PROGRESS REPORT ON A CONTEMPORARY SURVEY OF THE FUSARIUM WILT FUNGUS IN THE UNITED STATES

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Abstract

Knowledge of the genetic and pathogenic diversity present in a pathogen population is required to effectively deploy resistant cultivars. The only pathogenic survey of *Fusarium oxysporum* f. sp. *vasinfectum* in the U.S. was conducted in 1983. Since then, new distributions of races 3, 4, and 8, and four novel genotypes of this fungus, have been discovered in the U.S. A multi-state collaboration was initiated with the objective of conducting a comprehensive contemporary survey of the genotypic and pathogenic diversity of *F. oxysporum* f. sp. *vasinfectum* in the United States. Preliminary data are presented. A 618-bp fragment of the translation elongation factor gene (*EF-1α*) was sequenced from 39 isolates collected from eight states. These sequences were compared to those obtained from six isolates from the Ivory Coast and 70 sequences from GenBank. Maximum parsimony analysis yielded six shortest trees with topologies consistent with previous reports. Three isolates from Alabama and two isolates from Mississippi were in Lineage IV (race 4). Other isolates from southeastern states were in Lineages II (race 1) and III (race 8), a clade with genotypes 127 and 140, and an unresolved group related to genotypes 108 and 110. All isolates from Texas and most isolates from the Ivory Coast were in Lineage II. None of the isolates sequenced was in Lineages I (race 3) or V (Australian genotypes). These results support previous reports suggesting *F. oxysporum* f. sp. *vasinfectum* in the southeastern U.S. have a high level of genotypic diversity.

Introduction

Effective deployment of host plant resistance against Fusarium wilt of cotton (*Gossypium* spp. L.) requires an improved understanding of the pathogenic diversity of *Fusarium oxysporum* Schltd.:Fr. f. sp. *vasinfectum* (Atk.) W.C. Snyd. and H. N. Hans. Recent reports, suggesting more genotypic and pathogenic diversity is present in the U.S. than previously believed (Kim et al., 2005; Holmes et al., 2009; O'Donnell et al., 2009), point to the need for a current and comprehensive survey of the Fusarium wilt pathogen of cotton. Nearly thirty years have passed since the only nation-wide survey of this fungus was conducted (Kappelman, 1983). That survey found *F. oxysporum* f. sp. *vasinfectum* present in all of the seven states sampled (Alabama, California, Mississippi, Missouri, North Carolina, South Carolina, and Texas). In addition, pathogenicity tests on tobacco were used to differentiate the isolates into races 1 or 2. Prior to the survey, race 2 had been found only in South Carolina (Armstrong and Armstrong, 1958). Of the 53 isolates screened in the 1983 survey, 11 isolates from four states (Alabama, California,

North Carolina, and South Carolina) were identified as race 2. These results suggested a significant increase in the known distribution of race 2.

More changes have occurred in the diversity and distribution of *F. oxysporum* f. sp. *vasinfectum* since Kappelman's survey, within and outside the U.S. Race 4, thought to occur only in Asia, appeared in California in 2001 and has caused significant problems for the San Joaquin Valley cotton industry (Kim et al., 2005). Kim and colleagues also discovered races 3 and 8 were present in California but had been undetected for years. More recently, race 8 was found in Arkansas, Louisiana, Georgia, and Missouri, and race 3 was found in Louisiana (Holmes et al., 2009; O'Donnell et al., 2009). In addition to new distributions of known races, novel genotypes of *F. oxysporum* f. sp. *vasinfectum* have been discovered in the southeastern U.S., Australia, and the Ivory Coast (Holmes et al., 2009; O'Donnell et al., 2009; Davis et al., 1996; Abo et al., 2005). New genotypes and changes in distribution of known genotypes may not necessarily pose significant threats to current cotton production in the U.S. For example, the novel genotype 112 and races 3 and 8 have not been especially virulent to date to U.S. commercial cultivars in the field or in greenhouse assays (Kim et al., 2005; Holmes et al., 2009). However, concern may be warranted for other genotypes. Race 4, the Australian genotypes, and genotypes 108 and 110 from the southeastern U.S. may be quite virulent (Constable, 2007; Holmes et al., 2009). The Australian biotypes have not been found outside of that country, and in the U.S., the presence of race 4 has been confirmed only in California. However, isolates from Alabama with sequence similarity to race 4 in the nuclear ribosomal DNA internal transcribed spacer 2 region (ITS2) were recently reported (Castillo et al., 2010). These isolates have yet to be confirmed as race 4 through pathogenicity tests or analyses of other gene sequences. In a sample of 61 isolates from four states, genotype 108 was found only in Arkansas and Georgia and 110 was found only in Arkansas (Holmes et al., 2009). The complete geographic distribution of genotypes 108 and 110 is unknown.

