyptian race 3 and from Indian race 4 or against other cotton pathogenic fungi and related formae speciales that originated from Greece. These assays were also of high sensitivity, allowing the absolute quantification by real-time PCR of the two representative Australian *FOV* isolates *FOV 1.2* and *FOV 2.1*. These isolates were selected for additional real-time based PCR quantitative analysis because after nucleotide alignment of the sequenced portions of the IGS regions, isolates *FOV 1.2* and *FOV 2.1* revealed unique and representative SNP patterns common to the four other *FOV* isolates (*FOV 1.1*, *FOV 1.3*, *FOV 2.2*, *FOV 2.3*), capable of distinguishing them from all the other fungal isolates used. We were able to detect amplification products by real-time PCR at the lower range of 5 and 50 pg/μl of target DNA, respectively. Both pairs of the Australian *FOV*-specific primers under optimized PCR conditions amplified single amplicons of 125 and 165 bp, respectively, without amplifying nontarget genomic DNA extracted from nonsterile soil substrates. Two standard curves with R^2 values from 0.994 to 0.993 were constructed, confirming the linearity of the quantification process between exponential increases of DNA concentrations and real-time PCR threshold cycles. Because C_T values may vary slightly between experiments, we included parallel runs of three separate

dilution series of pure standard DNA in all experiments.

Field soils harbor microbial complexes, making it tedious and time-consuming to isolate soilborne pathogens such as *FOV* by traditional agar plating. High-quality purified DNA of *FOV* isolates extracted from infested soil substrates was obtained using the commercially available UltraClean Soil DNA Kit (MoBio Laboratories, Inc). The real-time PCR assays were highly sensitive, which allowed for detection of low levels of fungal propagules (less than 10^4 conidia/g infested soil substrate, in case of *FOV 1.2*).

Regardless of the treatment, the 10-fold increase in conidia concentration was not linearly proportional to increases in amplified genomic DNA from both *FOV* isolates in the two soil types, particularly with samples of 10^5 and 10^6 conidia. Recently, Cullen et al. (13) and Fillion et al. (22), using PCR primers targeting the ribosomal region, also showed that the amount of *Helminthosporium solani* and *Glomus intraradices* DNA extracted from different soil types increased with spore concentration, but not linearly. Since fungi are known to exist in multinucleate forms (11), this nonlinear increase may be attributed to the fact that there might be multiple and different copies of ribosomal sequences present in a given fungal cell. This well-demonstrated phenomenon of ribosomal sequence polymorphism (44) could interfere with primer binding and reduce the overall efficiency of PCR that in turn would also be proportionally reduced as the amount of total target genomic DNA increases. Furthermore, we could assume that even soil composition and structure could affect the amounts of genomic DNA detected. This may be the case for *FOV 1.2* DNA amount detected in soil A, which had a lighter structure and probably less co-extracted humic acids and PCR inhibitors. In this case, a 20-fold decrease in DNA amount was observed after the inoculation of 10^7 to 10^6 conidia/g. This might also have occurred because of the lower standard curve efficiency found in independent experiments conducted. Nevertheless, the real-time PCR approach described in this study was statistically appropriate to quantify *FOV* genomic DNA and discriminate between different conidia quantities incor-

Table 4. Quantification of *Fusarium oxysporum* f. sp. *vasinfectum* (*FOV 1.2* and *FOV 2.1*) genomic DNA in infected cotton tissues by real-time polymerase chain reaction

Cultivars tested	DNA amount (ng) / 100 ng total plant genomic DNA ^y					
	<i>FOV 1.2</i>		<i>FOV 2.1</i>		<i>FOV 1.2</i>	<i>FOV 2.1</i>
	Root	Hypocotyl	Root	Hypocotyl	Mean of root and hypocotyl tissues	
Emerald	0.03 ± 0.003	0.03 ± 0.002	0.13 ± 0.014	0.08 ± 0.003	0.03 ± 0.002 a ^z	0.11 ± 0.013 a
Lacta	0.04 ± 0.006	0.37 ± 0.021	0.52 ± 0.214	1.05 ± 0.053	0.20 ± 0.074 b	0.79 ± 0.155 b
Mean of cultivars	0.03 ± 0.003 a ^z	0.20 ± 0.076 a	0.33 ± 0.129 a	0.57 ± 0.219 b		

^y Values are the mean of three replicates ± standard error of mean.

^z Within rows and columns, values followed by a different letter are significantly different for each *FOV* isolate using Sidac's studentized range test ($P < 0.05$).

porated into soil samples. With regard to absolute quantification of Australian *FOV* isolates in plant tissues, higher amount of fungal genomic DNA was detected in hypocotyl tissues than in root tissues (Table 4). Furthermore, the amount of fungal genomic DNA detected was greater for susceptible cv. Lacta hypocotyls (a mean of 1.05 ng in the case of *FOV* 2.1) compared with hypocotyls from the resistant cv. Emerald (a mean of 0.08 ng).

In conclusion, this study has shown that IGS-RFLP analysis has generated unique restriction profiles for each *FOV* isolate, allowing the development of RFLP markers for the characterization of *FOV* isolates and distinguishing them from all other common fungal isolates occurring in cotton fields of Greece. By combining a commercial soil DNA extraction protocol and real-time PCR-based assay using the SYBR Green dye approach described in this study, the entire fungal DNA quantification process from soil substrates to PCR results could be adequately performed in less than 2 h.

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