EF-1α gene and IGS rDNA sequencing of *Fusarium oxysporum* f. sp. *vasinfectum* and *F. oxysporum* f. sp. *phaseoli* reveals polyphyletic origin of strains

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ABSTRACT

The fungi *Fusarium oxysporum* f. sp. *vasinfectum* (Fov) and *F. oxysporum* f. sp. *phaseoli* (Fop) are important pathogens that cause wilt on cotton and common bean, respectively. In the present study, phylogenetic trees constructed with partial sequences of the translation elongation factor gene and ribosomal intergenic spacer region of Brazilian Fov and Fop strains revealed polyphyletic origin of strains within both formae speciales. This creates an obstacle for the development of specific molecular diagnostic methods and explains the failure of supposedly specific methods described in the literature to correctly discriminate formae speciales of *F. oxysporum*.
Key words: *Gossypium* spp., *Phaseolus vulgaris*, Fusarium wilt, molecular diagnosis.

INTRODUCTION

Cotton (*Gossypium* spp.) and common bean (*Phaseolus vulgaris* L.) are important crops in Brazil and several countries worldwide. Cotton is the main natural fiber in the world and Brazil is the third largest exporter (EMBRAPA Algodão, 2003; MAPA 2012). Common bean plays an important role in the Brazilians' diet and Brazil is the world's largest producer of this crop (EMBRAPA Arroz e Feijão, 2005). These crops can be severely impacted by the fungal pathogens *Fusarium oxysporum* Schlechtend.:Fr. f. sp. *vasinfectum* (Atk.) Snyd. & Hans. (Fov) and *Fusarium oxysporum* f. sp. *phaseoli* Kendr. & Snyd. (Fop), that cause Fusarium wilt in cotton and common beans, respectively. Both pathogens can be inadvertently introduced in the field by contaminated seed, therefore, sensitive and specific methods for their detection are important tools for seed inspection needed to control the spread of these diseases.

Members of the *F. oxysporum* species complex (FOSC), both saprophytic and pathogenic, are commonly found in soil (Michielse & Rep, 2009). The pathogenic forms can be separated into formae speciales based on their ability to cause disease on different plant species hosts. Within formae speciales, subdivision into races has also been described based on the ability of distinct strains to infect different varieties of a plant host species (O'Donnell et al., 2009). *Fusarium oxysporum* strains that can cause infection in humans and animals have also been described which cause high mortality in immunodeprived patients (O'Donnell et al., 2004). Although morphological distinction of *F. oxysporum* from other species of the genus is attainable, infra-specific identification with such method is not feasible.

Phylogenetic studies of members of the FOSC using molecular tools such as multilocus sequencing typing (MLST) and amplified fragment length polymorphism (AFLP) have been allowed discrimination of formae speciales within FOSC, but many cases of polyphyletic origin of formae speciales have also been described (O'Donnell et al., 1998; Alves-Santos et al., 1999; Baayen et al., 2000; Lievens et al., 2007).

Fov strains are currently separated into 7 races worldwide according to their ability to infect varieties of cotton species and also related (okra – *Abelmoschus esculentus* L.) and unrelated species [alfalfa – *Medicago sativa* L., tobacco – *Nicotiana tabacum* L., and soybean – *Glycine max* (L.) Merr.] (Armstrong & Armstrong, 1978; 1981; Chen et al., 1985). Race 6 of Fov has been discovered in Brazil (Armstrong & Armstrong, 1978) and since then no further studies were made to characterize the races present in the country. Genetic diversity among Brazilian Fov isolates has been detected through RAPD, PCR-RFLP of rDNA, ERIC and REP PCR but occurrence of races was not investigated (Bibanco et al., 2010).

Seven races of Fop have been described based on the virulence to up to eight bean cultivars (Ribeiro & Hagedorn, 1979; Aloj et al., 1987; Woo et al., 1996; Alves-Santos et al., 2002a). Relationship between race and geographic origin has been observed with race 1 occurring in the USA (South Carolina) and Italy, race 2 in Brazil, race 3 in Colombia, race 4 in the USA (Colorado), race 5 and 7 in Greece, and race 6 in Spain. Recent studies in Brazil using differential common bean cultivars revealed the presence of Fop races 2, 3, 6, and other strains that could not be assigned to any known race (A. Wendland, *personal communication*).

Despite the complexity of the origin of host-specificity, molecular methods have been reported to be able to specifically detect formae speciales of *F. oxysporum*. Moricca et al. (1998) described primers based on differences in sequences of the internal transcribed spacer region (ITS) that specifically amplified for Fov. Alves-Santos et al. (2002b) developed specific primers for highly virulent Fop strains based on the sequence of a distinct fragment revealed by random amplified polymorphic DNA (RAPD).

