Diagnostic qPCR Assay to Detect *Fusarium brasiliense*, a Causal Agent of Soybean Sudden Death Syndrome and Root Rot of Dry Bean

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Abstract

Species within clade 2 of the *Fusarium solani* species complex (FSSC) are significant pathogens of dry bean (*Phaseolus vulgaris*) and soybean (*Glycine max*), causing root rot and/or sudden death syndrome (SDS). These species are morphologically difficult to distinguish and often require molecular tools for proper diagnosis to a species level. Here, a TaqMan probe-based quantitative PCR (qPCR) assay was developed to distinguish *Fusarium brasiliense* from other closely related species within clade 2 of the FSSC. The assay displays high specificity against close relatives and high sensitivity, with a detection limit of 100 fg. This assay was able to detect *F. brasiliense* from purified mycelia, infected dry bean roots, and soil samples throughout Michigan. When multiplexed with an existing qPCR assay specific to *Fusarium virguliforme*,

accurate quantification of both *F. brasiliense* and *F. virguliforme* was obtained, which can facilitate accurate diagnoses and identify coinfections with a single reaction. The assay is compatible with multiple qPCR thermal cycling platforms and will be helpful in providing accurate detection of *F. brasiliense*. Management of root rot and SDS pathogens in clade 2 of the FSSC is challenging and must be done proactively, because no midseason management strategies currently exist. However, accurate detection can facilitate management decisions for subsequent growing seasons to successfully manage these pathogens.

Keywords: field crops, fungi, oilseeds and legumes, pathogen detection

The *Fusarium solani* species complex (FSSC) is composed of three major clades designated 1, 2, and 3 (Chitrapalam and Nelson 2016; O'Donnell 2000). Some *Fusarium* species within clade 2 are soilborne plant pathogens that cause root rot of soybean (*Glycine max*) and dry bean (*Phaseolus vulgaris*) (Aoki et al. 2014; Chitrapalam and Nelson 2016). Macroscopic and microscopic morphological features of the species within clade 2 are very challenging to differentiate, because all produce similar pigments, and conidia lengths and widths overlap (Aoki et al. 2003). Of the species within this clade, six are known to cause sudden death syndrome (SDS) on soybean (Aoki et al. 2003, 2005, 2012a, b; Tewoldemedhin et al. 2017). In South America, the SDS-causing pathogens include *Fusarium virguliforme* O'Donnell & T Aoki, *Fusarium brasiliense* T Aoki & O'Donnell, *Fusarium tucumaniae*, and *Fusarium crassistipitatum*, whereas in North America, SDS is caused by *F. virguliforme* and *F. brasiliense* (Wang et al. 2019b). *Fusarium brasiliense* and an undescribed *Fusarium* sp. cause soybean SDS in South Africa (Tewoldemedhin et al. 2017). The sixth SDS-causing species, *Fusarium azukicola*, has only been isolated from adzuki bean (*Vigna angularis*), but when inoculated on soybeans, it caused foliar SDS symptoms (Aoki et al. 2012b).

Soybean SDS is an annual threat in both the United States and Brazil (Aoki et al. 2003; O'Donnell et al. 2010). These countries dominate soybean production in North and South America, respectively, both producing an estimated 120 million metric tons of soybeans in 2017 to 2018 (USDA 2018). Managing these SDS-causing FSSC

clade 2 species is challenging, because few fungicides demonstrate efficacy (Kandel et al. 2018; Sang et al. 2018; Wang et al. 2017), genetic resistance is partial (Chang et al. 2018), and crop rotation practices take up to 3 or 4 years of nonhost crops to lower SDS pressure (Leandro et al. 2018). However, some SDS-causing species, like *F. tucumaniae*, show increased sensitivity to certain chemistries (Sang et al. 2018), and therefore, it is important to know the species present to recommend proper management.

