Multilocus Analysis Using Putative Fungal Effectors to Describe a Population of *Fusarium oxysporum* from Sugar Beet

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Accepted for publication 29 January 2014.

ABSTRACT

Covey, P. A., Kuwitzky, B., Hanson, M., and Webb, K. M. 2014. Multilocus analysis using putative fungal effectors to describe a population of *Fusarium oxysporum* from sugar beet. *Phytopathology* 104:886–896.

Sugar beet (*Beta vulgaris*) Fusarium yellows is caused by *Fusarium oxysporum* f. sp. *betae* and can lead to significant reductions in root yield, sucrose percentage, juice purity, and storability. *F. oxysporum* f. sp. *betae* can be highly variable and many *F. oxysporum* strains isolated from symptomatic sugar beet are nonpathogenic. Identifying pathogenicity factors and their diversity in the *F. oxysporum* f. sp. *betae* population could further understanding of how this pathogen causes disease and

potentially provide molecular markers to rapidly identify pathogenic isolates. This study used several previously described fungal effector genes (*Fmk1*, *Fow1*, *Pda1*, *PelA*, *PelD*, *Pep1*, *Prt1*, *Rho1*, *Sge1*, *Six1*, *Six6*, *Snf1*, and *Ste12*) as genetic markers, in a population of 26 pathogenic and nonpathogenic isolates of *F. oxysporum* originally isolated from symptomatic sugar beet. Of the genes investigated, six were present in all *F. oxysporum* isolates from sugar beet (*Fmk1*, *Fow1*, *PelA*, *Rho1*, *Snf1*, and *Ste12*), and seven were found to be dispersed within the population (*Pda1*, *PelD*, *Pep1*, *Prt1*, *Sge1*, *Six1*, and *Six6*). Of these, *Fmk1*, *Fow1*, *PelA*, *Rho1*, *Sge1*, *Snf1*, and *Ste12* were significant in relating clade designations and *PelD*, and *Prt1* were significant for correlating with pathogenicity in *F. oxysporum* f. sp. *betae*.

Fusarium oxysporum strains are common soilborne fungi, many of which cause wilting and root or bulb rot in a wide range of plant species (17). Individual strains of *F. oxysporum* usually infect one of a few host species; therefore, they have been grouped into host-specific forms (formae speciales) based on pathogenicity to a particular plant host species (2). *F. oxysporum* also contains many nonpathogenic strains, which are very efficient saprophytes of plant tissues but do not cause symptoms on their hosts (11,17,57,61). *F. oxysporum* is a species complex of morphologically indistinguishable strains (3,44), and isolates of different formae speciales may be more closely related to each other than isolates belonging to the same forma specialis (39,44, 61). To date, there have been >120 formae speciales described (13,50). Currently, the most dependable method to confidently classify strains of *F. oxysporum* to formae speciales is the use of a bioassay, in which putative host plants are exposed to the fungus and are assessed for the development of associated pathogenic symptoms (2,61). Rapid and reliable methods to differentiate pathogenic isolates from nonpathogenic *F. oxysporum* generally are unavailable for all formae speciales (61).

F. oxysporum f. sp. *betae* (70,73,74) is an important pathogen in sugar beet (*Beta vulgaris* L.) and is the primary causal agent for Fusarium yellows, which can lead to significant reductions in root yield, sucrose percentage, juice purity, and storability (4,24, 74). Fusarium yellows is characterized by interveinal yellowing, chlorosis, wilting and necrosis of the leaves, and a gray to reddish or red-brown discoloration in the vascular tissue of the roots

(24,70,74). *F. oxysporum* f. sp. *betae* can be highly variable in growth, pigmentation, conidia production, and virulence (22,25, 70). A complicating factor for the identification of pathogenic *F. oxysporum* f. sp. *betae* isolates is that a high number of *F. oxysporum* isolates recovered from symptomatic sugar beet are nonpathogenic (22,70,81). Variability of the sugar beet *F. oxysporum* population is increased further by the wide geographic distribution of sugar beet production in the United States, initial diversity of the fungal population, and environmental influences (23–26).

In *F. oxysporum*, many methodologies have been used to characterize phylogenetic relationships and the classification of pathogenicity to a host species (3,39,55,60,61). Many studies characterizing the population structure of fungal pathogens, including *F. oxysporum*, have utilized conserved homologous gene markers (76). The methods that have been used to characterize the genetic diversity and evolutionary origin of *F. oxysporum* f. sp. *betae* from sugar beet include vegetative compatibility groups (25,80), restriction fragment length polymorphisms (52), random amplified polymorphic DNA (7), and comparisons of DNA sequences from conserved genomic regions (27,81). Although these technologies have been useful in distinguishing *F. oxysporum* from other *Fusarium* spp., and have been used to further group strains into some formae speciales (1,44,55), they have been insufficient in describing the regional variation of the population of *F. oxysporum* from sugar beet and are unable to differentiate between pathogenic *F. oxysporum* f. sp. *betae* and nonpathogenic isolates of *F. oxysporum* (7,25,27,81).

Effector genes are described as genes necessary for disease development but are not necessary for a pathogen to complete its lifecycle in vitro (28,32). Fungal effector genes fall into several classes, including coding for infection structures, cuticle- and cell-wall-degrading enzymes, ability to respond to environmental cues, fungal toxins, signal cascade components, and suppression of plant immunity (28,32,34). Fungal plant pathogens, including *F. oxysporum*, utilize a wide array of secreted molecules often referred to as elicitors or virulence and avirulence factors (34,78)

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*The e-Xtra logo stands for “electronic extra” and indicates that the online version contains one supplementary table.

<http://dx.doi.org/10.1094/PHYTO-09-13-0248-R>

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as well as specific genes that encode host-determining factors, which are used to gain access into plants and contribute to disease development in the host (5,28,71,76).

Utilizing these putative effector genes, it is possible to use patterns of nucleotide diversity within and among populations to infer the molecular basis of pathogen relatedness and coevolution with a plant host (76). Stukenbrock and McDonald (75) used the haplotypic diversity of *SnToxA* in a population of *Phaeosphaeria nodorum* to support the hypothesis that evolution of the gene is driven by the host. Another example is the study of *Rhynchosporium secalis*, which causes leaf scald in barley, where the polymorphism of deletion mutations within the avirulence gene *NIP1* was used to characterize the population diversity as well as determine whether that particular gene was under corresponding diversifying selection (72). In *F. oxysporum*, Lievens et al. (43) used the presence of seven secreted-in-xylem (*Six*) genes to identify and discriminate isolates to the forma specialis of *F. oxysporum* f. sp. *lycopersici* pathogenic to tomato.