In the present study, we verified the possibility of designing Fov- or Fop-specific primers through genetic studies based on sequencing of the ITS (internal transcribed spacer) region and partial sequences of translation elongation factor (EF-1α) gene and 28S-18S ribosomal intergenic spacer region (IGS) from a collection of 15 Fov strains (14 Brazilian and one Argentine) and 15 Fop strains (all Brazilians). The performance of primers for the specific detection of Fov and Fop described in the literature was also evaluated.

MATERIAL AND METHODS

Strains and pathogenicity tests

Fifteen strains identified as Fov and fifteen as Fop from the collections of Instituto Biologico (São Paulo, Brazil) and Instituto Agronômico (Campinas, Brazil) (Table 1) were grown on potato-dextrose agar (PDA) for ten days at 25ºC. Spores suspensions in water at a concentration of 10^6/mL were prepared to inoculate cotton (cultivar Fibermax 966) and common bean plants (cultivar IPA6) grown on sterilized natural soil for approximately three weeks (first true leaves set) in 16 cm diam pots . The terminal third part of the roots were cut and the exposed cut ends were submerged in the spore suspensions for 10 min. Three plants per pot were inoculated with each strain. Controls were submitted to the same procedure but using sterile water. The plants were kept in a greenhouse under natural sunlight and watered twice every day. The symptoms were evaluated 15, 20, 25 and 30 days after inoculation. This evaluation aimed to confirm pathogenicity of strains and was limited to the observation of growth reduction of inoculated plants compared to non-inoculated controls, appearance of symptoms and plant death. After symptoms evaluation, the fungus was re-isolated from the stem of the inoculated plants, by placing tissue fragments on PDA plates.

DNA extraction

Mycelia of the fungi grown on PDA for one week at 25ºC was collected with a scalpel and ground in a microtube with a plastic pestle with liquid nitrogen. Genomic DNA was extracted according to a protocol described by Dellaporta et al. (1983). 600 µL of a cetyl trimethylammonium bromide (CTAB) extraction solution (2% CTAB, 100 mM Tris HCl pH 8.0, 20 mM EDTA, and 1.4 M NaCl) were added to the ground mycelia and incubated at 65ºC for 45 min under moderate agitation. 600 µL of chloroform-isoamyl alcohol (24:1) were added and mixed by inversion for 10 min. After centrifugation at 12,000 g for 10 min at 4ºC, the water phase was transferred to a new microtube and extracted with chloroform-isoamyl alcohol again. The water phase was transferred to a new microtube, 0.6 vol of isopropanol was added and the microtubes were kept at -20ºC overnight. The DNA was sedimented by centrifugation at 12,000 g for 10 min at 4ºC and washed with 1 mL of 70% ethanol. The DNA pellet was dried at 37ºC for 20 min and re-suspended in 30 µL of sterile water.

PCR amplifications and sequencing of ITS region, partial EF-1α gene, and IGS rDNA

Amplification of the internal transcribed spacer (ITS) region was performed with the primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATTGC-3') (White et al., 1990). Partial EF-1α gene was amplified with the primers EF1 (5'-ATGGGTAAGGA(A/G)GACAAGAC-3') and EF2 (5'-GGA(G/A)GTACCAGT(G/C)ATCATGTT-3') (O'Donnell et al., 1998). The entire IGS rDNA was amplified with the primers NL11 (5'- CTGAACGCCTCTAAGTCAG -3') e CNS1 (5'-GAGACAAGCATATGACTAC -3') (Aoki et al., 2003). Platinum Taq DNA polymerase (Life Technologies) was used for the PCR amplifications according to the manufacturer's instructions. The thermocycler (PTC-100, MJ Research) was used and the program for the ITS region and EF-1α gene consisted of initial denaturation at 94ºC/2 min, 40 cycles of 94ºC/30 s – 54ºC/30 s – 72ºC/40 s, final extension at 72ºC/4 min. The same program was used for the IGS rDNA, using a 2 min extension time in each cycle instead. Amplification products were verified by electrophoresis in 2% agarose gel and visualized under UV light with ethidium bromide staining

The PCR products were purified by polyethylene glycol (PEG) 6000 precipitation according to the protocol described by Schmitz & Riesner (2006). 1.6 µL of EDTA 0.5 M, pH 8.0, 21 µL of 50% PEG 6000, and 8.1 µL of 5 M NaCl were added to each 50 µL of PCR product. After a brief agitation in a vortex apparatus, the mixture was incubated at room temperature for 10 min and centrifuged at 12,000 g for 10 min at room temperature. The supernatant was discarded and the pellet washed with 125 µL of 70% ethanol. The pellet was dried at 37ºC for 30 min and re-suspended in 30 µL of autoclaved MilliQ water.

The purified PCR products were sequenced by the dideoxy chain terminator method using Big Dye 3.1 (Applied Biosystems) and a capillary DNA sequencer (ABI 3500 XL, Applied Biosystems).

Phylogenetic analysis

Approximately 600 nt of the EF-1α gene and 720 nt of the 5' region of IGS were separately employed for the phylogenetic analysis using Clustal W for sequences alignment and the Maximum Likelihood method for tree

TABLE 1 - List of strains used in this study.

