Pathogenic and Phylogenetic Analysis of *Fusarium oxysporum* from Sugarbeet in Michigan and Minnesota

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ABSTRACT

Fusarium yellows of sugarbeet (*Beta vulgaris*), caused by *Fusarium oxysporum* f. sp. *betae*, can lead to a significant reduction in root yield, sucrose percentage, and juice purity. Fusarium yellows has become increasingly common in both Michigan and Minnesota sugarbeet production areas, and although genetic resistance provides some control, growers have reported failures when resistant varieties are grown in different parts of the country, potentially due to the variability of local *F. oxysporum* populations. Previous research has demonstrated that the *F. oxysporum* population collected from symptomatic sugarbeet can be highly variable in pathogenicity but that this is not solely due to the wide geographic distribution of sugarbeet production. *F. oxysporum* isolates were collected from symptomatic sugarbeet throughout the production region of Michigan and Minnesota and were characterized utilizing pathogenicity and phylogenetic analyses. The *F. oxysporum* population from Michigan and Minnesota was found to be inconsistent in pathogenicity to sugarbeet and was polyphyletic. Therefore, the population from Michigan and Minnesota could not be classified into distinct races, but rather was described adequately by three previously reported phylogenetic clades.

Additional key words: *Beta vulgaris*, Fusarium yellows, gene sequencing, genetic diversity

Fusarium yellows, caused by *Fusarium oxysporum* Schlechtend:Fr. f. sp. *betae* (Stewart) Snyd & Hans (Stewart, 1931; Snyder and Hansen, 1940; Ruppel, 1991), is a disease of sugarbeet (*Beta vulgaris* L.), which can lead to a significant reduction in root yield, sucrose percentage and juice purity in affected plants (Hanson and Jacobsen, 2009). Fusarium yellows was first reported and described by Stewart (1931) from symptomatic sugarbeets from the Arkansas Valley in Southeastern Colorado. Symptoms include grayish-brown vascular tissue in roots, interveinal yellowing and wilting of leaves, and eventual death of the plant (Stewart, 1931; Schneider and Whitney, 1986; Franc et al., 2001). Since that time, Fusarium yellows has increased in significance in the Central High Plains of the United States including Colorado, Montana, Nebraska, and Wyoming as well as some parts of Texas (Harveson and Rush, 1997; Panella and Lewellen, 2005). Until recently, Fusarium yellows has had little impact on sugarbeet production in the Red River Valley of Minnesota and North Dakota or in Michigan. Fusarium yellows was first reported in the Red River Valley in 2002 (Windels et al., 2005) and in Michigan in 2005 (Hanson, 2006). Since these initial reports, Fusarium yellows has become increasingly common, particularly in Minnesota sugarbeet production areas, possibly due to increased planting of susceptible varieties (Khan et al., 2003; Burlakoti, 2007; Rivera et al., 2008). In Michigan, Fusarium yellows has not been reported widely; however, *Fusarium oxysporum* f. sp. *betae* has been found on plants from various areas (Hanson, unpublished).

Fusarium oxysporum is considered to be a species complex of morphologically indistinguishable strains (Lievens et al., 2008), containing pathogenic and non-pathogenic members assigned to *formae speciales* based on host specificity (Armstrong and Armstrong, 1981). Although *F. oxysporum* can be classified into *formae speciales*, this designation does not indicate that host specificity has resulted from a single (monophyletic) source but rather comprises a species complex with polyphyletic origin (Baayen et al., 2000). Many methods have been used to characterize the genetic diversity and evolutionary origin of *F. oxysporum* f. sp. *betae* from sugarbeet, including vegetative compatibility grouping (VCG) (Harveson and Rush, 1997), restriction fragment length polymorphism (RFLP) (Nitschke et al., 2009), random-amplified polymorphic DNA marker (RAPDs) (Cramer et al., 2003), and comparisons of DNA sequences from conserved genomic regions (Hill et al., 2011). While many of these technologies have been useful in distinguishing *F. oxysporum* from other *Fusarium* spp., they do little to describe the regional variation of populations of *F. oxysporum* f. sp. *betae* and are unable to differentiate between pathogenic and non-pathogenic isolates.

Previous work by Hill et al. (2011) utilized three conserved genetic regions; *β-tubulin* (Koenraadt et al., 1992), a translation elongation factor 1a (*TEF-1a)* (O'Donnell et al., 1998), and the internal tran-

scribed spacer (*ITS*) region of the rRNA 5.8S gene (White et al., 1990), to characterize a set of *F. oxysporum* isolates collected from sugarbeet. The authors found that genetic relatedness of *F. oxysporum* isolates collected from sugarbeet did not correlate with pathogenicity, preventing them from reliably identifying pathogenic *F. oxysporum* f. sp. *betae* isolates from non-pathogenic isolates. Additionally, they found that genetic variation within *F. oxysporum* f. sp. *betae* was organized loosely into multiple clades, very generally based on production region (Hill et al., 2011). However, few *F. oxysporum* isolates from Michigan and Minnesota production regions were included in this work. It is unknown how the diversity of *F. oxysporum* f. sp. *betae* isolates from this region with increasing instances of Fusarium yellows, compares with pathogen populations in other regions of sugarbeet production.

Studies with another *Fusarium* species complex, *F. solani*, have highlighted the advantage of developing multi-locus DNA sequencing schemes to characterize pathogen diversity (O'Donnell et al., 2008; Balmas et al., 2010). In addition to the *β-tubulin*, *TEF-1a*, and *ITS*; genomic sequences from mitochondrial rDNA (*mtSSU*) and Histone 3 genes (*H3*) have been reported to be effective in characterizing *F. oxysporum* isolates by pathogenicity for several other *formae speciales* (Donaldson et al., 1995; Baayen et al., 2000). Previous work has shown that the *ITS* gene sequence, while useful in characterizing other *Fusarium* spp., is ineffective for characterizing the *F. oxysporum* species complex (Donaldson et al., 1995; Baayen et al., 2000; Hill et al., 2011), therefore we did not use this locus in our studies. In this work, we utilized *β-tubulin, TEF-1a, mtSSU,* and *H3* gene sequences to characterize a population of *F. oxysporum* isolated from symptomatic sugarbeets from Michigan and Minnesota and associate their genetic variation with a previously described population of *F. oxysporum* f. sp. *betae*.