Monitoring the spread of fungal pathogens is also important to determine if management strategies are effective. Fungal pathogens can be spread through the transfer of infected materials, shared use of contaminated field equipment, and wind-blown spores. The mechanism of *F. virguliforme* spread remains unknown, although the sexual stage has never been observed, and only one mating type has been detected (Hughes et al. 2014). Therefore, spread through the discharge of sexual ascospores seems unlikely. A single mating type for *F. brasiliense* was identified in South Africa (Tewoldemedhin et al. 2017), but both mating types have been found in South America (Hughes et al. 2014) and Michigan isolates (K. A. Oudman et al., unpublished data). In addition, *F. brasiliense* isolates with different mating types were identified in the same field (K. A. Oudman et al., unpublished data). This indicates that conventional fungal sexual reproduction is probable in *F. brasiliense*, providing an opportunity for the pathogen to produce perithecia and spread through the discharge of sexual ascospores into the air. Only one isolate of *F. brasiliense* had been identified in the United States before 2014 from an unknown host in California (Aoki et al. 2005), and therefore, it is unlikely that it has spread from California to Michigan without being detected in soybean-growing states in between. Population genetic studies of *F. virguliforme* suggest that it originated in South America and spread to North America (Wang 2016). It may be possible that *F. brasiliense* also originated in South America and spread to the United States through unknown mechanisms, but a native Michigan population of *F. brasiliense* cannot be ruled out.

Because both soybeans and dry beans are hosts for *Fusarium* root rot pathogens, accurate identification of these species in these fields is critical to prevent significant inoculum increases, severe yield losses, and potential spread to neighboring fields. Accurate identification of *F. brasiliense* and monitoring its spread throughout the United States will allow growers to implement the few successful

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Funding: This work was funded in part by the Michigan Soybean Promotion Committee, the Michigan Dry Bean Commission, the Michigan Department of Agriculture and Rural Development, Bayer CropSciences, the Syngenta Agricultural Scholarship, and Project GREEN.

The author(s) declare no conflict of interest.

Accepted for publication 9 July 2019.

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management strategies available. Additionally, with consumer trends increasing the demand for non-genetically modified foods, many dry bean growers are beginning to rotate non-genetically modified soybeans with dry beans (B. Glass, *personal communication*), potentially leading to even more significant inoculum increases. Our primary objective was to develop a highly specific and sensitive quantitative PCR (qPCR) assay to detect *F. brasiliense*. The second objective was to validate the performance of this qPCR assay in detecting *F. brasiliense* from purified mycelia, infected plant roots, and soil. Also, we examined the compatibility of this assay with a *F. virguliforme* qPCR assay for multiplexing purposes, which provides fast and efficient diagnosis of closely related species or the presence of coinfections.

Materials and Methods

Assay design. The intergenic spacer (IGS) region of the ribosomal RNA gene has been used to distinguish species within the FSSC (Wang et al. 2011), including SDS- and brown root rot-causing fusaria (O'Donnell et al. 2010), and it was used as the target locus for this assay as in other qPCR assays (Chilvers et al. 2007; Okubara et al. 2013; Wang et al. 2015). IGS sequences from species within clade 2 of the FSSC were obtained from the NCBI: *F. azukicola* GenBank number JQ670149; *F. brasiliense* GenBank numbers KF706657 and FJ919512; *Fusarium cuneirostrum* GenBank numbers FJ919511 and FJ919548; *F. crassistipitatum* GenBank numbers FJ919521 and FJ919554; *Fusarium phaseoli* GenBank numbers FJ919498, FJ919510, and FJ919500; *F. tucumaniae* GenBank numbers FJ919507 and FJ919515; and *F. virguliforme* GenBank numbers FJ919499 and FJ919557. Sequences were aligned in MEGA7 (Kumar et al. 2016) using the MUSCLE algorithm (Edgar 2004).

A TaqMan probe using locked nucleic acids was designed to capitalize on the presence of two single-nucleotide polymorphisms (SNPs) specific to *F. brasiliense* within eight base pairs (bp) of each other (Fig. 1). Primer and probe sequences are reported in Table 1. Each qPCR reaction was performed with two technical replicates and contained 10 µl of 2× TaqMan Universal real-time PCR master mix (1× final concentration; Applied Biosystems), 250 nM Fb_Prb1, 500 nM Fb_F1 primer, 500 nM Fb_F2 primer, 400 ng of bovine serum albumin (BSA), 2 µl of DNA template, and molecular-grade

water up to a total reaction volume of 20 µl. All assays were performed on the StepOnePlus real-time PCR system (v2.3; Applied Biosystems), and they were set up in clear MicroAmp 96-well plates (Thermo-Fisher Scientific), except for multiplexed assays. Multiplexed reactions were performed with two technical replicates, each containing 10 µl of 2× TaqMan Universal real-time PCR master mix (Applied Biosystems), 250 nM Fb_Prb1, 500 nM Fb_F1 primer, 500 nM Fb_F2 primer, 100 nM FvPrb3, 500 nM F6-3, 500 nM R6, 400 ng of BSA, 2 µl of each DNA template, and molecular-grade water up to a total reaction volume of 20 µl.