Strain code in the present study	Original strain designation	Geographic origin (State or Province/Country)	Isolation year
Fusarium oxysporum f. sp. *vasinfectum*			
Fov 1	IB 230	São Paulo/Brazil	1961
Fov 2	IB 492	Castelar/ Argentina	1959
Fov 3	IB 3/96	São Paulo/Brazil	1996
Fov 4	IAC 12803	Goiás/Brazil	2003
Fov 5	IAC 12976	São Paulo/Brazil	2004
Fov 6	IAC 12911	São Paulo/Brazil	2001
Fov 7	IAC 12047	São Paulo/Brazil	2001
Fov 8	IAC 12344	Tocantins/Brazil	2002
Fov 9	IAC 13824	São Paulo/Brazil	1997
Fov 10	IAC 12401	São Paulo/Brazil	2002
Fov 11	IAC 12345	Tocantins/Brazil	2002
Fov 12	IAC 12325	São Paulo/Brazil	2001
Fov 13	IAC 696	São Paulo/Brazil	1972
Fov 14	IAC 3463	Paraná/Brazil	1981
Fov 15	IAC 21-4	São Paulo/Brazil	2009
Fusarium oxysporum f. sp. *phaseoli*			
Fop 1	IB 595	São Paulo/Brazil	1967
Fop 2	IB 4/96	São Paulo/Brazil	1989
Fop 3	IAC 12872	São Paulo/Brazil	2003
Fop 4	IAC 12802	São Paulo/Brazil	2003
Fop 5	IAC 11747	São Paulo/Brazil	2000
Fop 6	IAC 11749	São Paulo/Brazil	2000
Fop 7	IAC 11750	São Paulo/Brazil	2000
Fop 8	IAC 11848	São Paulo/Brazil	2000
Fop 9	IAC 11849	São Paulo/Brazil	2000
Fop 10	IAC 13029	São Paulo/Brazil	2004
Fop 11	IAC 9760/1	São Paulo/Brazil	1996
Fop 12	IAC 13349	São Paulo/Brazil	2005
Fop 13	IAC 13368	São Paulo/Brazil	2005
Fop 14	IAC 2556	Rio de Janeiro/Brazil	1977
Fop 15	IAC 8278	Minas Gerais/Brazil	1968
Fusarium solani from cotton			
Fsa 1	IAC 8450	Paraná/Brazil	1994
Fusarium solani from common bean			
Fsf 1	IAC 13030	São Paulo/Brazil	2004
Fsf 2	IAC 10241	Minas Gerais/Brazil	1997

IB - Instituto Biológico Collection, São Paulo, Brazil
IAC - Instituto Agronômico Collection, Campinas, Brazil

construction with the best fitting Kimura 2-parameter (for EF-1α) and Hasegawa-Kishino-Yano (for IGS) nucleotide substitution models and 1000 bootstrap repetitions using the MEGA 5.2 software (Tamura et al., 2011). ITS sequences did not show significant variation therefore were not submitted to phylogenetic analysis. The FOSC sequence types (STs) described by O'Donnell et al. (2009) that had the highest sequence similarity to the strains of the present study were retrieved from the GenBank and Fusarium ID (Park et al., 2011) databases and included in the analyses. *Fusarium foetens* and *Fusarium commune* sequences for EF-1α gene (FJ985444 and AF362263, respectively) and IGS region (GU170581 and HM057285) were used as outgroup taxa to root the trees.

Verification of Fov and Fop specific primers

The specificities of the primers for Fov described by Moricca et al. (1998), Fov1 (5'-CCCCTGTGAACATACCTTACT-3') and Fov2 (5'-ACCAGTAACGAGGGTTTTACT-3'), and the highly virulent Fop-specific primers described by Alves-Santos et al. (2002b), A280 (5'-TATACCGGACGGGCGTAGTGACGATGG-3') and B310 (5'-CAGCCATTCATGGATGACATAACGAATTTC-3')

were verified using the PCR protocols described by these authors.

RESULTS

Pathogenicity tests

Symptoms induced by Fov and Fop strains, on cotton and common bean plants respectively, were growth reduction, leaf yellowing, wilting and plant death. (Table 2). Although these inoculations were made to confirm pathogenicity of the strains, differences in the severity of symptoms was also observed. The most severe Fov strains (Fov 3, 4, 8, and 11) caused death within 15 days after inoculation while milder strains (Fov 2 and 13) caused slight growth retardation, wilting, and yellowing 25-30 days after inoculation. Only two Fop strains (Fop 1 and 6) caused plant death, most induced wilting and the mildest ones (Fop 10 and 11) caused mild wilting and yellowing.