To better understand the pathogenic diversity and distribution of *F. oxysporum* f. sp. *vasinfectum* in the U.S., a multi-state collaborative project was initiated. The goal of this project is to comprehensively survey *F. oxysporum* f. sp. *vasinfectum* in the U.S., identifying isolates using a two-locus sequence type. Two loci, translation elongation factor (*EF-1*α) and nuclear ribosomal DNA intergenic spacer region (IGS rDNA), were used to identify 23 sequence-types in *F. oxysporum* f. sp. *vasinfectum* (O'Donnell et al., 2009). Preliminary analysis of *EF-1*α sequence data of isolates collected to date is presented here.

Materials and Methods

Isolates Used

Thirty-nine isolates with morphology typical of *F. oxysporum* were obtained from cotton plants showing symptoms of Fusarium wilt from eight states (Alabama, Arkansas, California, Florida, Louisiana, Mississippi, South Carolina, and Texas) (Table 1). The 15 isolates from A. Bell were collected in 1992-1995. The 11 isolates from K. Lawrence were collected in 2009 from cotton breeding plots of Auburn University's E. V. Smith Research Center. Isolates from J. Woodward were collected in 2008-2010 from commercial fields in four Texas counties. The five isolates from G. Lawrence originated from four diseased plants collected in 2009 from a Mississippi State University research plot. Six isolates from the Ivory Coast, some with putatively novel vegetative compatibility groups (Abo et al., 2005), were also obtained from the Centraalbureau voor Schimmelcultures (Table 1). Single-spore cultures were made of each isolate as described previously (Bennett et al., 2008).

DNA Manipulations

Isolates were grown on 4 x 4-cm pieces of sterile cellophane placed on the surface of ¼-strength potato dextrose agar (BD Difco, Franklin Lakes, NJ). After four days of incubation in the dark, mycelium was harvested from the cellophane and lyophilized overnight. DNA was obtained from lyophilized mycelium with the FastDNA Kit and FastPrep Instrument (QBiogene, Irvine, CA) following manufacturer protocols. Partial sequences of the *EF-1*α gene were amplified in final volumes of 20 µl, containing 5-20 ng of genomic DNA, 0.2 mM of each dNTP, 0.2 µM of each primer, and 0.25 units of GoTaq DNA polymerase (Promega, Madison, WI). Previously described PCR primers, EF-1 and EF-2 (O'Donnell et al., 1998), and thermocycler conditions (Kim et al., 2005) were used. PCR products were visualized on 1.5% agarose gels stained with SYBR-Safe (Invitrogen, Carlsbad, CA). Gel fragments containing bands of expected size were excised with a clean scalpel, dissolved in 200 µl of 5.5M guanidine thiocyanate at 50°C, and purified through silica membrane tubes (Epoch Life Science, Missouri City, TX). Purified DNA was eluted from the silica membranes with 15 µl of 10 mM TRIS buffer. The BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems (ABI), Foster City, CA) was used to sequence both directions with primers

EF-3 and EF-22 (O'Donnell et al., 2009; O'Donnell et al. 1998). Extension products were purified using the ethanol-EDTA precipitation protocol of the sequencing kit. Samples were run on an ABI 3130 capillary sequencer.

Table 1. Isolates of *Fusarium oxysporum* f. sp. *vasinfectum* used.