qPCR assay specificity. *Fusarium* isolates used for testing the assay specificity were either collected from counties throughout Michigan or requested from the U.S. Department of Agriculture Agricultural Research Service (ARS) Culture Collection (NRRL) (Table 2). Isolates collected from Michigan were obtained from root tissues of infected soybean or dry bean plants. Briefly, plants displaying stunting or foliar SDS symptoms from fields throughout Michigan were uprooted and transported to the laboratory, where soil was thoroughly washed off roots. Root sections displaying severe discoloration and rot were soaked in 5% bleach solution (0.4% NaOCl) for 4 min, rinsed thoroughly in sterile deionized water for 4 min, blotted dry with a sterile paper towel, and plated on WMS agar (2% agar, 15 µg/ml metalaxyl, and 300 µg/ml streptomycin) to select for fungal growth. Plates were incubated for 7 days at room temperature and observed under a dissecting microscope (Leica Microsystems) for the formation of sporodochia. Using an insect pin, macroconidia from a sporodochium were transferred, spread on fresh WMS agar, and allowed to grow for 24 h. Finally, a single germinating macroconidium was aseptically transferred onto full-strength potato dextrose agar (Neogen). Isolates were stored at room temperature on synthetic nutrient agar slants (1 g/liter KH₂PO₄, 1 g/liter KNO₃, 0.5 g/liter MgSO₄, 0.5 g/liter KCl, 0.2 g/liter glucose, 0.2 g/liter sucrose, and 2% agar) in scintillation vials (Research Products International) for long-term storage.

Macroconidia from pure cultures were inoculated into potato dextrose broth and allowed to grow at room temperature for 48 h with shaking at 100 rpm. DNA was extracted with two similar methods. For testing specificity at a standard 5 ng per reaction, mycelia were

Fig. 1. Sequence alignment of 149 base pairs of the intergenic spacer (IGS) of species within clade 2 of the *Fusarium solani* species complex. Primers (blue) and probe sequences (green) are displayed as arrows at their annealing location within the IGS. Asterisks represent locked nucleic acids (LNA) designed in the probe.

filtered from broth with two layers of Miracloth (Millipore Sigma) and rinsed with 200 ml of sterile deionized water. The fresh rinsed mycelia were immediately transferred to a sterile, prechilled mortar and ground with a pestle in the presence of liquid nitrogen. After ~1 min of tissue disruption, the ground mycelia were immediately transferred into microcentrifuge tubes containing a DNA extraction buffer (200 mM Tris-HCl, 1 M NaCl, 25 mM EDTA, 25 mM sodium dodecyl sulfate, and 100 µg of RNaseA) and incubated for 30 min at room temperature, with gentle inversions every 5 min. Proteinase K (200 µg) was added to each sample, and they were incubated at room

temperature for 20 min, with gentle inversions every 5 min. The samples were chilled on ice for 5 min followed by an addition of 0.2 vol of ice-cold 5 M potassium acetate and another 5-min incubation; then, they were centrifuged at 5,000 × g for 12 min at 4°C. The supernatant was transferred to fresh tubes, and an equal volume of 25:24:1 phenol:chloroform:isoamyl alcohol (Millipore Sigma) was added, mixed gently by inversion, and centrifuged. The phenol:chloroform step was repeated followed by an additional step using chloroform only (Millipore Sigma). The DNA was precipitated by adding 0.1 vol of 5 M NaCl and 1 vol of isopropanol, incubated on ice for

Table 1. Primers and probes used in this study

Primer/probe name ^a	Sequence (5' to 3')	Length (base pairs)	Temperature (°C) ^b	Reference
Fb_F2	AGGTCAGATTTGGTATAGGTTAGGTGAGA	29	67.4	This study
Fb_R2	CGGACCATCCGTCTGGGAATTT	22	66.3	This study
Fb_Prb1	5HEX-TGGGATGCCCT+AATTTT+ACGG-3IABkFQ	22	64.7	This study
F6-3	GTAAGTGAGATTTAGTCTAGGTTAGGTGAC	30	57.8	Wang et al. 2015
R6	GGGACCACCTACCCTACACCTACT	24	59.6	Wang et al. 2015
FvPrb-3	6FAM-TTTGGTCTAGGTTAGGCCG-MGBNFQ	19	70.0	Wang et al. 2015

^a Probes contain 5' fluorophores "5HEX" or "6FAM" and 3' quenchers "3IABkFQ" or "MGBNFQ."