Phylogenetic analysis

ITS region sequences did not show significant differences between strains of Fov and Fop but revealed that two strains of the collections were misidentified: Fov1 and Fop3 were actually *Fusarium solani*, with similarities higher than 99% to GenBank sequences of vouchered specimens CBS109028 (JX435216) and CBS121450 (JX435211), respectively. The same results were obtained using EF-1α sequences and the Fusarium ID database, with Fov1 and Fop3 showing higher than 99.5% similarities to *F. solani* strains NRRL 28541 (*F. solani* species complex 26-a, according to O'Donnell et al., 2008) and 32720 (*F. solani* species complex 3+4 –pp), respectively.

In both EF-1α and IGS trees most Fov and Fop strains could be clearly distinguished in separated branches (Figures 1 and 2). Exceptions are Fop 10, 12, and 13 that grouped with Fovs in both EF-1α and IGS trees and Fop 11 that grouped with Fovs in the EF-1α tree but remained in a separated branch in the IGS tree.

TABLE 2 - Symptoms on cotton and common bean plants inoculated with *Fusarium oxysporum* f. sp. *vasinfectum* and *F. oxysporum* f. sp. *phaseoli*, respectively.

| Strains	Days after inoculation				Symptoms
	15	20	25	30	
Fov 1	X				Growth reduction
Fov 2			X		Growth reduction, yellowing, mild wilting
Fov 3	X				Death
Fov 4	X				Death
Fov 5	X				Growth reduction, mild wilt
Fov 6		X			Death
Fov 7		X			Growth reduction
Fov 8	X				Death
Fov 9		X			Growth reduction, yellowing, mild wilting
Fov 10		X			Wilt
Fov 11	X				Death
Fov 12	X				Mild wilt
Fov 13				X	Slight growth reduction
Fov 14		X			Growth reduction