Identifying putative effector genes and their diversity in the *F. oxysporum* f. sp. *betae* population could lead to further understanding of how this pathogen causes disease on sugar beet, characterize the diversity of the pathogen population, offer genetic markers to identify pathogenic isolates, and, ultimately, provide insight into host susceptibility and resistance. The objectives of this study were to (i) determine the genetic diversity of the *F. oxysporum* population from symptomatic sugar beet using described effector genes and (ii) determine whether this diversity could be associated with the pathogenicity of *F. oxysporum* f. sp. *betae* to sugar beet.

MATERIALS AND METHODS

Fusarium isolates and culture maintenance. *F. oxysporum* isolates were obtained from the *Fusarium* long-term culture collection at the United States Department of Agriculture–Agricultural Research Service Sugar Beet Research Unit (SBRU) in Fort Collins, CO. Each isolate was originally recovered from a single conidium or via hyphal-tip transfer from original cultures obtained from infected sugar beet, and stored on filter papers at -20°C in the SBRU culture collection using modified protocols described by Fong et al. (14) and Peever and Milgroom (59). Briefly, each isolate was plated onto water agar and sterile glass microfiber filter paper pieces of $\approx 1\text{ cm}^2$ placed equidistant from the point of transfer. Plates were grown at 25°C for 10 to 14 days or until the fungus had grown through all paper sections, after which filter paper pieces were removed, placed in sterile paper envelopes, and dried overnight over desiccant in a biocontainment hood (14,22,59). Working cultures of all isolates were maintained on potato dextrose agar (PDA) (Becton, Dickinson and Co., Sparks, MD) grown at 25°C for 7 days, then maintained at room temperature, and transferred a maximum of two times using established protocols, as described by Leslie and Summerell (42). In total, 26 isolates were originally isolated from symptomatic sugar beet and represent both pathogenic (*F. oxysporum* f. sp. *betae*) and nonpathogenic (*F. oxysporum*) isolates (Table 1). Pathogenicity for most isolates had been reported previously (27,81). For isolates in which pathogenicity to sugar beet had not been previously described, pathogenicity testing was performed as described by Hanson and Hill (22) and Hanson and Jacobsen (23). All isolates were inoculated on a *Fusarium* susceptible sugar beet germplasm ‘FC716’ that had been grown in a greenhouse for 5 weeks and inoculated with prepared inoculum via a root dip assay, as described by Hanson and Hill (22). Plants were rated weekly for *Fusarium* yellows symptoms for 6 weeks after inoculation using a 0-to-5 rating scale, as described by Hanson and Jacobsen (23). Selected isolates represented the phylogenetic diversity of each of the three clades found in the United States sugar beet *F. oxysporum* population, as previously described (27,81). Three *F. oxysporum* f. sp. *spinaciae* isolates

(FUS001, FUS003, and FUS004) originally collected from spinach but that have been reported previously to be pathogenic to sugar beet and characterized to be members of the phylogenetic clade C (27) were also included in this study (Table 1). Two isolates of *F. proliferatum* (F182 and Fob273a) and one isolate of *F. avenaceum* (F20), all originally isolated from symptomatic sugar beet, were used as outgroup isolates (Table 1). In silico genomic sequences were obtained from 11 additional *F. oxysporum* isolates (FOL4287, HDV247, FOSC3-a, Fo47, MN25, PHW808, PHW815, CL57, II5, NRRL26406, and NRRL25433), 1 *F. graminearum* isolate (PH-1), and 1 *F. verticillioides* isolate (Strain7600) using the comparative genomic sequence datasets available from the BROAD Institute database (http://www.broadinstitute.org/annotation/genome/fusarium_group/MultiHome.html) (Table 1).

Genomic DNA extractions. *F. oxysporum* cultures were grown in potato dextrose broth (PDB) (Becton, Dickinson and Co.) by inoculating with a 7-mm mycelial plug from a working PDA culture of each isolate. Cultures were grown in the dark for 5 days at 25°C on a rotary shaker at 100 rpm. Mycelial masses were collected by pouring PDB cultures through sterilized double-layered cheesecloth, then rinsing the mass five times with sterile distilled water. Mycelial masses were placed into 1.5-ml microcentrifuge tubes which were then frozen by placing the at -20°C for 24 h, and lyophilized at -50°C for 48 h using a Lyph Lock 4.5 lyophilizer (Labconco Co, Kansas City, MO). Lyophilized tissue was ground using a mortar and pestle and DNA was extracted using the manufacturer’s protocol number 3 of a EasyDNA extraction kit (Invitrogen, Carlsbad, CA).

Primer design for putative pathogenicity and virulence effector genes. The DNA sequences of 12 previously reported fungal effector genes and virulence effectors (*Fmk1*, *Fow1*, *Pda1*, *PelA*, *PelD*, *Pep1*, *Rho1*, *Sge1*, *Six1*, *Six6*, *Snf1*, and *Ste12*) (9,21, 29,33,47,49,58,62,66,67,79) were obtained from either the National Center for Biotechnology Information (NCBI) database or the BROAD Institute *Fusarium* Comparative Genome database. A 13th gene, which we dubbed “*Pep*-restless like transposon” (*Prt1*), was based on homology to an open reading frame (*ORF4*) found on the pea pathogenicity island of *F. solani* (teleomorph *Nectria haematococca*) which also contains *Pep* and *Pda* genes (21). This ORF has similarity to the transposase of the *restless* transposon of *Tolypocladium inflatum* (38). Single-gene or multiple-sequence alignments were used to design primers for each gene using the primer design software Primer 3 (69). Specific primer pairs were designed for each gene to eliminate paralogs of gene sequences present in our *F. oxysporum* population (Table 2). Primers previously described by O’Donnell et al. (56) were used to identify sequences of the translation elongation factor 1- α (*Tef1*- α) gene (Table 2) and used for the phylogenetic analysis to describe clade designation for each isolate. Clade assignments were performed as previously described (27,81).

Genotyping. Polymerase chain reaction (PCR) was carried out in a Mastercycler gradient thermal cycler (Eppendorf, Westbury, NY) using genomic DNA with PCR conditions optimized for each primer combination. PCR conditions consisted of an initial denaturing at 94°C for 2 min; followed by 32 cycles of 94°C for 45 s, primer-specific annealing temperature (Table 2) for 45 s, and 72°C for 45 s; and a final extension step of 72°C for 5 min. PCR products were held at 4°C until visualized. PCR reactions for each isolate–gene combination were repeated twice. PCR amplicons were visualized on 1.5% agarose gels stained with ethidium bromide. Single amplicons were directly purified from each PCR reaction, using the GenCatch PCR Cleanup Kit (Epoch, Sugarland, TX). Sequencing was performed by Eurofin, MWG/Operon (Huntsville, AL) and both the 5’ and 3’ primers (Table 2) were used for paired end sequencing for each purified amplicon. Novel gene sequences from sugar beet isolates discovered in this study were submitted to GenBank and are listed in supplemental materials (Supplementary Table 1).