Name[z]	Other Names[y]	Source	Geographic Origin	Reference
AL_ABWICS61	Bell#951	A. Bell	Shorter, AL	(Bell, 1995; Bell et al., 2003)
AL_ABWICS66	Bell#166	A. Bell	Hufford, AL	(Bell and Decker, 1993; Bell et al., 2003)
AL_ABWICS67	Bell#950	A. Bell	Shorter, AL	(Bell, 1995; Bell et al., 2003)
AL_ABWICS71	Bell#944	A. Bell	Shorter, AL	(Bell, 1995; Bell et al., 2003)
AL_KL1	301A	K. Lawrence	Milstead, AL	this study
AL_KL3	1025A	K. Lawrence	Milstead, AL	this study
AL_KL5	2076A	K. Lawrence	Milstead, AL	this study
AL_KL7	2085A	K. Lawrence	Milstead, AL	this study
AL_KL9	3006A	K. Lawrence	Milstead, AL	this study
AL_KL11	3035A	K. Lawrence	Milstead, AL	this study
AL_KL13	3046A	K. Lawrence	Milstead, AL	this study
AL_KL15	3106A	K. Lawrence	Milstead, AL	this study
AL_KL17	4025A	K. Lawrence	Milstead, AL	this study
AL_KL20	4075B	K. Lawrence	Milstead, AL	this study
AL_KL21	4085A	K. Lawrence	Milstead, AL	this study
AR_ABWICS51	Bell#237	A. Bell	Lafayette Co., AR	(Bell and Decker, 1993; Bell et al., 2003)
AR_ABWICS52	Bell#260	A. Bell	Hempstead Co., AR	(Bell and Decker, 1993; Bell et al., 2003)
AR_ABWICS70	Bell#245	A. Bell	Lafayette Co., AR	(Bell and Decker, 1993; Bell et al., 2003)
CA_ABWICS49	Bell#4	A. Bell	CA	(Bell and Decker, 1993; Bell et al., 2003)
FL_ABWICS62	Bell#194	A. Bell	Jay, FL	(Bell and Decker, 1993; Bell et al., 2003)
LA_ABWICS56	Bell#528	A. Bell	Baton Rouge, LA	(Bell and Decker, 1993; Bell et al., 2003)
MS_ABWICS68	Bell#5	A. Bell	MS	(Bell and Decker, 1993; Bell et al., 2003)
MS_GL3	-	G. Lawrence	Mississippi State, MS	this study
MS_GL10	-	G. Lawrence	Mississippi State, MS	this study
MS_GL18	-	G. Lawrence	Mississippi State, MS	this study
MS_GL53	-	G. Lawrence	Mississippi State, MS	this study
MS_GL58	-	G. Lawrence	Mississippi State, MS	this study
SC_ABWICS57	Bell#203	A. Bell	Lee Co., SC	(Bell and Decker, 1993; Bell et al., 2003)
TX_ABWICS63	Bell#1023	A. Bell	Chillicothe, TX	(Bell, 1995; Bell et al., 2003)
TX_ABWICS64	Bell#930	A. Bell	Big Spring-Holland, TX	(Bell, 1995; Bell et al., 2003)
TX_ABWICS69	Bell#1034	A. Bell	Brownfield, TX	(Bell, 1995; Bell et al., 2003)
TX_JW3	-	J. Woodward	Lynn Co., TX	this study
TX_JW7	19	J. Woodward	Lubbock Co., TX	this study
TX_JW8	22	J. Woodward	Lubbock Co., TX	this study
TX_JW10	29	J. Woodward	Dawson Co., TX	this study
TX_JW12	40	J. Woodward	Dawson Co., TX	this study

Table 1 (continued).

Name[z]	Other Names[y]	Source	Geographic Origin	Reference
TX_JW13	50	J. Woodward	Terry Co., TX	this study
TX_JW14	51	J. Woodward	Terry Co., TX	this study
TX_JW15	58	J. Woodward	Gaines Co., TX	this study
IVC_WICS73	CBS 116612, Fov2	CBS	Bouaké, Ivory Coast	this study, (Abo et al., 2005)
IVC_WICS74	CBS 116613, Fov3	CBS	Tiéningboué, Ivory Coast	this study, (Abo et al., 2005)
IVC_WICS75	CBS 116614, Fov4	CBS	Bouaké, Ivory Coast	this study, (Abo et al., 2005)
IVC_WICS77	CBS 116619, Fov9	CBS	Tiéningboué, Ivory Coast	this study, (Abo et al., 2005)
IVC_WICS78	CBS 116620, Fov10	CBS	Béoumi, Ivory Coast	this study, (Abo et al., 2005)
IVC_WICS80	CBS 116622, Fov13	CBS	Béoumi, Ivory Coast	this study, (Abo et al., 2005)
BBA (25), NRRL (5)	same as in reference	GenBank	-	(Skovgaard et al., 2001)
127, 140, 031665, Aus (2)	same as reference	GenBank	-	(Holmes et al., 2009)
CA1,3,10,11,14	same as reference	GenBank	-	(Holmes et al., 2009)
ATCC (5)	same as reference	GenBank	-	(Holmes et al., 2009)
ST (23), Ffo (2)	same as reference	GenBank	-	(O'Donnell et al., 2009)

[z] BBA = Biologische Bundesanstalt für Land-und Forstwirtschaft, Berlin, Germany; NRRL = National Center for Agricultural Utilization Research, Peoria, IL; ATCC = American Type Culture Collection, Manassas, VA; ST = sequence type; Ffo = *Fusarium foetens*, used as outgroup. Numbers in parentheses after abbreviations are the numbers of isolates for which sequences were obtained from GenBank.