MATERIALS AND METHODS

Isolates

Twenty nine isolates of *F. oxysporum* were used in this study. All isolates were collected originally from symptomatic sugarbeet, single-spored or hyphal-tipped, and stored with proper maintenance, either in the Sugar Beet Research Unit culture collection located at Ft. Collins, CO, or the Sugarbeet and Bean Research Unit culture collection located at East Lansing, MI. Isolates are maintained in culture collections either as filter paper stocks, silica gel stocks, or lyophilized culture stocks, using accepted protocols and appropriately maintained as described by Leslie and Summerell (2006) (Table 1). Fourteen of the 29 isolates were previously described in Hill et al. (2011) and were used here to anchor our results to previously reported results (Table

1). Working cultures of all isolates used for this study, were maintained on potato dextrose agar plates (PDA; Becton, Dickinson and Co.) at room temperature, and transferred only 2-3 times to maintain viability of isolates, using established protocols as described by Leslie and Summerell (2006). One *F. avenaceum* isolate (F20), that is moderately virulent to sugarbeet, was used as an outgroup for building phylogenetic trees.

Pathogenicity Testing

Pathogenicity had been determined on some of the isolates previously (Hill et al., 2011), using the same described protocol, and therefore, were not repeated. Isolates that had undetermined pathogenicity, were tested at USDA-ARS facilities in either Fort Collins, CO or East Lansing, MI (Table 1). A susceptible sugarbeet cultivar 'FC716' (Panella et al., 1995) was grown in a greenhouse at approximately 28°C and 16 h daylight, as previously described (Hanson and Hill, 2004). Six weeks after sowing, plants were gently removed from the soil, rinsed under running tap water, and placed in a conidial spore suspension (~1X10^{5}CFU per mL) prepared as described by Hanson and Hill (2004) for 8 min with intermittent agitation. Control sugarbeet roots were placed in sterile distilled water. Two pathogenic *F. oxysporum* f. sp. *betae* isolates (F19 and Fob220a) were included during all pathogenicity testing as positive controls. Five to 10 individual beets per isolate were then replanted into 10 cm x 25 cm cone-tainers (Steuwe and Sons, Inc.) containing premoistened, pasteurized potting mix (Farfard #2-SV, American Clay Works). Plants were then placed back into a greenhouse, in a randomized complete block design for 2 days at approximately 22°C to reduce transplant shock, after which temperatures were raised to 28°C with 16 h of daylight for the remainder of the incubation period. Isolates were tested for pathogenicity by repeating the experiment twice. Plants were rated weekly for Fusarium yellows symptoms for 6 weeks after inoculation using a 0 to 5 rating scale as described by Hanson et al. (2009). A rating of 0 = no disease; 1 = leaves wilted, small chlorotic areas on lower leaves, most of leaf green; 2 = leaves showing interveinal yellowing; 3 = leaves have small areas of necrosis or becoming necrotic and dying, less than half of the leaves affected; 4 = more than half of leaves dead, plant stunted, most living leaves showing symptoms; 5 = plant death. Pathogenicity was determined using the most severe disease rating, which occurred on the sixth week after inoculation. Isolates with a mean rating at the sixth week of 2 or above were considered to be pathogenic (Ruppel, 1991).

DNA Isolation

Isolates were grown in 50 mL potato dextrose broth (PDB; Becton, Dickinson and Co.) by inoculating with a 7 mm diameter mycelia plug. Cultures were grown in the dark for 5 days at 25°C on a rotary

Table 1. Geographic origin and pathogenicity on sugarbeet (cv. FC716) of *Fusarium oxysporum* isolates included in phylogenetic studies.

Isolate	Geographic Origin	Year of Isolation	Pathogenicityy[†]	Source of PathogenicityData[‡]
F02-105	MN	2002	NP (0.4)	Ft. Collins, CO
F02-78	MN	2002	NP (0.4)	Ft. Collins, CO
F05-157	MN	2005	NP (0.5)	Ft. Collins, CO
F05-77	MN	2005	NP (0.5)	Ft. Collins, CO
F07-35	MI	2007	NP (0.9)	Ft. Collins, CO
F07-43	MI	2007	NP (0.9)	Ft. Collins, CO
F07-52	MI	2007	NP (1.0)	Ft. Collins, CO
F08-10	**MI**	**2008**	**NP/P (1.1/2.0)**	**Ft. Collins, CO/East Lansing, MI**
F08-11	MI	2008	NP (0.7)	Ft. Collins, CO
F08-13	**MI**	**2008**	**NP/P (1.1/2.1)**	**Ft. Collins, CO/East Lansing, MI**
F08-174	MI	2008	NP (0.7)	Ft. Collins, CO
F08-184	MI	2008	NP (0.6)	Ft. Collins, CO
F08-49	MI	2008	NP (0.8/1.8)	Ft. Collins, CO/East Lansing, MI
F17	OR	2001	P (3.0)	Hill et al (2011)[¶]
F19	OR	2001	P (3.8)	Ft. Collins, CO
F28	CO	2001	P (2.1)	Hill et al (2011)[¶]
Fo17	MN	2004	NP (1.7)	Hill et al (2011)[¶]
Fo22/Fusarium #1	MN	1998	NP (0.7)	Hill et al (2011)[¶]