Data analysis. *Sequence alignment and homology.* Sequences were first edited by hand using Geneious Software v. 5.5.6 (37) and any ambiguously aligned sequences were not included in subsequent analysis. Multiple sequence alignments were performed using the neighbor-joining clustering algorithm of CLUSTALX 2.1 (40). Consensus nucleotide sequences for each gene were used to search available *Fusarium* spp. genomes, using the comparative genomic sequences datasets available at the BROAD institute using BLASTn (BLOSUM62). In general, each gene was aligned as a single hit; however, if multiple paralogs were identified, the most similar paralog, based on pairwise analysis (cut-off E-value 1e-3; pairwise identity [PWI] > 70%), was included in the nucleotide sequence alignments and used for subsequent phylogenetic analysis.

Phylogenetics. Phylogenetic analysis was performed on all isolates (including the in silico isolates) using the *Tef1-α* sequences available from the BROAD *Fusarium* database. *Tef1-α* sequence for all isolates were aligned as previously described by Hill et al. (27) and used as input data for generating a phylogenetic tree using the MrBayes plugin of Geneious (30). The General Time Reversible substitution model was performed using a γ -distributed variation consisting of two simultaneous runs with 1,100,000 generations, a sample frequency of 200, and a burn-in of 110,000 generations, resulting in 4,951 sample trees. All isolates were then assigned to clade using previously reported criteria (27,81).

Phylogenetic analysis was also performed on each gene from all isolates included in the study. Sequences were aligned as previously described and used as input alignment data for gen-

TABLE 1. Description of *Fusarium oxysporum* isolates used to characterize the population from symptomatic sugar beet

Isolate ^w	Species	forma specialis	Isolated from (host)	State collected ^x	Original donor ^y	Pathogenicity ^z	Year collected
F05-157	<i>F. oxysporum</i>	...	Sugar beet	Minnesota	L. E. Hanson	NP‡	2005
F14	<i>F. oxysporum</i>	...	Sugar beet	Oregon	A. L. Hill and L. E. Hanson	NP	2001
F17	<i>F. oxysporum</i>	<i>betae</i>	Sugar beet	Oregon	A. L. Hill and L. E. Hanson	P†	2001
F19	<i>F. oxysporum</i>	<i>betae</i>	Sugar beet	Oregon	L. E. Hanson	P†	2001
F29	<i>F. oxysporum</i>	...	Sugar beet	Oregon	A. L. Hill and L. E. Hanson	NP	2001
F30	<i>F. oxysporum</i>	...	Sugar beet	Oregon	A. L. Hill and L. E. Hanson	NP	2001
F38	<i>F. oxysporum</i>	<i>betae</i>	Sugar beet	Oregon	A. L. Hill and L. E. Hanson	P†	2001
FOB13/F180	<i>F. oxysporum</i>	<i>betae</i>	Sugar beet	Oregon	R. M. Harveson	P†	1994
FOB216c	<i>F. oxysporum</i>	<i>betae</i>	Sugar beet	Colorado	H. F. Schwartz	P†	1998
F28	<i>F. oxysporum</i>	<i>betae</i>	Sugar beet	Colorado	A. L. Hill and L. E. Hanson	P†	2001
Fo37	<i>F. oxysporum</i>	<i>betae</i>	Sugar beet	Minnesota	C. Windels	P†	2004
F174	<i>F. oxysporum</i>	<i>betae</i>	Sugar beet	California	R. M. Harveson	P†	1995
FOB220a	<i>F. oxysporum</i>	<i>betae</i>	Sugar beet	Colorado	H. F. Schwartz	P†	1998
FOB257a	<i>F. oxysporum</i>	<i>betae</i>	Sugar beet	Colorado	H. F. Schwartz	P†	1998
H7	<i>F. oxysporum</i>	<i>betae</i>	Sugar beet	Montana	B. J. Jacobsen	P†	2004
H8	<i>F. oxysporum</i>	<i>betae</i>	Sugar beet	Montana	B. J. Jacobsen	P†	2004
F02-78	<i>F. oxysporum</i>	...	Sugar beet	Minnesota	A. Cattanaach	NP‡	2002
F05-284	<i>F. oxysporum</i>	...	Sugar beet	Michigan	L. Hanson	NP	2005
F05-77	<i>F. oxysporum</i>	...	Sugar beet	Minnesota	L. E. Hanson	NP‡	2005
F15	<i>F. oxysporum</i>	...	Sugar beet	Oregon	A. L. Hill and L. E. Hanson	NP	2001
F33	<i>F. oxysporum</i>	...	Sugar beet	Oregon	A. L. Hill and L. E. Hanson	NP	2001
Fo17	<i>F. oxysporum</i>	<i>betae</i>	Sugar beet	Minnesota	C. Windels	P†	2004
Fo23/ <i>Fusarium</i> 2	<i>F. oxysporum</i>	...	Sugar beet	Minnesota	J. J. Weiland	NP†	1998
Fo25/ <i>Fusarium</i> 4	<i>F. oxysporum</i>	...	Sugar beet	Minnesota	J. J. Weiland	NP†	1998
Fo27/ <i>Fusarium</i> 6	<i>F. oxysporum</i>	...	Sugar beet	Minnesota	J. J. Weiland	NP†	1998
Fo29/ <i>Fusarium</i> 8	<i>F. oxysporum</i>	...	Sugar beet	Minnesota	J. J. Weiland	NP†	1998
FUS001	<i>F. oxysporum</i>	<i>spinaciae</i>	Spinach	Washington	L. du Toit	P†	2002
FUS003	<i>F. oxysporum</i>	<i>spinaciae</i>	Spinach	Washington	L. du Toit	P†	2002
FUS004	<i>F. oxysporum</i>	<i>spinaciae</i>	Spinach	Washington	L. du Toit	P†	2002
F182	<i>F. proliferatum</i>	...	Sugar beet	Oregon	R. M. Harveson	P†	1995
FOB273a	<i>F. proliferatum</i>	...	Sugar beet	Nebraska	H. F. Schwartz	NP†	1998
F20	<i>F. avenaceum</i>	...	Sugar beet	Oregon	L. E. Hanson	P†	2001
FOL4287/34936	<i>F. oxysporum</i>	<i>lycopersici</i> race 2	Tomato	in silico	...	ND	...
HDV247/37622	<i>F. oxysporum</i>	<i>pisi</i>	Pea	in silico	...	ND	...
FOSC3-a/32931	<i>F. oxysporum</i>	(human)	Human blood	in silico	...	ND	...
Fo47/54002	<i>F. oxysporum</i>	(biocontrol)	Soil	in silico	...	ND	...
MN25/54003	<i>F. oxysporum</i>	<i>lycopersici</i> race 3	Tomato	in silico	...	ND	...
PHW808/54008	<i>F. oxysporum</i>	<i>conglutinans</i>	Cabbage	in silico	...	ND	...
PHW815/54005	<i>F. oxysporum</i>	<i>raphani</i>	Radish	in silico	...	ND	...
CL57/26381	<i>F. oxysporum</i>	<i>radicis-lycopersici</i>	Tomato	in silico	...	ND	...
II5/54006	<i>F. oxysporum</i>	<i>cubense</i>	Banana	in silico	...	ND	...
/26406	<i>F. oxysporum</i>	<i>melonis</i>	Melon	in silico	...	ND	...
/25433	<i>F. oxysporum</i>	<i>vasinfectum</i>	Cotton	in silico	...	ND	...
PH-1/31084	<i>F. graminearum</i>	...	...	in silico	...	ND	...
Strain7600/20956	<i>F. verticillioides</i>	...	...	in silico	...	ND	...