^b Calculated with the OligoAnalyzer tool from IDT using the default quantitative PCR parameter settings.

Table 2. Panel of isolates used to determine specificity of *Fusarium brasiliense* quantitative PCR assay

Isolate ^a	Species ^b	Host	Location isolated	Assay result ^c	Ct with 5 ng of starting DNA ^d
54361	<i>Fusarium azukicola</i>	<i>Vigna angularis</i>	Obihiro, Hokkaido, Japan	–	n.d.
54362	<i>Fusarium azukicola</i>	<i>Vigna angularis</i>	Obihiro, Hokkaido, Japan	–	n.d.
34938	<i>Fusarium brasiliense</i>	Unknown	Vila Maria, Rio Grande do Sul, Brazil	+	14.92
22678	<i>Fusarium brasiliense</i>	<i>Phaseolus vulgaris</i>	California, U.S.A.	+	
MI-Mtc-C1	<i>Fusarium brasiliense</i>	<i>Glycine max</i>	Montcalm County, MI, U.S.A.	+	14.21
MI-Mtc-A13	<i>Fusarium brasiliense</i>	<i>Glycine max</i>	Montcalm County, MI, U.S.A.	+	14.43
F-14-12	<i>Fusarium brasiliense</i>	<i>Phaseolus vulgaris</i>	Ingham County, MI, U.S.A.	+	
F-14-42	<i>Fusarium brasiliense</i>	<i>Phaseolus vulgaris</i>	Montcalm County, MI, U.S.A.	+	14.71
F-14-44	<i>Fusarium brasiliense</i>	<i>Phaseolus vulgaris</i>	Montcalm County, MI, U.S.A.	+	
F-15-33	<i>Fusarium brasiliense</i>	<i>Phaseolus vulgaris</i>	Montcalm County, MI, U.S.A.	+	
F-15-101	<i>Fusarium brasiliense</i>	<i>Phaseolus vulgaris</i>	Huron County, MI, U.S.A.	+	
F-15-102	<i>Fusarium brasiliense</i>	<i>Phaseolus vulgaris</i>	Huron County, MI, U.S.A.	+	
F-15-144	<i>Fusarium brasiliense</i>	<i>Phaseolus vulgaris</i>	Montcalm County, MI, U.S.A.	+	
F-15-158	<i>Fusarium brasiliense</i>	<i>Phaseolus vulgaris</i>	Saginaw County, MI, U.S.A.	+	14.47
F-15-162	<i>Fusarium brasiliense</i>	<i>Phaseolus vulgaris</i>	Saginaw County, MI, U.S.A.	+	
F-15-174	<i>Fusarium brasiliense</i>	<i>Phaseolus vulgaris</i>	Gratiot County, MI, U.S.A.	+	
F-15-192	<i>Fusarium brasiliense</i>	<i>Phaseolus vulgaris</i>	Michigan, U.S.A.	+	
F-16-21	<i>Fusarium brasiliense</i>	<i>Glycine max</i>	Montcalm County, MI, U.S.A.	+	
F-16-59	<i>Fusarium brasiliense</i>	<i>Glycine max</i>	Montcalm County, MI, U.S.A.	+	13.94
F-16-118	<i>Fusarium brasiliense</i>	<i>Phaseolus vulgaris</i>	Saginaw County, MI, U.S.A.	+	
F-16-119	<i>Fusarium brasiliense</i>	<i>Phaseolus vulgaris</i>	Saginaw County, MI, U.S.A.	+	
F-16-124	<i>Fusarium brasiliense</i>	<i>Phaseolus vulgaris</i>	Saginaw County, MI, U.S.A.	+	
F-16-125	<i>Fusarium brasiliense</i>	<i>Phaseolus vulgaris</i>	Saginaw County, MI, U.S.A.	+	
F-16-127	<i>Fusarium brasiliense</i>	<i>Phaseolus vulgaris</i>	Saginaw County, MI, U.S.A.	+	
F-16-128	<i>Fusarium brasiliense</i>	<i>Phaseolus vulgaris</i>	Saginaw County, MI, U.S.A.	+	
F-16-131	<i>Fusarium brasiliense</i>	<i>Phaseolus vulgaris</i>	Saginaw County, MI, U.S.A.	+	
F-16-136	<i>Fusarium brasiliense</i>	<i>Phaseolus vulgaris</i>	Saginaw County, MI, U.S.A.	+	
F-16-137	<i>Fusarium brasiliense</i>	<i>Phaseolus vulgaris</i>	Saginaw County, MI, U.S.A.	+	14.52
31949	<i>Fusarium crassispitatum</i>	<i>Glycine max</i>	Cristalina, Goias, Brazil	–	n.d.
46170	<i>Fusarium crassispitatum</i>	<i>Glycine max</i>	Las Lajitas, Salta, Argentina	–	
46175	<i>Fusarium crassispitatum</i>	<i>Glycine max</i>	Las Lajitas, Salta, Argentina	–	
F-14-52	<i>Fusarium cuneirostrum</i>	<i>Phaseolus vulgaris</i>	Montcalm County, MI, U.S.A.	–	n.d.
Fsp3	<i>Fusarium cuneirostrum</i>	<i>Phaseolus vulgaris</i>	Uganda, Africa	–	