[y] Equivalent names given by collectors, culture collections, or reference; CBS = Centraalbureau voor Schimmelcultures, Utrecht, Netherlands.

[x] GenBank-sourced sequences were previously published and downloaded from the GenBank website (http://www.ncbi.nlm.nih.gov/genbank/).

Phylogenetic Analyses

Previously published *EF-1*α sequences of *F. oxysporum* f. sp. *vasinfectum* (Skovgaard et al. 2001; Holmes et al., 2009; O'Donnell et al., 2009) were downloaded from GenBank for comparison to the newly sequenced isolates (Table 1). Downloads included sequences of 25 isolates from the culture collection BBA, 5 from NRRL, and 5 from ATCC (Skovgaard et al., 2001; Holmes et al., 2009). These included reference isolates ATCC16421 (race 1), ATCC16611 (race 2), ATCC16612 (race 3), ATCC16613 (race 4), 031665 (race 8), and AUS16 and 19 (Australian races). *EF-1*α sequences from each representative of the 23 two-locus sequence types of *F. oxysporum* f. sp. *vasinfectum* defined by O'Donnell et al. (2009) were also obtained from GenBank. Sequence data (618 bp) were edited in SeqMan Pro and aligned using the ClustalW algorithm in MegAlign (DNASTAR, Madison, WI). Phylogenetic analysis was conducted in PAUP v. 4.0b (Sinauer Associates, Sunderland, MA). All characters were unordered and given equal weight. Alignment gaps were considered as missing data. *EF-1*α sequences from two isolates of *Fusarium foetens* Schroers, a sister taxon to *F. oxysporum* (Schroers et al., 2004), were used as outgroups (O'Donnell et al., 2009). Maximum parsimony trees were inferred, using the heuristic search option and 1,000 random addition sequences with tree bisection-reconnection branch swapping. Support for the internal nodes was measured with 1,000 parsimony bootstrap replications.

Results and Discussion

The 618-bp *EF-1*α dataset had 58 polymorphic sites, 38 of which were phylogenetically informative. Maximum parsimony analysis of the data generated six equally parsimonious trees with a length of 43 steps. Tree topologies