^w Isolate or strain name. Isolate name followed by original donor's name. Last 13 isolates are in silico genomic sequences obtained from the BROAD Institute sequence database with original isolate (or strain name) listed first, followed by the NRRL number.

^x In silico genomic sequence available from the BROAD Institute *Fusarium* Comparative database (http://www.broadinstitute.org/annotation/genome/fusarium_group/MultiHome.html).

^y Current institution of isolate donors: L. E. Hanson, United States Department of Agriculture–Agricultural Research Service (USDA-ARS), East Lansing, MI, 48824; A. L. Hill, Ohio State University, Columbus 43210; R. M. Harveson, University of Nebraska, Scottsbluff 69361; H. F. Schwartz, Colorado State University, Fort Collins 80523; C. Windels, University of Minnesota, Crookston 56716; B. J. Jacobsen, Montana State University, Bozeman 59717; A. Cattanaach, American Crystal Sugar Company, Moorhead, MN, 56560; John J. Weiland, USDA-ARS, Fargo, ND, 58102; Lindsey du Toit, Washington State University, Mount Vernon.

^z Pathogenicity to sugar beet as determined by † = Hill et al. (27) and ‡ = Webb et al. (81). Pathogenicity determinations indicated in bold are reported in this article. NP = nonpathogen, P = pathogen, and ND = not determined.

erating a phylogenetic tree using MrBayes performed as described above. Phylogenetic data for *Sge1*, *PelD*, and *Prt1* can be found at TreeBASE under study IDS number 13754.

Population structure analysis. The population structure of the isolates was performed for two data sets (or groups). Each group was defined by organizing isolates into populations based on designation of clade or pathogenicity. The first group, based on clade designation, was determined for each isolate by using the phylogenetic analysis of the reference gene *Tef1-α* and assignment into one of four populations (A, B, C, or outgroup [OG]), as described by Hill et al. (27). All sequences for *Fusarium* spp. downloaded from the BROAD Institute were included in the clade group analysis. The second group, pathogenicity, was based on known pathogenicity to sugar beet, where isolates were assigned as either a pathogen or nonpathogen based on pathogenicity testing (Table 1). Sequences for the *Fusarium* spp. downloaded from the BROAD Institute were not included in this analysis because pathogenicity to sugar beet is unknown.

Analysis of population structure was performed using Arlequin 3.5.1.3 (12). Each of the 13 genes independently, as well as a concatenated gene sequence with all 13 genes for each isolate sequentially concatenated end to end, was used as “haplotypic” input data. The two groups, clade and pathogenicity, were analyzed separately for all 13 genes, as well as the concatenated gene sequence. For each group, standard molecular diversity indices were determined and included total number of loci, total number of polymorphic loci, total number of usable loci, allele frequencies, and expected heterozygosity of loci. The total amount of differentiation among the groups was evaluated using this pairwise distance matrix in a global hierarchical analysis of molecular variance (AMOVA) using 1,023 permutations (82) and a significance level of *P* value ≤ 0.01. Tamura and Nei’s distance model was used to calculate a pairwise Euclidean squared genetic distance matrix of fixation statistics (*Fst*) between population of a group and between isolates of a group using 100 permutations (77). Population pairwise genetic distance analysis was also performed using Arlequin, by computing the isolate pairwise *Fst*

matrix for each population of a group (e.g., A by B, B by OG, and so on) to a linearized distance between population using the Tamura and Nei’s distance metric with 110 permutations and a significance level of *P* value ≤ 0.01. Negative *Fst* values were interpreted as zero.

RESULTS

Presence or absence of putative effector genes. Of the 13 genes investigated, six were present in all *F. oxysporum* isolates from sugar beet (*Fmk1*, *Fow1*, *PelA*, *Rho1*, *Snf1*, and *Ste12*), and seven were dispersed within the *F. oxysporum* population from sugar beet (*Pda1*, *PelD*, *Pep1*, *Prt1*, *Sge1*, *Six1*, and *Six6*). The number of alleles varied from gene to gene not only within the sugar beet *F. oxysporum* population but also among the in silico sequences representing the other *Fusarium* isolates. For example, *Six6* had 3 alleles, all of which were 468 bp in size, while *Ste12* had 26 alleles, all of which were 1,618 bp in size (Table 3).

In our population of *F. oxysporum* from sugar beet, one isolate amplified a homolog of the *Six1* gene (Fo37) (Table 3). Fo37 is pathogenic to sugar beet; therefore, it has been classified as *F. oxysporum* f. sp. *betae*. The *Six1* allele amplified from Fo37 (*Six1-a*) had 78.8% PWI to the *Six1* gene of *F. oxysporum* f. sp. *lycopersici* race 2 (FOL4287). The *Six1* allele found in the genome of *F. oxysporum* f. sp. *lycopersici* race 3 isolate (MN25, *Six1-f*) shares 99.8% PWI to the allele found in FOL4287. *Six1* alleles were also found in the genomic sequences of *F. oxysporum* f. sp. *pisi* (HDV247), *F. oxysporum* f. sp. *conglutinans* (PHW808), *F. oxysporum* f. sp. *melonis* (NRRL26406), and *F. oxysporum* f. sp. *cubense* (II5) (Table 3). Collectively, the alleles from these isolates showed 74.6% PWI to the *F. oxysporum* f. sp. *lycopersici* allele from FOL4287. PCR amplification of the *Six6* gene did not occur from any of the *F. oxysporum* isolates from sugar beet; however, sequences for *Six6* were found in *F. oxysporum* f. sp. *lycopersici* race 3 (MN25), *F. oxysporum* f. sp. *melonis* (NRRL26406), and *F. oxysporum* f. sp. *cubense* (II5), which had a combined 80.2% PWI to the allele from FOL4287 (Table 3).