(Continued on next page)

^a Isolate identifications with only numbers were obtained from the U.S. Department of Agriculture Agricultural Research Service (ARS) Culture Collection (NRRL). All other isolates were obtained by J. L. Jacobs and M. I. Chilvers.

^b *Fusarium solani* species complex 5 (FSSC 5) is named *Fusarium solani*, whereas FSSC 11 is an unnamed species within clade 3.

^c + indicates positive amplification, whereas – indicates no amplification.

^d Ct, cycle threshold; n.d., not determined.

10 min, and pelleted at $10,000 \times g$ in a refrigerated centrifuge at 4°C . The pelleted DNA was washed twice with freshly prepared 80% ethanol and resuspended in 10 mM Tris buffer (pH 8.0) overnight. DNA quality was determined via 260/280- and 260/230-nm readings obtained from a Nanodrop 1000 (Thermo-Fisher Scientific), and quantity was determined via the Qubit dsDNA BR Assay Kit (Thermo-Fisher Scientific). An aliquot of DNA for each strain was diluted to 2.5 ng/ μl , with 2 μl used in qPCR assays for specificity testing (Fig. 2). For testing specificity of other isolates at various starting DNA concentrations (Table 2), mycelia were harvested with sterilized wooden stirrers (STR Supplies Inc.) into sterile 1.5-ml tubes. The tube was plugged with a sterile cotton ball, and mycelia were lyophilized for 48 h. Approximately 20 mg of lyophilized mycelia were ground with three to five 2-mm glass beads and subjected to the same extraction protocol as described above.

qPCR assay sensitivity. Extracted DNA from four *F. brasiliense* isolates was tested for sensitivity via serial dilutions. Concentrations tested were 10-fold dilutions beginning with 1,000,000 fg (1 ng) of *F. brasiliense* DNA to 10 fg (Fig. 3A). The cycle threshold (Ct) values for each concentration were averaged across isolates, because ribosomal DNA copy number can vary among strains of the same species (Balajee et al. 2009; Wang et al. 2015). The qPCR efficiency was calculated by finding the line of best fit on a plot with the logarithm of the DNA concentrations and their respective Ct value (Fig. 3B and Table 3). The limit of detection was determined by samples showing >95% amplification at the lowest DNA concentration tested (Bustin et al. 2009).