Fo23/Fusarium #2	MN	1998	NP (0.6)	Hill et al (2011)[¶]
Fo25/Fusarium #4	MN	1998	NP (0.7)	Hill et al (2011)[¶]
Fo27/Fusarium #6	MN	1998	NP (1.0)	Hill et al (2011)[¶]
Fo29/Fusarium #8	MN	1998	NP (0.7)	Hill et al (2011)[¶]
Fo37	MN	2004	NP (1.7)	Hill et al (2011)[¶]
FOB13/F180	OR	1994	P (2.5)	Hill et al (2011)[¶]
F174	CA	1995	NP (1.8)	Hill et al (2011)[¶]
Fob220a	CO	1998	P (3.3)	Hill et al (2011)[¶]
Fob257a	CO	1998	P (3.4)	Hill et al (2011)[¶]
H8	MT	2004	P (2.9)	Hill et al (2011)[¶]
F20[§]	OR	2001	P (2.5)	Hill et al (2011)[¶]

[†] Those isolates with an average disease severity rating of greater than 2 are considered to be pathogenic (P); isolates with an average disease severity rating less than 2 are non-pathogenic (NP) (Ruppel, 1991); isolates that were tested during multiple experiments are indicated with multiple pathogenicity designations; bold indicates isolates that had discrepancies in pathogenicity testing.

[‡] Source (or the location/researcher) that performed pathogenicity testing; USDA-ARS, Ft. Collins, CO (Kimberly Webb), USDA-ARS, East Lansing, MI (Linda Hanson), or previously reported in Hill et al (2011) at USDA-ARS, Ft. Collins, CO.

[§] Indicates single *F. avenaceum* isolate that was included as an out-group to anchor phylogenetic trees. [¶]Indicates isolates previously reported in Hill et al (2011) and included to root our results to published phylogenetic trees.

Table 2. Primer sequences and melting temperatures (TM) for polymerase chain reactions.

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')	Reference	TM
TEF-1a	EF1-ATGGGTAAGGA(A/G)GACAAGAC	EF2-GGA(G/A)GTACCAGT(G/C)ATCATGTT	O'Donnell et. al., 1998	59°C
ß-tubulin	C-GAGGAATTCCCAGACCGTATGATG	D-GCTGGATCCTATTCTTTGGGTCGAACAT	Koenraadt et. al., 1992	58°C
mtSSU	MS1-CAGCAGTCAAGAATATTAGTCAATG	MS2-GCGGATTATCGAATTAAATAAC	White et. al., 1990	52°C
Histone-3	H31a-ACTAAGCAGACCGCCCGCAGG	H31b-GCGGGCGAGCTGGATGTCCTT	Glass and Donaldson 1995	55°C

shaker at 100 rpm. Mycelia masses were collected by filtering through sterile cheese cloth, rinsed with de-ionized water, and then lyophilized at -50°C for 48 h. Lyophilized tissue was ground into a fine powder using a spatula, and DNA extracted using the Invitrogen Easy-DNA extraction kit (Carlsbad, CA) utilizing the protocol for small amounts of plant tissues.

DNA Amplification and Sequencing

Primers for PCR amplification of *TEF1-a, β-tubulin, mtSSU,* and H*3* were used as previously described (Table 2), as were corresponding PCR conditions (O'Donnell et al., 1998; Koenraadt et al., 1992; White et al., 1990; Glass and Donaldson, 1995). Fermentas brand (Glen Burnie, MD) *Taq* polymerase was used for all PCR amplifications. Briefly, one cycle of 94°C for 2 min followed by 32 cycles of 94°C for 45 sec, gene specific target melting temperatures (Tm) (Table 2) for 45 sec, and an extension cycle of 72°C for 1 min, followed by final extension cycle of 72°C for 5 min. PCR products were held at 4°C until they could be removed from a Mastercyler gradient thermo cycler (Eppendorf, Hamburg, Germany). All reactions were repeated at least twice. PCR amplicons were visualized on a 1.5% agarose gel and purified using either the Epoch Genecatch PCR Clean up kit (Sugarland, TX) or extracted from the agarose gels and purified using the Epoch Genecatch Gel Extraction Clean up kit (Sugarland, TX). Products were sequenced in both directions by Eurofins, MWG/Operon (Huntsville, AL).

Phylogenetic Analysis

Gene sequences from the amplified PCR products for each gene (*TEF1-a, β-tubulin, mtSSU*, and *H3*), were manually edited using Sequencher v. 4.1 (Gene Codes Corp). ClustalX v 1.83 (Thompson et al., 1997) was used to align sequences from all isolates for each gene. An individual data set was generated for each gene using the sequence data from all isolates for that gene. Parsimony bootstrap analysis was carried out in PAUP 4.0b10 (Swofford, 1999) with 1000 random stepwise replicates, the tree-bisection-reconnection branch-swapping procedure, and MULTREES off (Debry and Olmstead, 2000) for each gene data set. *F. avenaceum* isolate F20 was used as an outgroup to anchor all trees. Ambiguously aligned flanking sequences were not included in phylogenetic analyses. To determine the ability to combine the four individual gene datasets, parsimony analysis utilizing a partition-homogeneity test (Farris et al., 1995; Hill et al., 2011) was implemented using PAUP, with 1000 homogeneity replicates and MAXTREES set to 1000. Additionally, Bayesian MCMC phylogenetic analysis using MrBayes 3.0 (Ronquist and Huelsenbeck, 2003) was used to assess the combined data set and compare with the parsimony analysis. The General Time Reversible (GTR) model (Felsenstein, 2004) was used and a proportion of invari-

able sites and a gamma-shaped distribution of rates across sites utilizing two simultaneous chains of 1.5 x 10^7 generations and a sample frequency of 300, for a total of 50000 sample trees, were used as parameters from each chain replicate. The same parameters were applied for all four data partitions.