TABLE 2. Polymerase chain reaction primers for amplification of targeted pathogenicity genes

Gene	Sequence accession ID ²	Forward and reverse primers	Primer annealing temperature (°C)
<i>Fmk1</i>	AF286533 and FOXG_08140.2	5' AGCGACTGCGCATTCTTCTCTACA 3' 5' TGAGATCACAGTTGGCGTTGAGGA 3'	60
<i>Fow1</i>	AB078975.1	5' ACTATCCGTACCGATCTTCCAGCTCCC 3' 5' ATGTACCTGAAGGTGTTGCCGACCG 3'	61
<i>Pda1</i>	AY487143.1	5' CACGAGTGTGGGAGATGAGGAAGGC 3' 5' AGGCAGAAGTGAGGAGAGCCATAACC 3'	60
<i>PelA</i>	AF080485 and FOXG_12264.2	5' CTGTCAAGGGCAGCTACGATGGTGG 3' 5' GAGCTGGTGATCTTGCAGGTGTCTCC 3'	60
<i>PelD</i>	U13050.1 and FOXG_13103.2	5' GTGAAGTGCTCTGGTCAGGCTGAGG 3' 5' TGGACTGTGCGCAGCCCTTGTAAACC 3'	60
<i>Pep1</i>	AF315804	5' TGCCTCCAACGCCTCTACAACCTATTCC 3' 5' ACCGTGTGGTTTCTCCTTGACGTGC 3'	60
<i>Prt1</i>	AF315315 and <i>Fusarium oxysporum</i> 4287 supercontig 2.22 [DNA] 610812–615000+	5' CTAAGTCCACCTCGCCAGATTCC 3' 5' AGGTAAGGACGGAGTTTGATCGC 3'	60
<i>Rho1</i>	AY884607.1 and FOXG_13835.2	5' GAAAGCTCGTCATTGTCGGCGATGG 3' 5' CTCGATGGTCTTCTGGTTCGTATCGC 3'	60
<i>Sge1</i>	FOXG_10510.2	5' TCTCTCTGGCAAACCTGTTTCATGTCCC 3' 5' GGTTGTGGTCGGTACTGTTGTTGTGG 3'	60
<i>Six1</i>	AJ608702 and FOXG_16418.2	5' GCGCTTCATGATCTGTGCGTTGAA 3' 5' ATCGCAACTTAGTGTGGGCTGGTA 3'	61
<i>Six6</i>	FJ755835.1 and FOXG_14246.2	5' AGAAGAGTTGAACCACCGAAGGCG 3' 5' ACTGGAATTGTCGCTGCTGAAGTACG 3'	61
<i>Snf1</i>	AF420488.1	5' TTTCTCAAGACAAGCTGTGGCAGTCCC 3' 5' TGTCTGTTGTGTTGGGAGGTTTCGTCG 3'	61
<i>Ste12</i>	FJ609797.1 and FOXG_02103.2	5' GTGAGATGCCTGTCTGTTCCGATTCC 3' 5' ATCAGAGTGTGGTAGGCTTCTTCG 3'	61
<i>Tef1-α</i>	O'Donnell et al. (56)	5' ATGGGTAAGGA(A/G)GACAAGAC 3' 5' GGA(G/A)GTACCAGT(G/C)ATCATGTT 3'	55

² Reference gene sequences for design of primers obtained from National Center for Biotechnology Information (NCBI) database or the BROAD Institute *Fusarium* Comparative Genome database. NCBI accession numbers or BROAD identifications are shown.

Another gene of interest was the pisatin demethylase (*Pda1*) gene, which was amplified from 14 of the *F. oxysporum* isolates from sugar beet and found in 3 of the in silico isolates in the BROAD Institute database (Table 3). PWI for all alleles of the *Pda1* gene was 96% represented by an ≈569-bp sequence, with three exons (97% PWI) and two introns where the majority of variability was observed (65 and 67% PWI, respectively; data not shown). None of the sugar beet *F. oxysporum* alleles of *Pda1* shared sequence identity to the allele that was obtained from the *F. oxysporum* f. sp. *pisi* isolate included in this study (HDV247) (Table 3). However, allele *Pda1-a*, which was found in several of the *F. oxysporum* isolates from sugar beet, was 100% identical to the previously characterized *FoPda1* gene from the *F. oxysporum* f. sp. *pisi* isolate T415 (6). This allele was found in all pathogenic isolates but was not in the nonpathogenic isolates of clade A.

Amplicons and their nucleotide sequences were obtained from all sugar beet *F. oxysporum* isolates for *Fmk1*, *Fow1*, *PelA*, *Rho1*, *Snf1*, and *Ste12* genes, although there was allelic diversity in the sequences (Table 3), not all of which caused a change to the predicted amino acid sequence. For example, although *Fmk1* showed some diversity in synonymous mutations at the nucleotide level, all alleles, except for alleles amplified from the *F. avenaceum* isolate (F20) and the genomic sequence from *F. graminearum* (PH-1) (data not shown), had the same putative amino acid sequence.

Population structure. Results from AMOVA showed that the *Fst* values were significant for more genes when analyzed by clade than by pathogenicity (Fig. 1). *Fmk1*, *Fow1*, *PelA*, *Rho1*, *Sge1*, *Snf1*, and *Ste12* had *Fst* values that were significant when analyzed by clade, and *PelD* and *Prt1* had significant *Fst* values when analyzed by pathogenicity (Fig. 1). The *Fst* value for the

TABLE 3. Allele designation and population indices for sample genes from a population of *Fusarium oxysporum* (outgroup [OG]) isolates from sugar beet^a