Assay validation. A field site in Ingham County, MI was planted with a randomized complete block design containing two dry bean cultivars (Zenith and Red Hawk) and three treatments (noninoculated, *F. brasiliense* inoculated, and *F. oxysporum* inoculated), with

four plot replicates per cultivar $\times$ treatment combination. Each plot was 6.1-m (20-ft) long, with four rows of plants with 76-cm (30-inch) row spacing. The plots were planted on 8 June 2018 at 172,100 seeds ha^{-1} for Red Hawk and 193,700 seeds ha^{-1} for Zenith using a cone planter. Inoculum was produced on autoclaved sorghum grains as

Fig. 2. Amplification results of 5 ng of DNA of select isolates across different species within the *Fusarium solani* species complex (FSSC). Isolates represented are *Fusarium azukicola* isolates NRRL 54361 and 54362; *Fusarium brasiliense* isolates F-14-42, F-15-158, F-16-137, NRRL 34938, and MI-Mtc-C1; *Fusarium solani* (FSSC 5) isolates MI-Mtc-B10 and F-15-118; *Fusarium crassistipitatum* isolate NRRL 31949; no template (water) control; *Fusarium cuneirostrum* F-14-52; *Fusarium phaseoli* isolates MI-Mtc-A12 and NRRL 22411; *Fusarium tucumaniae* isolates NRRL 43334 and 31777; and *Fusarium virguliforme* isolates DB P28 R13 and Vb-2a.

Table 2. (Continued from previous page)

Isolate ^a	Species ^b	Host	Location isolated	Assay result ^c	Ct with 5 ng of starting DNA ^d
F5	<i>Fusarium oxysporum</i>	<i>Solanum tuberosum</i>	Michigan, U.S.A.	–	
F-16-16	<i>Fusarium phaseoli</i>	<i>Glycine max</i>	Montcalm County, MI, U.S.A.	–	
MI-Mtc-A12	<i>Fusarium phaseoli</i>	<i>Glycine max</i>	Montcalm County, MI, U.S.A.	–	n.d.
22158	<i>Fusarium phaseoli</i>	<i>Phaseolus vulgaris</i>	Rockville, MD, U.S.A.	–	
22276	<i>Fusarium phaseoli</i>	<i>Phaseolus vulgaris</i>	Rockville, MD, U.S.A.	–	
MI-Mtc-A4	<i>Fusarium phaseoli</i>	<i>Glycine max</i>	Montcalm County, MI, U.S.A.	–	
22411	<i>Fusarium phaseoli</i>	<i>Phaseolus vulgaris</i>	California, U.S.A.	–	n.d.
36549	<i>Fusarium proliferatum</i>	<i>Gladiolus</i> sp.	The Netherlands	–	
MI-Mtc-C3FS (Wht)	FSSC 11	<i>Glycine max</i>	Montcalm County, MI, U.S.A.	–	
MI-Mtc-A2FS	FSSC 11	<i>Glycine max</i>	Montcalm County, MI, U.S.A.	+	21.13
F-15-118	<i>Fusarium solani</i> (FSSC 5)	<i>Phaseolus vulgaris</i>	Montcalm County, MI, U.S.A.	–	n.d.
MI-Mtc-C17FS	<i>Fusarium solani</i> (FSSC 5)	<i>Glycine max</i>	Montcalm County, MI, U.S.A.	–	
MI-Mtc-B16FS	<i>Fusarium solani</i> (FSSC 5)	<i>Glycine max</i>	Montcalm County, MI, U.S.A.	–	
MI-Mtc-B10	<i>Fusarium solani</i> (FSSC 5)	<i>Glycine max</i>	Montcalm County, MI, U.S.A.	+	30.43
31777	<i>Fusarium tucumaniae</i>	<i>Glycine max</i>	Vila Maria, Rio Grande do Sul, Brazil	–	n.d.
43334	<i>Fusarium tucumaniae</i>	<i>Glycine max</i>	Armstrong, Sante Fe, Argentina	–	n.d.
31096	<i>Fusarium tucumaniae</i>	Unknown	Argentina	–	
DB P28 R13	<i>Fusarium virguliforme</i>	<i>Phaseolus vulgaris</i>	Van Buren County, MI, U.S.A.	–	n.d.
Vb-2a	<i>Fusarium virguliforme</i>	<i>Glycine max</i>	Van Buren County, MI, U.S.A.	–	n.d.
F-14-77	<i>Fusarium virguliforme</i>	<i>Glycine max</i>	Ingham County, MI, U.S.A.	–	
DB P27 R13	<i>Fusarium virguliforme</i>	<i>Phaseolus vulgaris</i>	Van Buren County, MI, U.S.A.	–	
DB P30 R5	<i>Fusarium virguliforme</i>	<i>Phaseolus vulgaris</i>	Van Buren County, MI, U.S.A.	–	
22292 (Mont-1)	<i>Fusarium virguliforme</i>	<i>Glycine max</i>	Illinois, U.S.A.	–	
36897	<i>Fusarium virguliforme</i>	<i>Glycine max</i>	Argentina	–	
54291	<i>Fusarium virguliforme</i>	<i>Glycine max</i>	Argentina	–	
VB-1	<i>Fusarium virguliforme</i>	<i>Glycine max</i>	Van Buren County, MI, U.S.A.	–	
	<i>Rhizoctonia solani</i> (AG2-2)	<i>Glycine max</i>		–	
	<i>Rhizoctonia solani</i> (AG4)	<i>Glycine max</i>		–	
	<i>Phialophora gregata</i> (genotype A)	<i>Glycine max</i>		–	
	<i>Phialophora gregata</i> (genotype B)	<i>Glycine max</i>		–	
	<i>Macrophomina phaseolina</i>	<i>Glycine max</i>		–	
	<i>Sclerotinia sclerotiorum</i>	<i>Glycine max</i>		–	