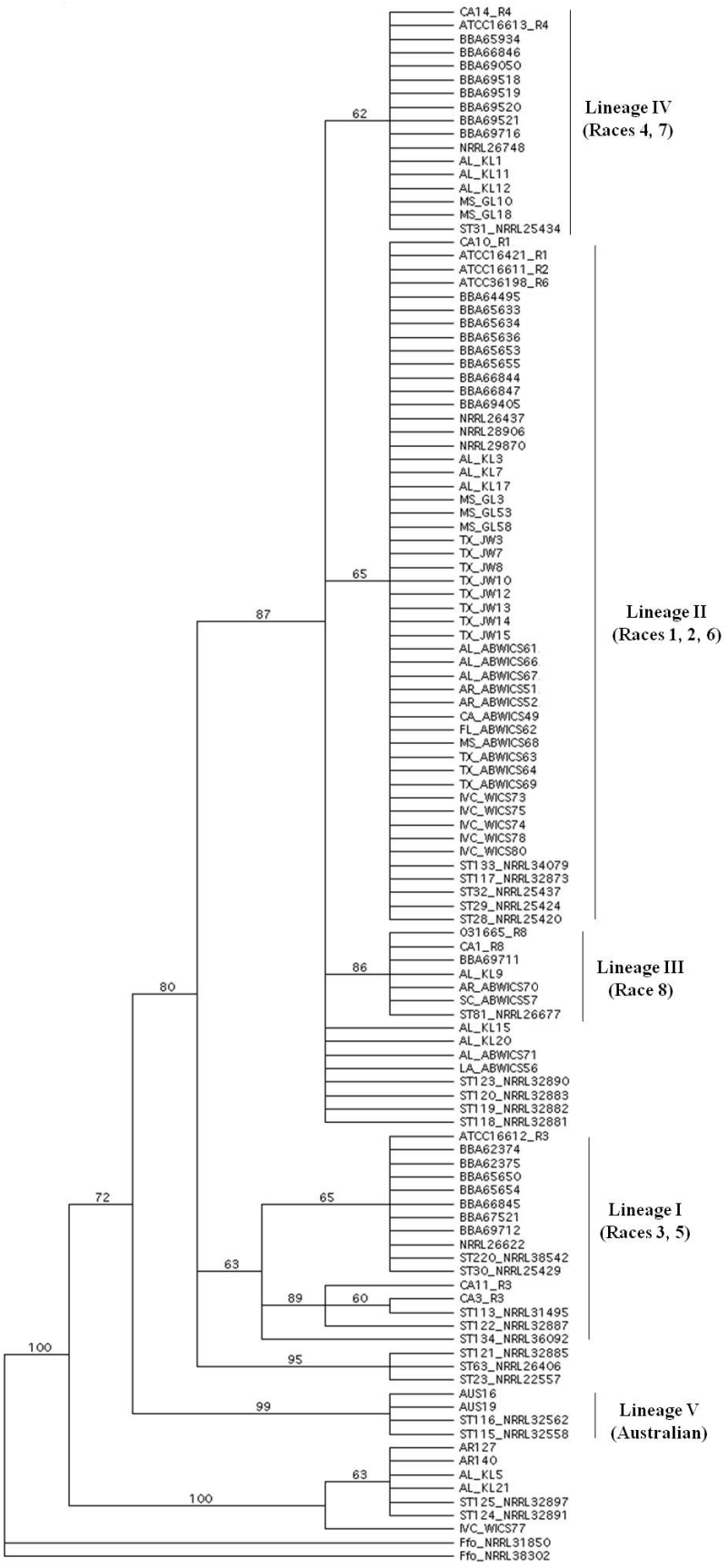
CA14_R4
ATCC16613_R4
BBA65934
BBA66846
BBA69050
BBA69518
BBA69519
BBA69520
BBA69521
BBA69716
NRRL26748
AL_KL1
AL_KL11
AL_KL12
MS_GL10
MS_GL18
ST31_NRRL25434
Lineage IV
(Races 4, 7)
CA10_R1
ATCC16421_R1
ATCC16611_R2
ATCC36198_R6
BBA64495
BBA65633
BBA65634
BBA65636
BBA65653
BBA65655
BBA66844
BBA66847
BBA69405
NRRL26437
NRRL28906
NRRL29870
AL_KL3
AL_KL7
AL_KL17
MS_GL3
MS_GL53
MS_GL58
TX_JW3
TX_JW7
TX_JW8
TX_JW10
TX_JW12
TX_JW13
TX_JW14
TX_JW15
AL_ABWICS61
AL_ABWICS66
AL_ABWICS67
AR_ABWICS51
AR_ABWICS52
CA_ABWICS49
FL_ABWICS62
MS_ABWICS68
TX_ABWICS63
TX_ABWICS64
TX_ABWICS69
IVC_WICS73
IVC_WICS75
IVC_WICS74
IVC_WICS78
IVC_WICS80
ST133_NRRL34079
ST117_NRRL32873
ST32_NRRL25437
ST29_NRRL25424
ST28_NRRL25420
Lineage II
(Races 1, 2, 6)
031665_R8
CA1_R8
BBA69711
AL_KL9
AR_ABWICS70
SC_ABWICS57
ST81_NRRL26677
Lineage III
(Race 8)
AL_KL15
AL_KL20
AL_ABWICS71
LA_ABWICS56
ST123_NRRL32890
ST120_NRRL32883
ST119_NRRL32882
ST118_NRRL32881
ATCC16612_R3
BBA62374
BBA62375
BBA65650
BBA65654
BBA66845
BBA67521
BBA69712
NRRL26622
ST220_NRRL38542
ST30_NRRL25429
CA11_R3
CA3_R3
ST113_NRRL31495
ST122_NRRL32887
ST134_NRRL36092
Lineage I
(Races 3, 5)
ST121_NRRL32885
ST63_NRRL26406
ST23_NRRL22557
AUS16
AUS19
ST116_NRRL32562
ST115_NRRL32558
Lineage V
(Australian)
AR127
AR140
AL_KL5
AL_KL21
ST125_NRRL32897
ST124_NRRL32891
IVC_WICS77
Ffo_NRRL31850
Ffo_NRRL38302
62
87
65
86
80
72
65
63
89
60
100
95
99
63
100