Isolate	CI ^v	Fmk1	Fow1	Pda1	PelA	PelD	Pep1	Prt1	Rho1	Sge1	Six1	Six6	Snf1	Ste12	Clade ^w
F05-157	a	a	a	a	a	...	...	...	a	a	...	...	a	a	A
F14	b	a	b	...	a	...	...	...	b	a	...	...	a	b	A
F17	c	a	a	a	a	...	b*	a	a	a	...	...	a	c	A
F19	d	a	a	a	a	...	a*	a	a	a	...	...	a	c	A
F29	e	a	c	...	a	...	...	b	b	a	...	...	b	c	A
F30	f	a	d	...	a	...	...	b	b	a	...	...	b	c	A
F38	g	a	a	a	a	...	b*	a	a	a	...	...	a	d	A
FOB13/F180	c	a	a	a	a	...	b*	a	a	a	...	...	a	c	A
FOB216c	h	a	a	a	b	...	...	b	a	a	...	...	a	d	A
F28	i	b	e	...	c	...	...	...	c	...	...	...	c	f	B
Fo37	j	c	f	...	d	...	...	b	d	...	a	...	d	g	B
F174	k	b	e	...	c	...	...	c	c	...	...	...	c	h	B
FOB220a	m	c	f	...	d	...	...	b	d	...	...	...	d	g	B
FOB257a	n	e	h	...	f	b	...	d	f	b	...	...	f	j	B
H7	m	c	f	...	d	...	...	b	d	...	...	...	d	g	B
H8	p	c	f	...	a	...	...	b	d	...	...	...	d	g	B
F02-78	q	g	i	b*	g	c	...	e	g	c	...	...	g	l	C
F05-284	r	h	j	...	h	d	...	...	h	d	...	...	h	m	C
F05-77	s	e	h	...	f	d	c*	f	i	b	...	...	i	j	C
F15	t	i	k	c*	i	e	d*	g	j	e	...	...	j	n	C
F33	g	e	j	...	h	d	...	...	k	d	...	...	h	m	C
Fo17	v	e	l	a	f	d	e*	f	f	d	...	...	k	j	C
Fo23/Fusarium 2	w	j	m	d	b	f	c*	f	l	f	...	...	l	o	C
Fo25/Fusarium 4	x	j	m	...	j	f	c*	f	m	f	...	...	l	p	C
Fo27/Fusarium 6	y	e	l	...	k	d	f	f	f	d	...	...	m	j	C
Fo29/Fusarium 8	z	j	m	d	d	f	c*	f	l	f	...	...	l	q	C
FUS001	al	i	n	g*	m	p	d*	l	s	k	...	...	n	y	C
FUS003	am	i	n	g*	m	p	...	l	s	l	...	...	n	y	C
FUS004	al	i	n	g*	m	p	d*	l	s	k	...	...	n	y	C
F182	l	d	g	...	e	a	...	...	e	...	...	...	e	i	OG
Fob273a	o	f	g	...	e	...	...	...	e	...	...	...	e	k	OG
F20	an	r	r	...	r	q	...	...	t	...	...	...	r	z	OG
FOL4287/34936	ag	p	q	...	o	m	g*	i	r	j	d	b	k	w	C
HDV247/37622	ae	n	n	f	m	k	h	h	p	c	b	...	g	u	C
FOSC3-a/32931	ad	m	o	...	l	j	...	...	o	h	...	...	p	t	C
Fo47/54002	ac	e	j	...	h	i	...	...	n	d	...	...	o	m	C
MN25/54003	ai	e	j	...	h	i	...	k	n	d	f	b	h	m	C
PHW808/54008	aj	q	i	b*	g	n	...	...	g	c	g	...	g	u	C
PHW815/54005	ak	n	n	...	q	o	i	...	p	c	...	...	g	u	C
CL57/26381	ab	l	j	...	h	h	j	f	n	d	...	...	i	s	C
II5/54006	af	o	p	...	n	l	...	...	q	i	c	c	q	v	C
/26406	ah	p	q	...	p	m	...	j	r	j	e	a	k	x	C
/25433	aa	k	n	e*	i	g	...	...	j	g	...	...	n	r	C
PH-1/31084	ao	s	s	...	s	r	...	...	u	n	...	...	s	aa	OG
Strain7600/20956	ap	t	t	...	t	s	...	...	v	m	...	...	t	ab	OG
Alleles (<i>n</i>) ^x	42	20	20	7	20	19	10	12	22	14	7	3	20	28	...
PWI (%) ^y	75.2	96.6	97	96	95.3	93.8	71.5	97.2	94.9	96.9	77.1	80.2	96.8	96.4	...
Loci/length (bp) ^z	10,014	605	779	569	485	582	838	1,133	681	675	469	468	631	1,618	...

^a Presence of a gene is listed by the nucleotide allele (alphabetic) designation; * indicates predicted mutant allele (i.e., a premature stop codon).

^v Concatenated idiomorph (CI) of the gene sequences of each gene in each isolate.

^w Each isolate is listed with its clade designation for *Tef1-α*, as determined by Hill et al. (27) and Webb et al. (81). OG = isolates used as outgroup controls for phylogenetic analysis.

^x Total number of nucleotide alleles in the population for each gene.

^y Pairwise identity (PWI) was determined across all isolates for each gene.

^z Length in base pairs for each gene.

concatenated gene sequence was significant for both groups. Genes that did not have significant *Fst* values for either clade or pathogenicity were *Pda1*, *Pep1*, *Six1*, and *Six6*.

All genes with significant *Fst* values for clade were conserved across all *Fusarium* isolates included in the study, except for *Sge1*. The allelic patterns and phylogenetic analysis of these genes demonstrated topologies and genealogical concordance similar to that of *Tef1-α*, supporting the clade designations laid out by Hill et al. (27) (Table 3). Most of the genes found to be significant had *Fst* values near 0.5 (Fig. 1A), indicating that variance components portioned roughly equally among and between populations compared with the total variance (data not shown). *Sge1* demonstrated a higher *Fst* value compared with the *Fst* values for the other genes that were significant by clade (Fig. 1A). *Sge1* was dispersed across the population and showed discordance between its gene genealogy and the phylogenetic organization described by *Tef1-α*. A large proportion of the variance for *Sge1* was partitioned among the clades, and was explained mostly by an absence of *Sge1* alleles in clade B isolates (Fig. 2). Additionally, all clade A isolates contained only one *Sge1* allele (Table 3).

The pairwise genetic distance *Fst* values indicated that clade A has a greater genetic distance when compared with each of the other clades (Fig. 3). *Fmk1*, *Fow1*, *PelA*, *Rho1*, *Snf1*, and *Ste12*, as well as the concatenated gene sequence, were found to have significant *Fst* values in all pairwise clade comparisons (Fig. 3). The *Fst* values for three genes (*Sge1*, *PelD*, and *Pda1*) showed

differing significance depending on specific pairwise clade comparisons. *Sge1* was found to not be significant for comparison between clades B and C and clades B and OG, although the *Fst* values were high for all other clade comparisons (Fig. 3). For example, the *Sge1* *Fst* value for the pairwise comparisons between clades A and B and clades A and OG approached the maximum of 1 (Fig. 3) and, in this case, *Sge1* was absent in all but one isolate (FOB257a) of clade B (Table 3). The *Fst* values for *PelD* were significant for the pairwise comparison between clades C and OG, and *Pda1* was significant for the pairwise comparisons between clades A and C. *Prt1* was not significant for any of pairwise clade comparisons (Fig. 3).

Putative effector genes, when analyzed by pathogenicity in AMOVA, had an average *Fst* value of ≈ 0.26 (Fig. 1B) and lower variance partitioning among the populations compared with the total variance observed. Two of the genes tested (*PelD* and *Prt1*) had significant *Fst* values when analyzed by pathogenicity and demonstrated higher relative *Fst* values (0.42 and 0.35, respectively) (Fig. 1B). Both *PelD* and *Prt1* were genealogically dispersed across the *F. oxysporum* population from sugar beet. *Prt1* was present in more pathogenic (to sugar beet) *F. oxysporum* isolates than nonpathogens, as opposed to *PelD*, which was found mostly in nonpathogenic isolates (Fig. 4; Table 3). Despite being significant for pathogenicity, *PelD* alleles were absent from more than one clade; in this case, both clade A and clade B isolates. However, the remaining alleles of *PelD* in clades C and OG were not monopolized by any one allele and the variance partitioning

Fig. 1. Analysis of molecular variance fixation index (*Fst*) values for the concatenated gene sequence and each pathogenicity gene when describing clade and pathogenicity, respectively, in a *Fusarium oxysporum* population from sugar beet. **A**, *Fst* values $\pm$ *P* values analyzed by clade. In silico genome sequences were included in the analysis. **B**, *Fst* $\pm$ *P* value analyzed by pathogenicity. In silico genome sequences were not included in the analysis. Error bars indicate the amount of variation in *Fst* value for each gene. *P* values for significant genes are indicated as follows: ** and * = *P* < 0.001 and 0.01, respectively.