RESULTS AND DISCUSSION

F. oxysporum pathogenicity to sugarbeet

Of the 29 *F. oxysporum* isolates included in this study, 10 were considered to be pathogenic and either highly virulent or moderately virulent to sugarbeet (34%; Table 1). Only two of the isolates from Michigan or Minnesota were considered to be at least moderately virulent to sugarbeet (Table 1). A mean disease severity rating of 2, where plants are starting to show distinct Fusarium yellows symptoms (i.e. leaves showing interveinal yellowing) indicates that the inoculated isolate is capable of eliciting a pathogenic response in the host (Ruppel, 1991; Hanson and Hill, 2004). Therefore, isolates that had a mean disease severity rating of 2 or above were considered to be pathogenic to sugarbeet. To characterize virulence, pathogenic isolates with a rating of 2-3 are considered as moderately virulent and those with a rating of greater than 3-5 are highly virulent (Hanson and Hill, 2004). Many *F. oxysporum* f. sp. *betae* isolates with moderate or low virulence to sugarbeet have been reported to give variable disease severity ratings, which are not always significantly different from negative controls (water and/or uninoculated) over repeated experiments (Hanson and Hill, 2004; Hill et al., 2011). This is particularly evident when testing isolates in multiple locations, as we found here, where there may be environmental differences in growing and testing conditions (Stewart, 1931; Martyn et al., 1989; Ruppel, 1991; Hanson and Hill, 2004; Hill et al., 2011). In this study, there were two isolates (F08-10 and F08-13; Table 1) that had a discrepancy in pathogenicity at the two testing locations (Fort. Collins, CO and East Lansing, MI). Both of these isolates had disease severity ratings that ranged from 1.1 to 2.0 indicating that they may be moderately virulent isolates under appropriate testing conditions (Table 1). Experimental factors such as environment, soil medium or conditions, and plant growing conditions as well as the inherent variability of *F. oxysporum* f. sp. *betae*, can complicate the characterization of virulence and aggressiveness, and the determination of pathogenicity between laboratories. Additional studies to standardize environmental and testing factors, which influence *F. oxysporum* f. sp. *betae* virulence or Fusarium yellows symptom development should be undertaken to minimize these discrepancies, and used to develop a standard protocol for determining pathogenicity.

Ruppel (1991) reported that ~40% of *F. oxysporum* that were iso-

lated from symptomatic sugarbeet were indeed "pathogens" of the host. Hanson and Hill (2004) reported a slightly lower rate of recoverable pathogenic *F. oxysporum* isolates collected from symptomatic sugarbeet over the entire United States production region. Hanson and Hill (2004) also reported that ~55% of their pathogenic *F. oxysporum* isolates were considered highly virulent, and 44% moderately virulent. We found only 14% of the Michigan/Minnesota isolates tested in this study were pathogenic. Based on previous reports, we did not find that pathogenic *F. oxysporum* isolates from Michigan/Minnesota were being recovered at a higher rate and did not seem more virulent than those found in other sugarbeet production regions. This does not indicate that the *F. oxysporum* population in Michigan and Minnesota, which is displaying an increasing incidence of Fusarium yellows, is unique from other production regions. However, a more complete sampling of symptomatic sugarbeet from known infected fields may indicate an increased level of virulence and should be considered in the future.

Phylogenetic analysis of a set of *F. oxysporum* from symptomatic sugarbeet

Phylogenetic trees utilizing individual datasets for *H3* or *mtSSU* gene sequences, did not provide any additional resolution of genetic relatedness of *F. oxysporum* isolates from sugarbeet, than presented by Hill et al. (2011) utilizing *ITS, TEF1-a,* or *β-tubulin* (Fig. 1a-d). Each of the four gene trees identified the same putative primary groups (clades A-C) reported by Hill et al. (2011). *TEF1-a* resulted in the highest resolution among isolates, whereas mtSSU, *β-tubulin*, and finally *H3*, had decreasingly less resolution. Of the four datasets, *H3*, with the least resolution, indicated evidence of discordant groupings, particularly for some isolates. For example, Fo37 and F174, were found to group into clade C using *H3* but were grouped within clade B using *TEF1-a* , *β-tubulin*, and *mtSSU* (Fig1a-d). This incongruence is widespread among many fungi (Martin et al., 1999; Pantou et al., 2003; Mbofung et al., 2007). Previous research has shown that some isolates of *F. oxysporum* within the species complex, can have conflicting relationships depending on the gene being analyzed (O'-Donnell et al., 2009). O'Donnell et al (2009) speculated that some of this discordance may have resulted from either gene duplication with subsequent divergence or via horizontal gene transfer and/or introgressive hybridization events. One possibility is that *F. oxysporum* isolates obtained from sugarbeet may similarly contain members that have genetic contributions from more than one source. However, it is also possible that the discordance is due to other factors, such as variable numbers of sub-repeats or divergence of gene (O'Donnell, 2009). Future studies are needed to characterize the source of the discordant signal from the *H3* gene complex.

Assessment of the congruency of parsimony consensus indices,

Fig. 1. Parsimony bootstrap phylograms for each reference gene (a) *TEF1-α* (b) *β-tubulin* (c) *H3* and (d) *mtSSU*. Only Branches with bootstrap scores of 70% or higher are shown. *F. avenaceum* isolate (F20) was used as an out group for each phylogram. Isolates labeled with * have conflicting clade assignments when analyzed using *H3* relative to the clade designations when analyzed using other reference genes.