Fov 15		X			Death
Fop 1		X			Death
Fop 2	X				Wilt
Fop 3		X			Growth reduction, yellowing
Fop 4		X			Growth reduction, wilt
Fop 5	X				Wilt
Fop 6		X			Death
Fop 7	X				Wilt
Fop 8	X				Wilt
Fop 9	X				Wilt
Fop 10		X			Mild wilt
Fop 11		X			Yellowing
Fop 12		X			Growth reduction, yellowing
Fop 13		X			Growth reduction, yellowing
Fop 14	X				Wilt
Fop 15	X				Wilt
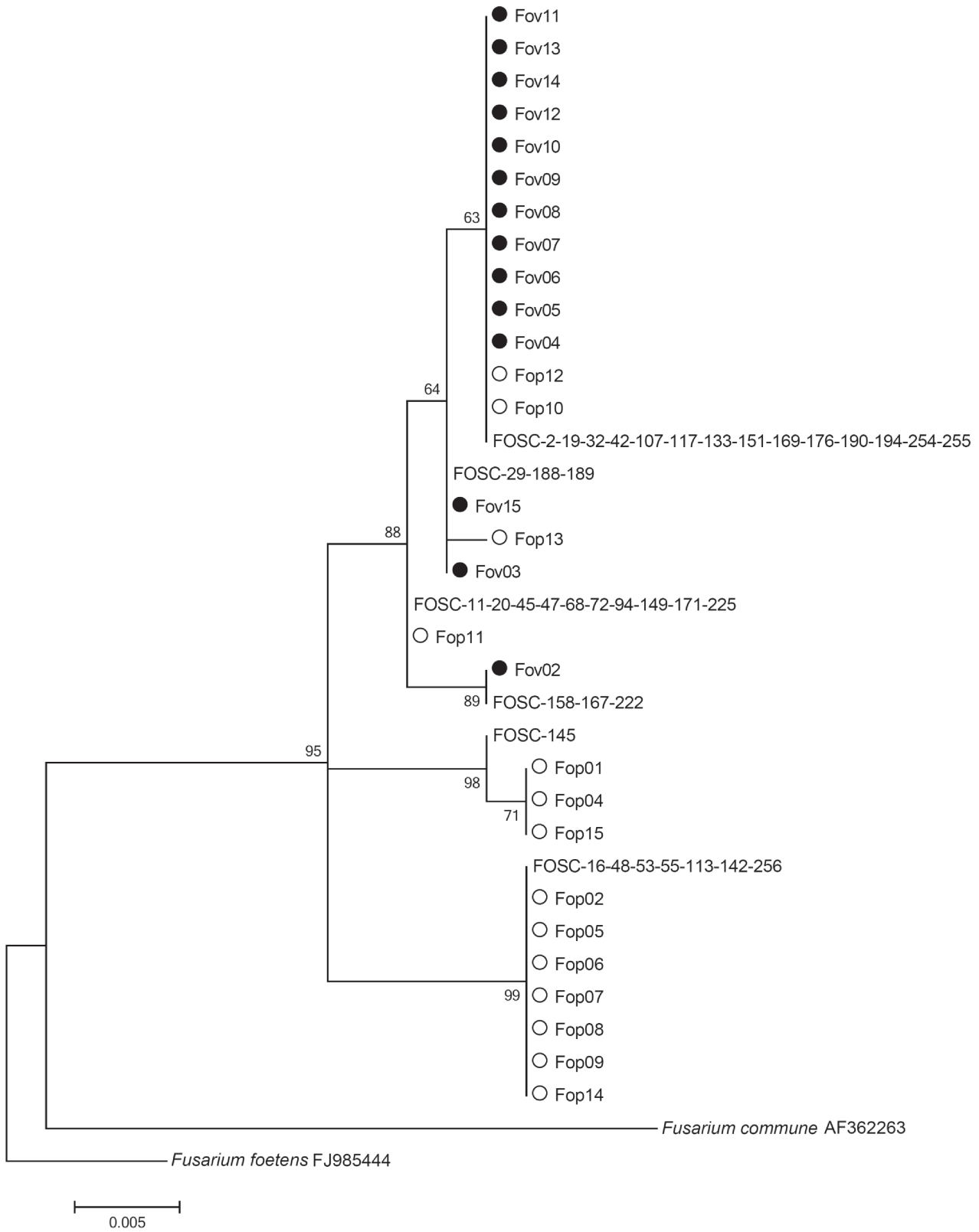
FIGURE 1 - Maximum Likelihood tree constructed with partial EF-1α gene sequences of strains of *Fusarium oxyporum* f. sp. *vasinfectum* and *F. oxysporum* f. sp. *phaseoli*. FOSC represent sequence types according to O'Donnell et al. (2009). Sequences of the following strains represent each FOSC sequence type group in the tree: FOSC2 (NRRL20433), FOSC29 (NRRL25424), FOSC11 (NRRL22543), FOSC158 (NRRL36356), FOSC145 (NRRL36228), FOSC16 (NRRL22549). *Fusarium foetens* (FJ985444) and *Fusarium commune* (AF362263) sequences were used as outgroup taxa to root the trees.
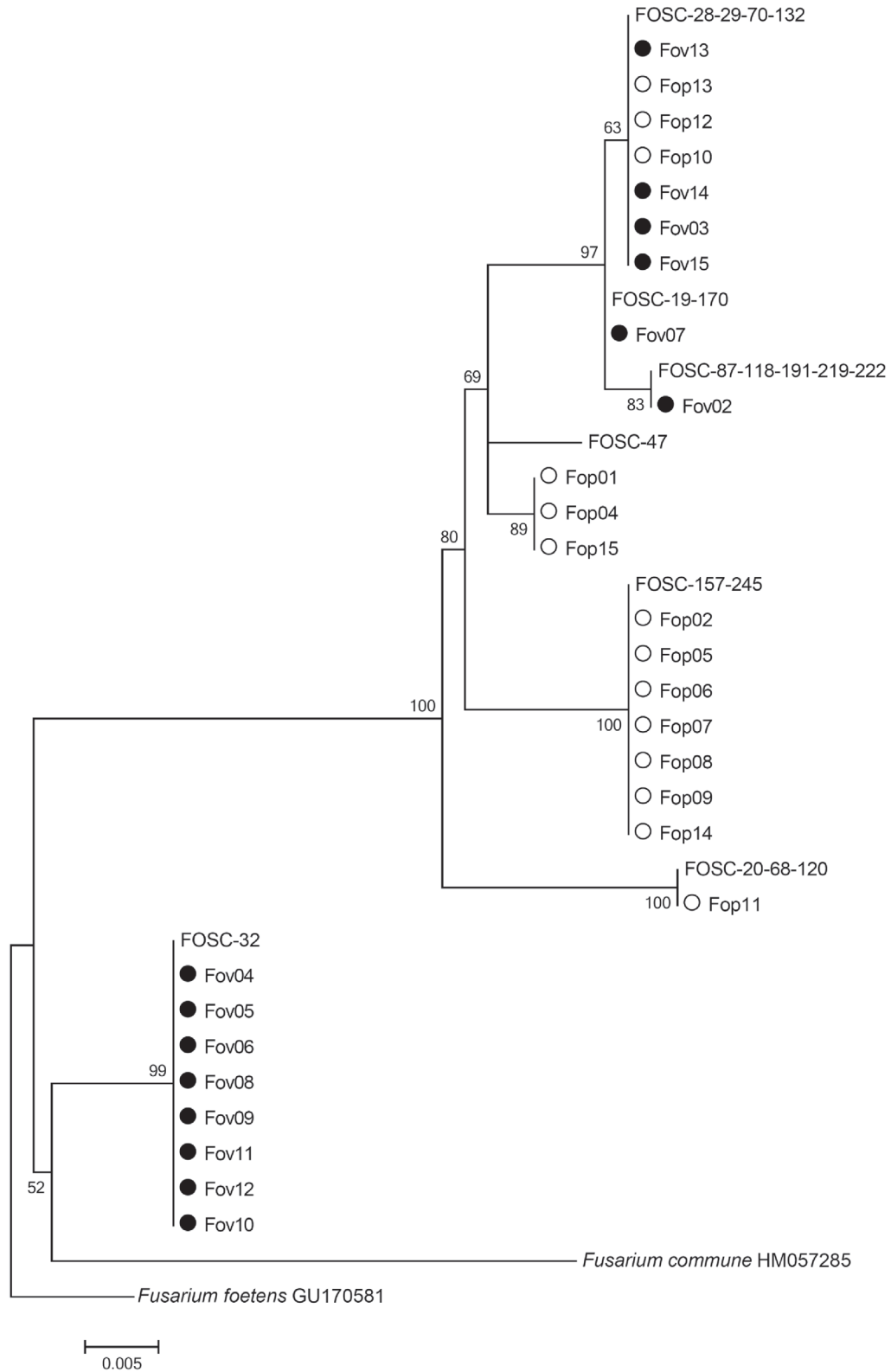
FIGURE 2 - Maximum Likelihood tree constructed with partial IGS rDNA of strains of *Fusarium. oxyporum* f. sp. *vasinfectum* and *F. oxysporum* f. sp. *phaseoli*. FOSC represent sequence types according to O'Donnell et al. (2009). Sequences of the following strains represent each FOSC sequence type group in the tree: FOSC28 (NRRL25420), FOSC19 (NRRL22553), FOSC87 (NRRL26960), FOSC47 (NRRL26225), FOSC157 (NRRL36355), FOSC20 (NRRL22554), FOSC32(NRRL25437). *Fusarium foetens* (GU170581) and *Fusarium commune* (HM057285) sequences were used as outgroup taxa to root the trees.