described in Wang et al. (2019a). Inoculum was packaged into envelopes directly with seeds, and the contents were poured into the cone planter, sowing the seeds and inoculum in furrow at an average rate of 7.1 ml of inoculum per foot. Three plants from each plot were sampled on 12 September 2018. The foliar tissues were removed from the roots in the field, and the roots were thoroughly washed clean of soil and allowed to dry at 55°C for 2 days. The dried roots from each sample were placed in a 50-ml plastic container (OPS Diagnostics) with two 1-cm-diameter steel balls and ground into a powder using a GenoGinder 2010 (SPEX Sample Prep) at 1,500 rpm for 5 min. DNA was extracted from these roots using the same phenol:chloroform procedure described above. Total DNA was quantified using a Nanodrop 1000 (Thermo-Fisher Scientific).

To validate the assay with soil samples, bulk soil was collected from 10 soybean fields throughout Michigan during the spring and summer of 2018 using a 3-cm-diameter, 15-cm-deep soil core. Soil was placed into a 3.8-liter (1-gallon) plastic bag, homogenized, subsampled into 2.3-kg (5-pound) paper bags, and dried at 55°C for 2 days. Approximately 500 mg of dried soil was used for DNA extraction using a FastDNA Spin Kit for Soil (MP Bio). Two technical DNA extraction replicates were performed, and a third technical replicate was extracted after being spiked with ~5,000 *F. brasiliense* conidia. An estimated number of conidia in nonspiked soil samples that showed positive qPCR results was obtained by performing the following calculation: $5,000 \text{ spores} \times z = x \text{ spores} \times y$, where 5,000 is the number of spores spiked into each soil sample, z is the Ct of the spiked sample, and y is the Ct of the nonspiked sample. Nonspiked samples that amplified at a Ct > 31 cannot be reliably quantified, because they are below the limit of detection. Therefore, an estimated number of conidia was not calculated for these samples (Table 4).

To test the multiplexing capacity and transferability of this assay on other platforms, serial dilutions of *F. virguliforme* DNA and *F. brasiliense* DNA were amplified in a single reaction containing primers F6-3 and R6 and probe FvPrb-3 (Wang et al. 2015) along with the primers and probe developed in this study (Table 1). Reactions were set up in LightCycler 480 96-well plates (Roche Diagnostics) and performed on a CFX96 Real-Time PCR Detection System v3.1 (BioRad Laboratories). Multiplexed reactions were set up as described above.

Results

Assay design. A 149-bp segment in the IGS was identified as containing *F. brasiliense*-specific SNPs, and the primers and probe were

designed in this region (Fig. 1). The forward primer encompasses one SNP shared by *F. brasiliense*, *F. cuneirostrum*, *F. crassistipitatum*, and *F. virguliforme* but excludes *F. phaseoli* and *F. tucumaniae*. The reverse primer is conserved among all species used in the IGS alignment. The probe encompasses two SNPs unique and specific to *F. brasiliense*, containing two thymines instead of two cytosines. The probe associated with these residues was designed with locked nucleic acid bases to enhance specific binding at these sites (Fig. 1, asterisks). The primers and probe are not

Table 3. Serial dilutions for four *Fusarium brasiliense* isolates used to determine assay sensitivity