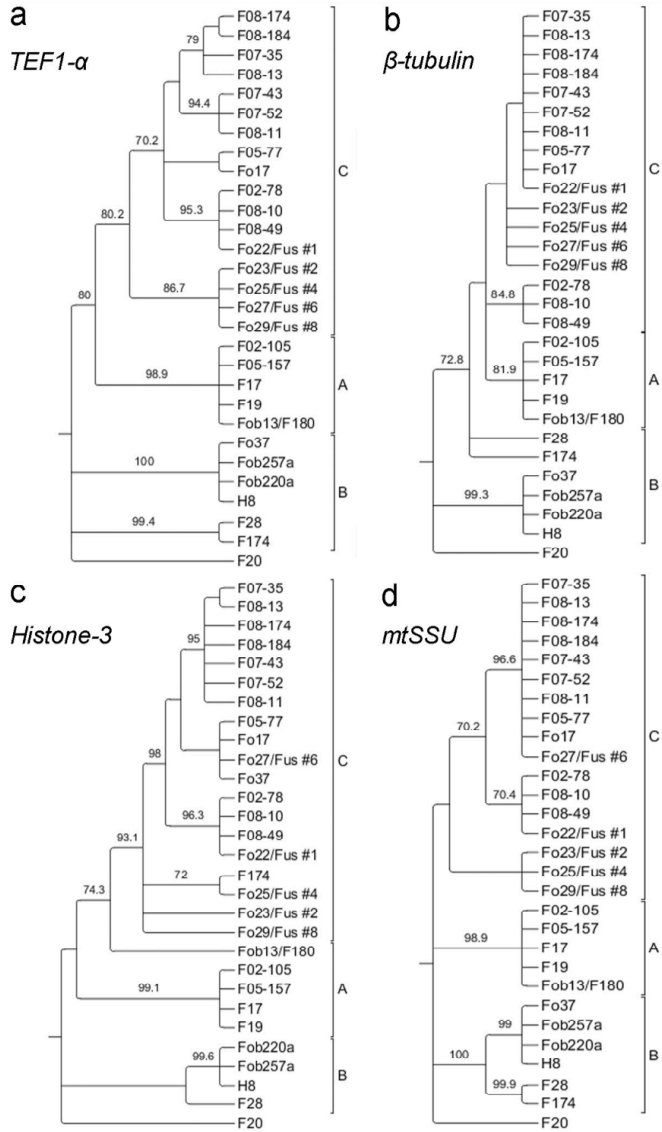
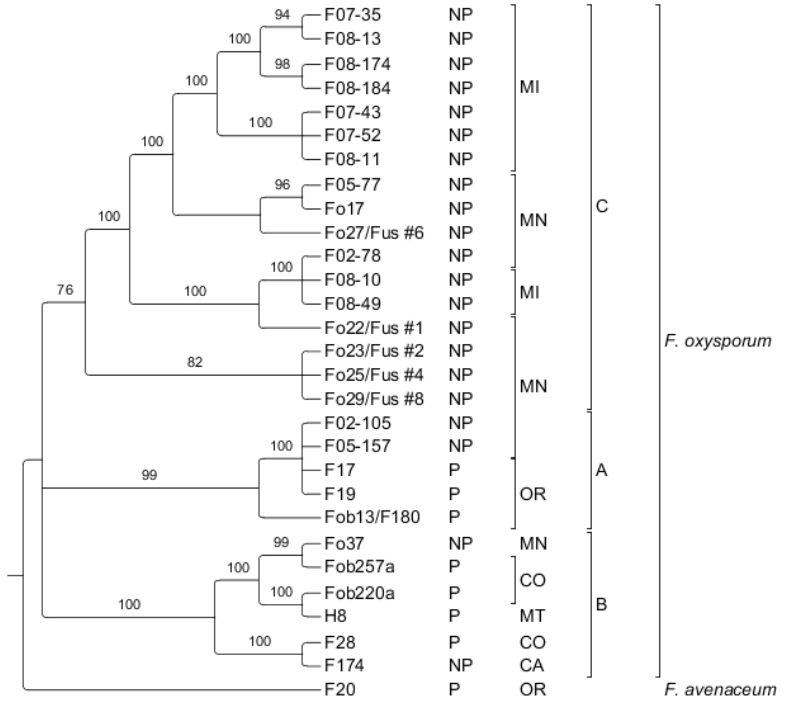


Fig. 2. Bayesian MCMC analysis of the combined data set from *TEF1-a, β-tubulin, mtSSU,* and *H3*. Only Bayesian posterior-probabilities (x100) above 50 are shown. *F. avenaceum* isolate (F20) was used as an out group for the phylogram. Pathogenicity and geographic origin for each isolate are shown. (NP) Non-pathogenic (P) Pathogenic.



using the partition-homogeneity test, (P=0.001) indicated that the *TEF-1a, β-tubulin, H3* and *MtSSU* consensus indices of the combined data sets are significantly heterogeneous and therefore could not be combined using parsimony bootstrap analysis. One explanation for this is the discordant groupings of some of the isolates within clade B (Fo37 and F174) of the *H3* dataset (Fig. 1c). Bayesian analysis therefore was used to analyze each gene as a separate data partition using the GTR model because this analysis does not require homogeneity of gene partitions. With this analysis, the three primary clades (A-C) again were supported (Fig. 2). The consensus analysis utilizing all four genes, does not suggest a link with pathogenicity to sugarbeet nor to geographic region, supporting the findings of Hill et al. (2011; Fig. 2). Isolates that represent the *F. oxysporum* population collected from symptomatic sugarbeet in the United States can be