EF-1α and IGS sequences obtained in the present study were deposited in the National Center for Biotechnology Information (NCBI) database under GenBank accession numbers KF030565 to KF030622.

Specificity of formae speciales-specific primers

The Fov specific primers described by Moricca et al. (1998) did not result in amplification product for any of the strains used in the present study (data not shown). The reason for this negative result was clarified by analyzing the sequences of the ITS region that revealed that the strains of the present study had differences in the forward primer (Fov1) annealing site when compared with the strains employed in the study of Moricca et al. (1998) (Figure 3). The two extra thymines in the primer precluded its annealing to the strains of the present study and would not amplify for several other Fov strains with sequences deposited in the GenBank.

Primers pair A280-B310 developed by Alves-Santos et al. (2002b) and reported to be specific to highly virulent Fop resulted in amplification products for most of Fop strains of the present study with exception of Fop 3, 11, 12, and 13 (Figure 4). Fop 3 has been identified as *F. solani* by ITS sequencing, therefore explaining its negative result. Fop 11, 12, and 13, although pathogenic to common bean, may represent atypical strains for they were placed in branches separated from most of the Fop strains in the EF and IGS trees and closer to Fov strains (Figures 1 and 2).

DISCUSSION

The inoculation of cotton and common bean plants confirmed the pathogenicity of all strains included in the present study. A wider range of symptoms was observed for strains of Fov than for Fop. Differences in aggressiveness are common among strains of *F. oxysporum* and can indicate the occurrence of races within formae speciales (Alves-Santos et al., 2002a; Kim et al., 2005). Since only one cultivar of each host species was used to evaluate the pathogenicity of Fov and Fop, the existence of different races among the strains in the present study could not be confirmed but the genetic diversity revealed by the phylogenetic analyses supports such possibility.

Comparison of the IGS sequences of Fov strains with the sequence types described by O'Donnell et al. (2009) showed that Fov 4, 5, 6, 8, 9, 10, 11, and 12 are identical to FOSC32, represented in their study by one Fov strain (NRRL25437), race 6, from Brazil, and Fov 3, 13, 14, and 15 are identical to FOSC28 and 29 that contains Fov strains of races 1 (NRRL25420) and 2 from the USA (NRRL25424) and race 2 from Peru (NRRL25428). These results confirm the occurrence of Fov race 6 (Armstrong and Armstrong, 1978) and suggest the presence of races 1 and/or 2 in Brazil. Although our pathogenicity tests were not conceived to rigorously discriminate differences in virulence among strains, the symptoms induced by members

within the same putative race varied from mild wilt to plant death, therefore, correlation between race and severity of symptoms could not be inferred.

Fop strains were separated in 5 and 4 branches in EF-1α and IGS phylogenetic trees, respectively. These groups might be associated to the diversity of Fop races detected in Brazil. Two groups consisting of Fop 1, 4, and 15 and Fop 2, 5, 6, 7, 8, 9, and 14 contained strains that caused wilting or plant death and may represent two highly virulent races. Future work using differential cultivars will allow verifying such possibility.