Figure 1. Consensus of six most parsimonious trees (Consistency Index = 0.71, Retention Index = 0.95) inferred from the *EF-1*α dataset (618 bp). Numbers above nodes indicate bootstrap intervals from 1000 replicates.

were similar to trees from previous analyses of the *EF-1*α gene (Skovgaard et al., 2001; Kim et al., 2005; Holmes et al., 2009) in that each of the five designated lineages of *F. oxysporum* f. sp. *vasinfectum* formed distinct clades (Figure 1). The placement of several isolates was unresolved, such as the four isolates below Lineages II (races 1, 2, and 6), III (race 8), and IV (race 4). The *EF-1*α data were also unable to distinguish among many of the 23 two-locus sequence types identified in *F. oxysporum* f. sp. *vasinfectum* (O'Donnell et al., 2009).

The 15 isolates from Alabama were quite diverse. Three isolates (AL_KL1, 11, and 12) were in Lineage IV, closely related to race 4 isolates from California and Asia. Isolates from Alabama were also found in Lineages II, III (race 8), and in the clade with genotypes 127 and 140. Three Alabama isolates (AL_KL15, 20, and AL_ABWICS71) were also among a poorly-resolved group of isolates including ST118 and ST120 (equivalent to genotypes 108 and 110, respectively, of Holmes et al. (2009)). Of the six Mississippi isolates, two (MS_GL10, 18) were in Lineage IV and four were in Lineage II. Isolates from other southeastern states were in Lineages II and III, and the unresolved ST120/ST118 group. All isolates from Texas were in Lineage II. Most isolates from the Ivory Coast were in Lineage II, but isolate IVC_WICS77 appeared unique, though related to the genotypes 127 and 140. None of the thirty-nine isolates sequenced were in Lineages I (race 3) or V (Australian genotypes).

Summary

The most significant finding from these preliminary data is that isolates genetically similar to race 4 are present in Alabama and Mississippi. This report substantiates a previous analysis of isolates from Alabama using ITS2 (Castillo et al., 2010) and is the first report of this race in Mississippi. Race 4 in California can cause significant losses in Pima (*G. barbadense* L.) and Upland (*G. hirsutum* L.) cultivars in the absence of nematodes, so the implications of this finding may be considerable. Pathogenicity assays are currently being conducted to determine if the Alabama and Mississippi isolates are as virulent as race 4 isolates from California. Additional sites in these states should be sampled to determine if the distribution of race 4 extends beyond research plots. While the 39 isolates were isolated from cotton plants with symptoms of Fusarium wilt, pathogenicity assays on cotton are still needed to confirm isolates are *F. oxysporum* f. sp. *vasinfectum*. These are also the first reports of isolates similar to race 8 in South Carolina and Alabama, and of isolates genetically similar to the virulent genotypes 108 and 110 (Holmes et al., 2009) in Alabama and Louisiana.

The *EF-1*α data were unable to distinguish among many of the 23 two-locus sequence types described thus far in *F. oxysporum* f. sp. *vasinfectum*. The IGS rDNA data currently being collected should clarify ambiguous relationships. Five times as many phylogenetically informative sites were found in IGS rDNA than in *EF-1*α (O'Donnell et al., 2009). While the exact sequence type could not be determined for many of the 39 isolates, most isolates appeared closely related to a two-locus sequence type. However, isolate IVC_WICS77 was distinct from all 23 sequence types described for *F. oxysporum* f. sp. *vasinfectum*. O'Donnell et al. (2009) found 256 sequence types among 850 isolates of *F. oxysporum*, but only sequence types containing forma specialis *vasinfectum* were included for this preliminary analysis. Since 21 of the 256 two-locus sequence types identified by O'Donnell et al. (2009) contained more than two *formae speciales*, IVC_WICS77 may belong to a two-locus sequence type excluded here.

Another objective of this project will be to identify susceptibilities and resistances in commercial germplasm to pathotypes of *F. oxysporum* f. sp. *vasinfectum*. Genetic characterization should simplify this task by narrowing the number of genotypes needed for pathogenicity assays. Genetic data may thus be used to create an updated panel of differential cultivars for race identification, or to develop alternative methods for diagnosing *F. oxysporum* f. sp. *vasinfectum* races or genotypes. Despite these preliminary findings, more isolates of *F. oxysporum* f. sp. *vasinfectum* from additional cotton production areas are needed to complete a robust survey and these downstream objectives.

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