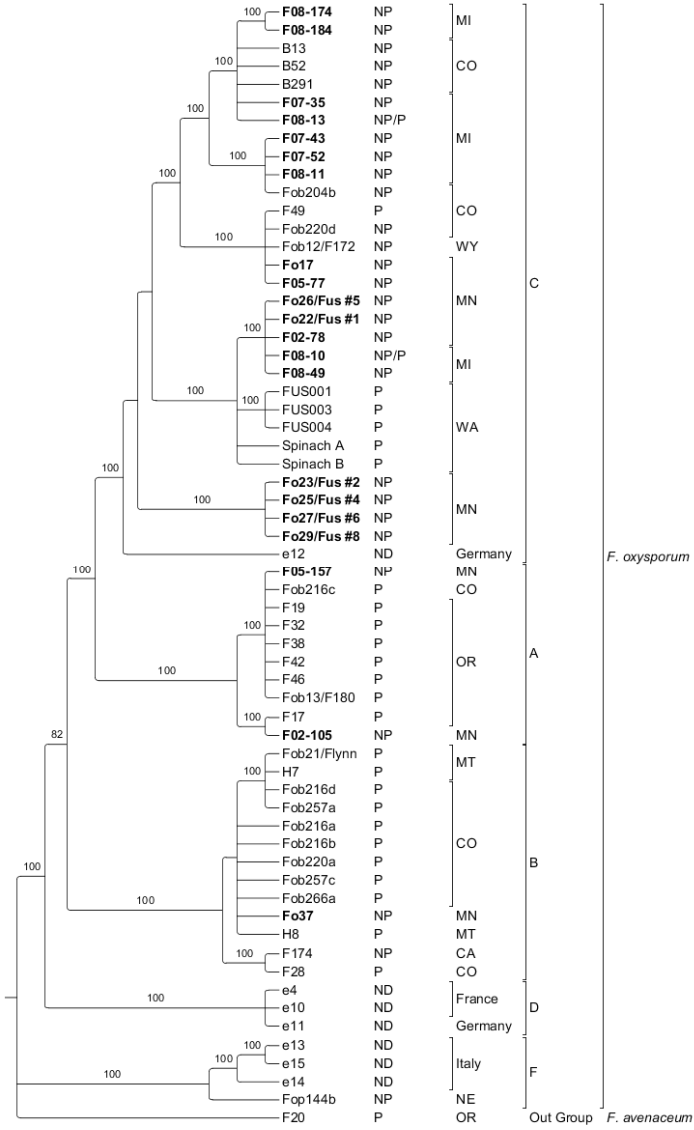
separated into three clades (A-B), regardless of production region that isolates were obtained. Adding additional isolates from a particular region, such as Michigan and Minnesota, did not strengthen weak correlations to geographic region previously seen by Hill et al (2011). While most of the Michigan and Minnesota isolates from this study grouped into clade C, several isolates from Minnesota fell into clade A, which primarily contains isolates from Oregon, and clade B (Fig. 2).

The continued inability to resolve pathogenic from non-pathogenic isolates, supports the conclusion that pathogenic *F. oxysporum* f. sp. *betae* isolates more likely evolved independently, multiple times. However due to the difficulty with assigning pathogenicity to some isolates (i.e. weakly virulent) and the influence of testing conditions and/or environment, it is possible that a genotype by environment interaction may be confounding phylogenetic associations. Correll (1991) hypothesized that endophytic, but non-pathogenic populations, of *F. oxysporum* may occur in a population. He speculates that this "basal" population would have a higher degree of diversity with many mutations towards virulence occurring among independent isolates. If such mutations occurred when an isolate was in close proximity to a susceptible host (i.e. the high sucrose environment of a sugar beet root), or under the correct environmental conditions, then this isolate could become pathogenic, favoring a more "opportunistic" pathogen population. It is clear that isolates with borderline pathogenic relationships require further study, particularly to characterize how environment (including host genotype) influences the *F. oxysporum* f. sp. *betae* population.

Because one gene dataset was not more informative than another in describing the *F. oxysporum* population from sugarbeet (Hill et al 2011, this work), we recommend use of only a single dataset (*TEF-1a*) for future studies. *TEF-1a* was chosen because it contains a high level of nucleotide diversity within the *F. oxysporum* species complex (O'Donnell et al., 1998; Baayen et al., 2000; O'Donnell et al., 2009) and is currently the primary dataset that is being used by the *F. oxysporum* research community for diagnostic identification to species, as well as phylogenetic analysis of the species complex (Geiser et al., 2004; Lievens et al., 2008). Utilizing only *TEF-1a*, we have built a phylogenetic tree, incorporating the published work of Hill et al (2011) with the findings reported here to show the diversity of the *F. oxyporum* population from sugarbeet (Fig. 3).

In sugarbeet, genetic resistance is the primary method of controlling Fusarium yellows (Hanson and Jacobsen, 2009) however, variability in the effectiveness of this resistance has been shown (MacDonald, 1975; Ruppel, 1991; Hanson et al., 2009). Due to the morphological, genetic, and phenotypic variability of *F. oxysporum* f. sp. *betae*, it is important that breeding programs screen with pathogenic isolates that represent the diversity found in the production re-

Fig. 3. Parsimony bootstrap analysis of the *TEF1-a* gene for the *F. oxysporum* population isolated from sugarbeet including those previously reported by Hill et al. (2011). Isolates in bold indicate new data being added from this work. Only branches with bootstrap scores of 50% or higher are shown. *F. avenaceum* isolate (F20) was used as an out group for each phylogram. Pathogenicity and geographic origin for each isolate are shown. (NP) Non-pathogenic (P) Pathogenic (ND) Not-determined.



gion of interest. For example, varieties for commercial production in Michigan and Minnesota should be screened with isolates that fall within clade C, but the addition of representative isolates from clade A is also recommended. Utilizing only a single gene (*TEF-1a*) the sugarbeet research community can continue to characterize the diversity of the *F. oxysporum* f. sp. *betae* population, and carefully select representative isolates for germplasm screening and cultivar deployment in the future.

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LITERATURE CITED

Armstrong, G.M., and J.K. Armstrong. 1981. Formae speciales and races of Fusarium oxysporum causing wilt diseases. p. 391-399. In P.E. Nelson, T.A. Toussoun, and R.J. Cook (ed.) Fusarium:Diseases, Biology, and Taxonomy. The Pennsylvania State University Press, University Park, PA.