Our phylogenetic analyses showed that strains from the same formae speciales can have very distinct genetic backgrounds, supporting their polyphyletic origin and confirming the observations of several studies on the origin of formae speciales of *F. oxysporum* (O'Donnell et al., 1998; Baayen et al., 2000; Alves-Santos et al., 2002a; O'Donnell et al., 2009). Determinants of host-specificity may be horizontally transferred between strains of *F. oxysporum* of different lineages, resulting in polyphyly. This genetic exchange makes it extremely difficult to develop primers for the specific detection of a given formae speciales. Molecular markers based on the genetic determinants of pathogenicity are more likely to succeed in distinguishing formae speciales (van der Does et al., 2008). Such is the case of the gene targeted by the A280-B310 primers developed by Alves-Santos et al. (2002b) that encodes a transcription factor from highly virulent Fop strains, expressed during early stages of host infection and located in a small chromosome (Ramos et al., 2007). These primers failed with three Fop strains in the present study, Fop 11, 12, and 13, that induce milder symptoms and resemble the weakly virulent strains described by Alves-Santos et al. (2002b) that did not result in amplification product with the same primers. Interestingly, these three strains are genetically more closely related to Fov strains (Figures 1 and 2). Fop 10 can be considered an exception since it has also shown closer relationship to Fov strains and induced milder symptoms but resulted in amplification product with the highly virulent Fop-specific primers.

Failure of the Fov-specific primers developed by Moricca et al. (1998) to detect Fov strains in the present study can be explained by the aforementioned polyphyletic origin of formae speciales associated with a limited number or limited geographic origin span of the strains included in their study.

Our results are consistent with recent discoveries that host specificity factors, such as Fop *ftf1*, and *F. oxysporum* f. sp. *lycopersici SIX* genes (Ramos et al., 2007; van der Does et al., 2008; Vega-Bartol et al., 2011) are concentrated in lineage specific entire chromosomes and chromosome segments of *F. oxysporum* that can be horizontally transferred between strains with distinct genetic background, transforming in a single event a non-pathogenic indigenous soilborne *F. oxysporum* into a highly aggressive and locally adapted host-specific formae