Fig. 2. Phylogenetic trees for *Sge1* that showed significant fixation index (*Fst*) values by clade. Bayesian posterior-probabilities ($\times 100$) >50 are shown above the branch and branch lengths (substitutions per site) below. Allele designations (lowercase) for each gene are labeled above the branch. Isolates with pathogenicity to sugar beet are in bold. Clade designation (A, B, C, or outgroup [OG]) and species are labeled for each isolate. *Fusarium graminearum* (PH-1) was used to root the tree for *Sge1*. Isolates where a gene was not amplified were not included in the Bayesian analysis but are shown as a separate branch for clarity.

remained low among the population (Fig. 4; Table 3). *Prt1*, while genealogically dispersed in all clades, primarily associated with pathogenic isolates (Fig. 5; Table 3).

DISCUSSION

Most studies characterizing the population structure of fungal pathogens, including *F. oxysporum*, have utilized single homologous gene markers that are found in many fungal species (76). An increasing number of studies are utilizing more than a single gene to characterize and describe populations of fungal species (36,41, 48,65). In *Fusarium* spp., O'Donnell et al. (54) used multiple gene markers to characterize the species complex of *Gibberella fujikuroi*. However, rapid diagnostic techniques are still mostly unavailable for identifying all strains of *F. oxysporum* to formae speciales (61). Although conserved gene markers have been useful in characterizing the *F. oxysporum* f. sp. *betae* population into phylogenetic clades, they have been ineffective in differentiating pathogenic from nonpathogenic isolates (27,81). Several researchers have started to use loci that are under balancing selection (such as effector genes) to describe fungal populations, some of which can also be effective in identifying pathogenic *F. oxysporum* (43,72,75).

In this study, we utilized 13 genes (*Fmk1*, *Fow1*, *Pda1*, *PelA*, *PelD*, *Pep1*, *Prt1*, *Rho1*, *Sge1*, *Six1*, *Six6*, *Snf1*, and *Ste12*), all but one of which have been previously described to contribute to pathogenicity of fungal plant pathogens (9,21,29,33,47,49,58, 62,66,67,79), to describe a population of *F. oxysporum* from sugar beet into phylogenetic clades and, potentially, be used to identify pathogenic isolates of *F. oxysporum* f. sp. *betae*. Of the genes investigated, six were present in all *F. oxysporum* isolates from sugar beet (*Fmk1*, *Fow1*, *PelA*, *Rho1*, *Snf1*, and *Ste12*) and seven were found to be dispersed within the *F. oxysporum* population (*Pda1*, *PelD*, *Pep1*, *Prt1*, *Sge1*, *Six1*, and *Six6*). Of those found in all *F. oxysporum* isolates from sugar beet, *Fmk1*, *Fow1*, *PelA*, *Rho1*, *Sge1*, *Snf1*, and *Ste12* had *Fst* values that were significant in describing clade designations while *PelD*, and *Prt1* had *Fst* values significant for describing pathogenicity to sugar beet. Although we were unable to identify a single marker that would allow us to clearly differentiate all pathogenic *F. oxysporum* f. sp. *betae* strains from nonpathogenic isolates, we were able to identify putative genes that may be contributing (or linked) to pathogenicity to sugar beet.

Within the population of *F. oxysporum* isolated from sugar beet, six conserved genes (*Fmk1*, *Fow1*, *PelA*, *Rho1*, *Snf1*, and *Ste12*) had *Fst* values that were significant for describing differences between the phylogenetic clade designations previously reported (27,81). As described, the presence of effector genes across the larger sample of not just *F. oxysporum* strains but also other *Fusarium* spp. supports the idea of shared common origins and raises the possibility of pleiotropic functions for many of these genes, not just in pathogenicity or host specificity (10,58, 66,68,83). However, a larger sample of isolates representing the broader diversity of the entire *Fusarium* species complex would be needed to assess specific relationships or potential functions. Because our results support the phylogenetic relationships previously reported by others (27,81) and do not provide any additional insights into the genetic diversity of the population of *F. oxysporum* from sugar beet, we suggest that the use of a single conserved gene (*TEF1α*) would adequately describe the phylogenetic relationships in the broader *F. oxysporum* species complex, as suggested by Webb et al. (81).

Two genes, *PelD* and *Prt1*, demonstrated significant genetic differences for analysis by pathogenicity within the sugar beet *F. oxysporum* population. *PelD* was present in 16 *F. oxysporum* isolates from sugar beet and had 19 differing alleles in the entire population tested. The *PelD* sequences in our study had a total of $\approx 94\%$ PWI to the *F. solani PelD* (NCBI: U13050) (data not

shown). Despite having a dispersed nature in the population, *PelD* was present almost exclusively in clade C and one isolate from clade B (Fob257a), as well as from *F. proliferatum*, *F. graminearum*, and *F. verticillioides*. It appears that the absence of *PelD* primarily is associated with pathogenicity to sugar beet, and its exclusion from the majority of pathogenic *F. oxysporum* f. sp. *betae* isolates (mostly in clades A and B) suggests that this gene does not contribute to pathogenicity on sugar beet. The *PelD* and *PelA* genes investigated in this article are members of a four-gene pectate lyase family found in *F. solani* (16,18,19,31,67). Presence of *PelB* and *PelC* paralogs in the sugar beet *F. oxysporum* population was not investigated but should be investigated in the future.

The other gene that demonstrated significant genetic differences for pathogenicity was *Prt1*, which is orthologous to the *F. solani* *ORF4* and is similar to the restless *Ac*-like transposon from *T. inflatum* (21,38). *Prt1* was present as a genealogically dispersed gene within the *F. oxysporum* population from sugar beet, and the majority of those isolates containing *Prt1* were pathogenic to sugar beet (Fig. 5; Table 3). Contribution of *Prt1*, if any, to pathogenicity of sugar beet is unknown and remains an avenue of future research. Its use as a marker for pathogenicity has strong research appeal, particularly due to its association with a previously described pathogenicity island (the *PEP* gene cluster) in the *F. solani* species complex, which contains genes required for causing disease in pea (*Pisum sativum* L.) (21). Additionally, in prokaryotes and in some fungi, pathogenicity islands are on traditionally mobile elements and, in the case of *Fusarium* spp., on supernumerary chromosomes that likely are transmitted horizontally and carry a number of disease-specific pathogenicity determinants (21,45,53,63). Whether these mobile elements are playing a role in the population of *F. oxysporum* f. sp. *betae* and pathogenicity to sugar beet is unknown but, based on some of our results, should continue to be evaluated.