Baayen, R.P., K. O'Donnell, P.J.M. Bonants, E. Cigelnik, L.P.N. Kroon, R.J.A. Roebroeck, and C. Waalwijk. 2000. Gene genealogies and AFLP analyses in the Fusarium oxysporum complex identify monophyletic and nonmonophyletic formae speciales causing wilt and rot diseases. Phytopathology 90:891-900. http://dx.doi.org/10.1094/PHYTO.2000.90.8.891

Balmas, V., Q. Migheli, B. Scherm, P. Garau, K. O'Donnell, G. Ceccherelli, S. Kang, and D.M. Geiser. 2010. Multilocus phylogenetics show high levels of endemic Fusaria inhabiting Sardinian soils (Tyrrhenian Islands). Mycologia 102:803-812. http://dx.doi.org/10.3852/09-201

Burlakoti, P. 2007. Fusarium species associated with sugarbeet grown in the Red River Valley: Pathogenicity, cultivar response, and baseline sensitivity to fungicides. MS North Dakota State University.

Correll, J.C. 1991. The relationship between formae speciales, races, and vegetative compatibility groups in Fusarium oxysporum. Phytopathology 81:1061-1064.

Cramer, R.A., P.F. Byrne, M.A. Brick, L. Panella, E. Wickliffe, and H.F. Schwartz. 2003. Characterization of Fusarium oxysporum isolates from common bean and sugar beet using pathogenicity assays and Random-amplified Polymorphic DNA markers. J. of Phytopathol. 151:352-360. http://dx.doi.org/10.1046/j.1439-0434.2003.00731.x

Debry, R.W., and R.G. Olmstead. 2000. A simulation study of reduced tree-search effort in bootstrap resampling analysis. Systemic Biol. 49:171-179. http://dx.doi.org/10.1080/10635150050207465

Donaldson, G.C., L.A. Ball, P.E. Axelrood, and N.L. Glass. 1995. Primer sets developed to amplify conserved genes from filamentous ascomycetes are useful in differentiating Fusarium species associated with conifers. App. Environ. Microbiol. 61:1331-1340.

Farris, J.S., M. Kallersjo, A.G. Kluge, and C. Bult. 1995. Testing of significance of incongruence. Cladistics 10:315-319. http://dx.doi.org/10.1111/j.1096-0031.1994.tb00181.x

Felsenstein, J. 2004. Inferring phylogenies. Sinauer Associates, Sunderland, Mass.

Franc, G. D., R. M. Harveson, E. D. Kerr, and B. J. Jacobsen, 2001. Disease Management Sugarbeet Production Guide. Lincoln, NE, University of Nebraska.

Geiser, D.M., M.M. Jimenez-Gasco, S. Kang, I. Makalowska, N. Veeraraghavan, T.J. Ward, N. Zhang, G.A. Kuldau, and K. O'Donnell. 2004. FUSARIUM-ID v. 1.0: A DNA sequence database for identifying Fusarium. Eur. J. Plant Pathol. 110:473-479. http://dx.doi.org/10.1023/B:EJPP.0000032386.75915.a0

Glass, N.L., and G.C. Donaldson. 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. App. Environ. Microbiol. 61:1323-1330.

Hanson, L.E. 2006. First Report of Fusarium yellows of sugar beet caused by Fusarium oxysporum in Michigan. Plant Dis. 90:1554. http://dx.doi.org/10.1094/PD-90-1554B http://dx.doi.org/10.1094/PD-90-0686A

Hanson, L.E., and A.L. Hill. 2004. Fusarium species causing Fusarium yellows oof sugarbeet. J. Sugar Beet Res. 41:163-178. http://dx.doi.org/10.5274/jsbr.41.4.163

Hanson, L.E., A.L. Hill, B.J. Jacobsen, and L. Panella. 2009. Response of sugar beet lines to isolates of Fusarium oxysporum f. sp. betae from the United States. J. Sugar Beet Res. 46:11-26. http://dx.doi.org/10.5274/jsbr.46.1.11

Hanson, L.E., and B.J. Jacobsen. 2009. Fusarium Yellows. p. 28-29. In R.M.Harveson, L.E. Hanson, and G.L. Hein (ed.) Compendium of Beet Diseases and Pests. American Phytopathological Society, St. Paul, Minnesota.

Harveson, R.M., and C.M. Rush. 1997. Genetic variation among Fusarium oxysporum isolates from sugarbeet as determined by vegetative compatibility. Plant Dis. 81:85-88. http://dx.doi.org/10.1094/PDIS.1997.81.1.85

Hill, A.L., P.A. Reeves, R.L. Larson, A.L. Fenwick, L.E. Hanson, and L. Panella. 2011. Genetic variability among isolates of Fusarium oxysporum from sugar beet. Plant Pathol. 60:496-505. http://dx.doi.org/10.1111/j.1365-3059.2010.02394.x

Khan, M., C. A. Bradley, and C. E. Windels, Fusarium yellows of sugarbeet. 2003. PP-1247. Univ. Minn. Ext. Serv. and North Dakota State Univ. Ext. Serv.

Koenraadt, H., S.C. Somerville, and A.L. Jones. 1992. Characterization of mutations in the Beta-tubulin gene of benomyl-resistant field strains of Venturia inaequalis and other plant pathogenic fungi. Phytopathology 82:1348-1354. http://dx.doi.org/10.1094/Phyto-82-1348

Leslie, J.F., and B.A. Summerell. 2006. The Fusarium Laboratory Manual. First ed. Blackwell Publishing, Ames, Iowa. http://dx.doi.org/10.1002/9780470278376

Lievens, B., M. Rep, and B.P. Thomma. 2008. Recent developments in the molecular discrimination of formae speciales of Fusarium oxysporum. Pest Manag. Sci. 64:781-788. http://dx.doi.org/10.1002/ps.1564

MacDonald, J.D. 1975. The pathogenicity, host specificity, and seed association of the sugarbeet stalk blight pathogen Fusarium oxysporum f. sp. betae. MS University of California, Davis.