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F_oxysporum_fsp_vasinfectum_X78258       : ACAACTCCCAAACCCCTGTGAACATACCTTACTTGTTGCCTCGGCGGATCAGCCCGGCTCCCGGTAAAACGGGACGG
F_oxysporum_fsp_vasinfectum_X78259       : ACAACTCCCAAACCCCTGTGAACATACCTTACTTGTTGCCTCGGCGGATCAGCCCGGCTCCCGGTAAAACGGGACGG
primer_Fov1                              : ------------CCCCTGTGAACATACCTTACT--------------------------------------------
F_oxysporum_fsp_dianthi_DQ452452         : ACAACTCCCAAACCCCTGTGAACATACC--ACTTGTTGCCTCGGCGGATCAGCCCGGCTCCCGGTAAAACGGGACGG
F_oxysporum_fsp_lilii_AY684920           : ACAACTCCCAAACCCCTGTGAACATACC--ACTTGTTGCCTCGGCGGATCAGCCCGGCTCCCGGTAAAACGGGACGG
F_oxysporum_fsp_cucumerinum_DQ452450     : ACAACTCCCAAACCCCTGTGAACATACC--ACTTGTTGCCTCGGCGGATCAGCCCGGCTCCCGGTAAAACGGGACGG
F_oxysporum_fsp_lycopersici_DQ452454     : ACAACTCCCAAACCCCTGTGAACATACC--ACTTGTTGCCTCGGCGGATCAGCCCGGCTCCCGGTAAAACGGGACGG
F_oxysporum_fsp_cubense_EF590328         : ACAACTCCCAAACCCCTGTGAACATACC--ACTTGTTGCCTCGGCGGATCAGCCCGGCTCCCGGTAAAACGGGACGG
F_oxysporum_fsp_vasinfectum_AF322074     : ACAACTCCCAAACCCCTGTGAACATACC--ACTTGTTGCCTCGGCGGATCAGCCCGGCTCCCGGTAAAACGGGACGG
F_oxysporum_fsp_vasinfectum_AF322075     : ACAACTCCCAAACCCCTGTGAACATACC--ACTTGTTGCCTCGGCGGATCAGCCCGGCTCCCGGTAAAACGGGACGG
F_oxysporum_fsp_vasinfectum_AF322076     : ACAACTCCCAAACCCCTGTGAACATACC--ACTTGTTGCCTCGGCGGATCAGCCCGGCTCCCGGTAAAACGGGACGG
F_oxysporum_fsp_vasinfectum_EU849584     : ACAACTCCCAAACCCCTGTGAACATACC--ACTTGTTGCCTCGGCGGATCAGCCCGGCTCCCGGTAAAACGGGACGG
Fov2                                     : ACAACTCCCAAACCCCTGTGAACATACC--ACTTGTTGCCTCGGCGGATCAGCCCGGCTCCCGGTAAAACGGGACGG
Fov3                                     : ACAACTCCCAAACCCCTGTGAACATACC--ACTTGTTGCCTCGGCGGATCAGCCCGGCTCCCGGTAAAACGGGACGG
Fov4                                     : ACAACTCCCAAACCCCTGTGAACATACC--ACTTGTTGCCTCGGCGGATCAGCCCGGCTCCCGGTAAAACGGGACGG
Fov5                                     : ACAACTCCCAAACCCCTGTGAACATACC--ACTTGTTGCCTCGGCGGATCAGCCCGGCTCCCGGTAAAACGGGACGG
Fov6                                     : ACAACTCCCAAACCCCTGTGAACATACC--ACTTGGTGCCTCGGCGGATCAGCCCGGCTCCCGGTAAAACGGGACGG
Fov7                                     : ACAACTCCCAAACCCCTGTGAACATACC--ACTTGTTGCCTCGGCGGATCAGCCCGGCTCCCGGTAAAACGGGACGG
Fov8                                     : ACAACTCCCAAACCCCTGTGAACATACC--ACTTGTTGCCTCGGCGGATCAGCCCGGCTCCCGGTAAAACGGGACGG
Fov9                                     : ACAACTCCCAAACCCCTGTGAACATACC--ACTTGTTGCCTCGGCGGATCAGCCCGGCTCCCGGTAAAACGGGACGG
Fov10                                    : ACAACTCCCAAACCCCTGTGAACATACC--ACTTGTTGCCTCGGCGGATCAGCCCGGCTCCCGGTAAAACGGGACGG
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FIGURE 3 - Alignment of ITS sequences from strains of *Fusarium oxysporum* f. sp. *vasinfectum* showing incompatibility of primer Fov1 developed by Moricca et al. (1998) to strains used in this study and to others with sequences deposited in the GenBank.
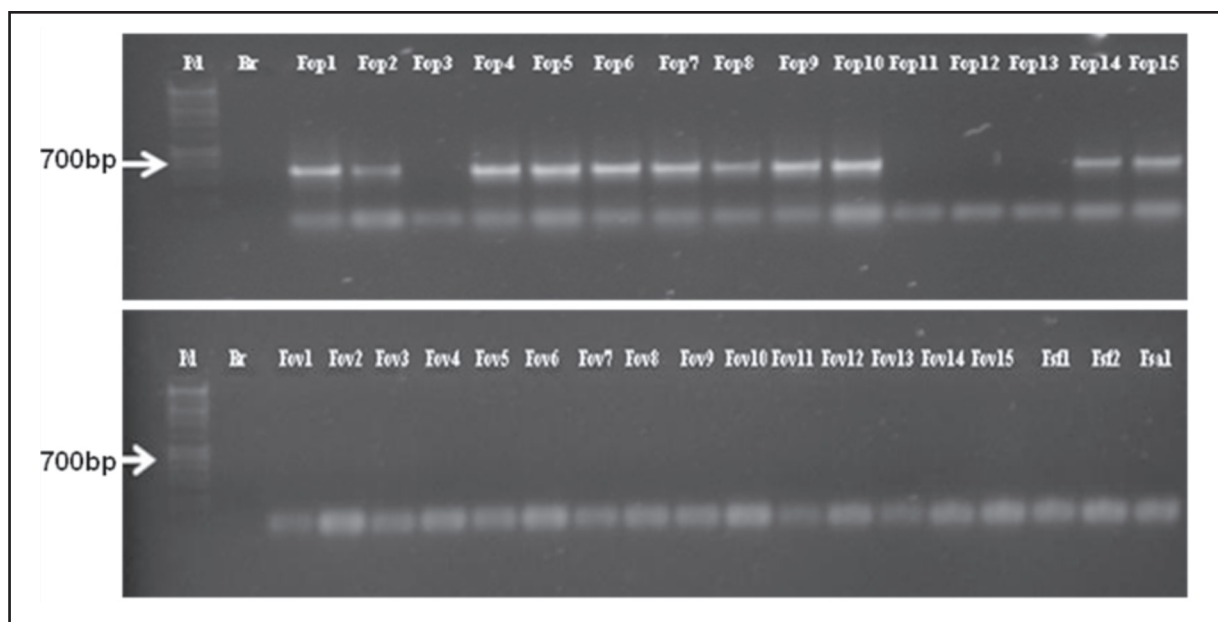
FIGURE 4 - Evaluation of the *Fusarium oxysporum* f. sp. *phaseoli*-specific primers A280-B310 developed by Alves-Santos et al. (2002). The expected amplification product is approximately 600 pb. Fov - *F. oxysporum* f. sp. *vasinfectum* strains, Fop - *F. oxysporum* f. sp. *phaseoli* strains, Fsa - *F. solani* strain from cotton, Fsf – *F. solani* strain from common bean. Pd – 1 Kb ladder (Life Technologies). Br – non-template control.

speciales (Ma et al., 2010). The genetic diversity of Fov and Fop strains revealed in the present study can have risen through the exchange of pathogenicity/virulence factors between exotic and indigenous lineages.

Development of highly specific molecular methods for detection of Fov and Fop requires a deeper knowledge of the pathogenicity and host specificity factors that allow these formae speciales to cause disease on their host plants. Ongoing whole genome sequencing projects of several formae speciales of *F. oxysporum* will soon reveal the genetic basis of these mechanisms (Park et al., 2011).

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