Several other genes were of general interest due to their previous use in describing other formae speciales of *F. oxysporum*. Lievens et al. (43) used the presence of several *Six* genes to discriminate isolates pathogenic to tomato and, therefore, to the formae speciales of *F. oxysporum* f. sp. *lycopersici*. All but one of the isolates from the sugar beet *F. oxysporum* population amplified a *Six1* homolog and none of them contained the *Six6* gene. However, the *Six1* allele that was amplified from Fo37 (a pathogenic isolate from sugar beet) only had a 78.8% PWI to the *Six1* gene found in *F. oxysporum* f. sp. *lycopersici* race 2 (FOL4287). Because of the low PWI identity to the *Six1* gene from FOL4287, we predict that isolate Fo37 is unlikely to be pathogenic to tomato, nor is it a member of *F. oxysporum* f. sp. *lycopersici*. However, pathogenicity testing on tomato has not been done to confirm this hypothesis but could be done in future experiments.

The *Pda1* gene has been reported to be important for some fungal pathogens, including *F. oxysporum* f. sp. *pisi*, to degrade the phytoalexin pisatin and contributes to its pathogenicity to pea (6,79). *Pda1* has been used in previous phylogenetic analysis to identify strains of *F. solani* and *F. oxysporum* that cause disease of pea (51). We found that this gene was amplified from 14 of the *F. oxysporum* isolates from sugar beet; however, only one allele, *Pda1-a*, had 100% identity to the previously characterized *FoPda1* gene from the *F. oxysporum* f. sp. *pisi* isolate T415 (data not shown) (6). *Pda1-a* was found in all sugar-beet-pathogenic isolates but not in the nonpathogenic isolates of clade A. Coleman et al. (6) found that only particular alleles of *Pda* were able to degrade pisatin or contributed to pathogenicity. It is unknown whether the isolates in our study that contain the *Pda1-a* allele are able to degrade pisatin or if they are cross-pathogenic to pea but this should be tested in future experiments. Another possibility is that sugar beet has been reported to produce two phytoalexins, betagarin and betavulgarin, in response to pathogen infection (20,35,46,64). Betavulgarin ($C_{17}H_{12}O_6$) is an isoflavone similar to pisatin ($C_{17}H_{14-16}O_6$) (8,35,64) and future research could be undertaken to determine whether *Pda1* alleles found in our population are potentially interacting with and degrading these phytoalexins found in sugar beet and, therefore, contributing to pathogenicity to sugar beet.

A particularly interesting sequence result from this study was the discovery of *Pepl* alleles (*Pepl-a* and *Pepl-b*) which contain a putative *Folyt hAT type II* transposable element (TE) (15) inserted into the coding sequence of the *Pepl* genes of all but one pathogenic isolate of clade A. The TE was 316 bp long and contained inverted terminal repeat sequences characteristic of known *hAT* TE's (data not shown). How this putative TE relates to *hAT type II* TE's still needs to be investigated but, because this sequence was primarily in pathogenic isolates from clade A, it makes this a valuable avenue of future investigation as a potential identifying marker.

Several of the genes investigated did not result in amplification under our experimental conditions. Although the primer annealing sites were selected based on regions with highly conserved sequence, we concede that there is a risk in drawing the conclusion that those genes are not present in those isolates. It is possible that there have been mutations at primer annealing sites, an intron has made the gene too large to be amplified, or even the presence of homologous sequences elsewhere in the genome are competing for primer annealing. Future work, including Southern analysis on current gene selections, as well as including additional reported effector factors not tested here, are ongoing in order to resolve many of these possibilities.

Rapid and reliable methods to differentiate pathogenic isolates of *F. oxysporum* f. sp. *betae* from nonpathogenic isolates of

Fig. 3. Analysis of molecular variance fixation index (*Fst*) values for the concatenated gene sequence and each pathogenicity gene when comparing each pairwise clade designation. *Fst* values $\pm$ *P* values for each pairwise clade comparison. Only genes with significant *Fst* values are shown ($P < 0.01$, 110 permutations).

Fig. 4. Phylogenetic tree for *PelD* that showed significant fixation index (*Fst*) values by pathogenicity. Bayesian posterior-probabilities ($\times 100$) > 50 are shown above the branch and branch lengths (substitutions per site) below. Allele designations (lowercase) for each gene are labeled above the branch. Isolates with pathogenicity to sugar beet are in bold. Clade designation (A, B, C, or outgroup) and species are labeled for each isolate. *Fusarium avenaceum* (F20) was used to root the tree for *PelD*. Isolates where a gene was not amplified were not included in the Bayesian analysis but are shown as a separate branch for clarity.

Fig. 5. Phylogenetic tree for *Prt1* that showed significant fixation index (*Fst*) values by pathogenicity. Bayesian posterior-probabilities ($\times 100$) > 50 are shown above the branch and branch lengths (substitutions per site) below. Allele designations (lowercase) for each gene are labeled above the branch. Isolates with pathogenicity to sugar beet are in bold. Clade designation (A, B, C, or outgroup) and species are labeled for each isolate. *Fusarium oxysporum* isolates FUS001, FUS003, and FUS004 were used to root the tree for *Prt1*. Isolates where a gene was not amplified were not included in the Bayesian analysis but are shown as a separate branch for clarity.

F. oxysporum are currently unavailable to sugar beet researchers and producers. Utilizing gene genealogies of several previously described effector genes (*Fmk1*, *Fowl1*, *Pda1*, *PelA*, *PelD*, *Pep1*, *Rho1*, *Sge1*, *Six1*, *Six6*, *Snf1*, and *Ste12*), we found that many were not only significant in their ability to correlate with the phylogenetic relationships of a population of *F. oxysporum* from sugar beet but two were also significant in being associated with pathogenicity to sugar beet. How these genes can be used to characterize our population against the broader *F. oxysporum* species complex should be investigated further; however, preliminary analysis indicates that using a multilocus approach utilizing putative effector genes may be useful in differentiating pathogenic *F. oxysporum* f. sp. *betae*. This research also identified potential genes that may contribute to the pathogenicity of *F. oxysporum* f. sp. *betae* to sugar beet, and experiments to follow up with these findings will be performed in the future. Although only a subset of potential effector genes was investigated on a small sample of isolates from the sugar beet *F. oxysporum* population, we predict that expanding the numbers of both will clarify some of the preliminary relationships identified.

ACKNOWLEDGMENTS

Additional funding for this project was provided by the Beet Sugar Development Foundation. We thank C. Richards and P. Reeves from the Plant Germplasm Preservation Research Unit at the United States Department of Agriculture–Agricultural Research Service National Center for Genetics Resource Preservation for assistance in data analysis. Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the United States Department of Agriculture (USDA). USDA is an equal opportunity provider and employer.

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