Martin, F., M. Selosse, and F. Le Tacon. 1999. The nuclear rDNA intergenic spacer of the ectomycorrhizal basidiomycete Laccaria bicolor: structural analysis and allelic polymorphism. Microbiology 145:1605-1611. http://dx.doi.org/10.1099/13500872-145-7-1605

Martyn, R.D., C.M. Rush, C.A. Biles, and E.H. Baker. 1989. Etiology of a root rot disease of sugar beet in Texas. Plant Dis. 73:879-884. http://dx.doi.org/10.1094/PD-73-0879

Mbofung, G.Y., S.G. Hong, and B.M. Pryor. 2007. Phylogeny of *Fusarium oxysporum* f. sp. lactucae inferred from mitochondrial small subunit, elongation factor 1-a, and nuclear ribosomal intergenic spacer sequence data. Phytopathology 97:87-98. http://dx.doi.org/10.1094/PHYTO-97-0087

Nitschke, E., M. Nihlgard, and M. Varrelmann. 2009. Differentiation of eleven Fusarium spp. isolated from sugar beet, using restriction fragment analysis of a polymerase chain reactionamplified translation elongation factor 1a gene fragment. Phytopathology 99:921-929. http://dx.doi.org/10.1094/PHYTO-99-8-0921

O'Donnell, K., H.C. Kistler, E. Cigelnik, and R.C. Ploetz. 1998. Multiple evolutionary origins of the fungus causing Panama disease of banana: Concordant evidence fom nuclear and mitochondrial gene genealogies. Proc. Natl. Acad. Sci. 95:2044-2049. http://dx.doi.org/10.1073/pnas.95.5.2044

O'Donnell, K., D.A. Sutton, A. Fothergill, D. McCarthy, M.G. Rinaldi, M.E. Brandt, N. Zhang, and D.M. Geiser. 2008. Molecular phylogenetic diversity, multilocus haplotype nomenclature, and in vitro antifungal resistance within the Fusarium solani species complex. J. Clin. Microbiol. 46:2477-2490. http://dx.doi.org/10.1128/JCM.02371-07

O'Donnell, K., C. Gueidan, S. Sink, P.R. Johnston, P.W. Crous, A. Glenn, R. Riley, N.C. Zitomer, P. Colyer, C. Waalwijk, T.V.D. Lee, A. Moretti, S. Kang, H.S. Kim, D.M. Geiser, J.H. Juba, R.P. Baayen, M.G. Cromey, S. Bithell, D.A. Sutton, K. Skovgaard, R. Ploetz, H. Corby Kistler, M. Elliott, M. Davis, and B.A.J. Sarver. 2009. A two-locus DNA sequence database for typing plant and human pathogens within the Fusarium oxysporum species complex. Fungal Genet. Biol. 46:936-948. http://dx.doi.org/10.1016/j.fgb.2009.08.006

Panella, L., and R.T. Lewellen. 2005. Objectives of sugar beet breeding: Fusarium yellows. p. 93-95. In E.Biancarki, L.G. Campbell, G.N. Skaracis, and M. De Biaggi (ed.) Genetics and Breeding of Sugar Beet. Science Publishers, Inc., Enfield, New Hampshire.

Panella, L., E.G. Ruppel, and R.J. Hecker. 1995. Registration of four multigerm sugar beet germplasm resistant to Rhizoctonia root rot: FC716, FC717, FC718, and FC719. Crop Sci. 35:291-292. http://dx.doi.org/10.2135/cropsci1995.0011183X003500010071x

Pantou, M.P., A. Mavridou, and M.A. Typas. 2003. IGS sequence variation, group-I introns and the complete nuclear ribosomal DNA of the entomopathogenic fungus Metarhizium: excellent tools for isolate detection and phylogenetic analysis. Fungal Genet. Biol. 38:159-174.http://dx.doi.org/10.1016/S1087-1845(02)00536-4

Rivera, V., J. Rengifo, M. Khan, D.M. Geiser, M. Mansfield, and G. Secor. 2008. First Report of a novel Fusarium species causing yellowing decline of sugar beet in Minnesota. Plant Dis. 92:1589. http://dx.doi.org/10.1094/PDIS-92-11-1589B

Ronquist, F., and J.P. Huelsenbeck. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19:1572-1574. http://dx.doi.org/10.1093/bioinformatics/btg180

Ruppel, E.G. 1991. Pathogenicity of Fusarium spp. from diseased sugarbeets and variation among sugarbeet isolates of F. oxysporum. Plant Dis. 75:486-489. http://dx.doi.org/10.1094/PD-75-0486

Schneider, C.L., and E.D. Whitney. 1986. Fusarium yellows. p. 18. In E.D. Whitney, and J.E. Duffus (ed.) Compendium of Beet Diseases and Insects. APS Press, St. Paul, MN.

Snyder, W.C., and H.N. Hansen. 1940. The species concept in Fusarium. Am. J. Botany 27:64-67. http://dx.doi.org/10.2307/2436688

Stewart, D. 1931. Sugar-beet yellows caused by Fusarium conglutinans var. betae. Phytopathology 21:59-70.

Swofford, D. 1999. PAUP: Phylogenetic Analysis Using Parsimony. Version 4.Ob1. Sinauer Associates, Sunderland, MA, USA.

Thompson, J.D., T.J. Gibson, F. Plewniak, F. Jeanmougin, and D.G. Higgins. 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25:4876-4882. http://dx.doi.org/10.1093/nar/25.24.4876

White, T.J., T. Bruns, S. Lee, and J. Taylor. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. p. 315-322. In M.A.Innis, G.H. Gelfand, J.J. Sninsky, and T.J. White (ed.) PCR Protocols: A Guide to Methods and Applications. Academic Press, Inc.

Windels, C.E., J.R. Brantner, C.A. Bradley, and M.F.R. Khan. 2005. First Report of Fusarium oxysporum causing yellows on sugar beet in the Red River Valley of Minnesota and North Dakota. Plant Dis. 89:341. http://dx.doi.org/10.1094/PD-89-0341B