Isolate ^a	DNA concentration ^b	Ct ^c	Mean Ct	Standard error
F-14-42	1,000,000	17.20	17.22	0.099
F-15-158	1,000,000	16.82		
F-16-137	1,000,000	17.37		
NRRL 34938	1,000,000	17.50		
F-14-42	100,000	20.50	20.49	0.075
F-15-158	100,000	20.22		
F-16-137	100,000	20.57		
NRRL 34938	100,000	20.69		
F-14-42	10,000	24.08	24.08	0.097
F-15-158	10,000	23.72		
F-16-137	10,000	24.33		
NRRL 34938	10,000	24.17		
F-14-42	1,000	27.65	27.66	0.079
F-15-158	1,000	27.35		
F-16-137	1,000	27.92		
NRRL 34938	1,000	27.72		
F-14-42	100	30.96	31.18	0.139
F-15-158	100	30.86		
F-16-137	100	31.28		
NRRL 34938	100	31.62		
F-14-42	10	35.26	34.93	0.249
F-15-158	10	33.93		
F-16-137	10	35.36		
NRRL 34938	10	35.16		

^a Isolate 34938 was obtained from the U.S. Department of Agriculture Agricultural Research Service (ARS) Culture Collection (NRRL). All other isolates were obtained by J. L. Jacobs and M. I. Chilvers.

^b Concentration in femtograms of DNA per reaction.

^c Ct, cycle threshold (or cycle at which fluorescent signal crossed the detection threshold).

Fig. 3. A, Detection of serial dilutions of four *Fusarium brasiliense* (Fb) isolates to determine assay sensitivity. B, These isolates consistently amplified at all concentrations tested, with an average of 99.6% efficiency.

predicted to form any significant homo- or heterodimers ($\Delta G > -9$) and perform at an ideal anneal and extension temperature of 60°C.

qPCR assay specificity. Genomic DNA isolated from purified mycelia was used to show specificity across closely related species within clade 2 of the FSSC (Table 2). The only isolates displaying amplification were *F. brasiliense* and some members of FSSC clade 3 (Chitrampalam and Nelson 2016) (Fig. 2 and Table 2). However, when tested with a standard 5 ng of starting DNA, *F. brasiliense*

isolates consistently had Ct values within 1 Ct of each other (13.94 to 14.92) (Table 2). In contrast, FSSC clade 3 isolates with positive reactions had higher Ct values. These results indicate that the assay designed here can distinguish *F. brasiliense* from closely related clade 2 species and other common soilborne pathogenic fungi (Table 2). However, there may be some cross-reactivity with clade 3 species. With pure cultures, *F. brasiliense* can readily be distinguished from clade 3 species based on growth rate and morphology (Fig. 4).

Table 4. Amplification of *Fusarium brasiliense* from soils across Michigan

Location in Michigan	Type	Ct ^a	Quantity DNA detected ^b	Spores per 500 mg of soil ^c
Clinton County	Unknown	—	—	—
Clinton County	Spiked	21.26	57,083	5,000
Hillsdale County	Unknown	—	—	—
Hillsdale County	Spiked	18.10	346,132	5,000
Lenawee County	Unknown	36.92	7	n.d.
Lenawee County	Spiked	20.14	108,360	5,000
Montcalm County	Unknown	32.03	137	n.d.
Montcalm County	Spiked	18.90	226,150	5,000
Ingham County	Unknown	34.13	39	n.d.
Ingham County	Spiked	17.29	547,582	5,000
Saginaw County	Unknown	30.83	246	2,804
Saginaw County	Spiked	18.01	363,252	5,000
Sanilac County	Unknown	29.51	529	3,052
Sanilac County	Spiked	17.41	512,226	5,000
St. Joseph County	Unknown	32.95	74	n.d.
St. Joseph County	Spiked	19.72	137,118	5,000
Van Buren County, Field 1	Unknown	—	—	—
Van Buren County, Field 1	Spiked	19.90	123,579	5,000
Van Buren County, Field 2	Unknown	—	—	—
Van Buren County, Field 2	Spiked	21.97	70,167	5,000

^a Ct, cycle threshold (or cycle at which fluorescent signal crossed the detection threshold).

^b Quantity in femtograms.

^c Estimated by solving for x using the equation $5,000 \text{ spores} \times z = x \text{ spores} \times y$, where 5,000 is the number of spores spiked into each soil sample, z is the Ct of the spiked sample, and y is the Ct of the nonspiked sample. Dashes indicate no detection, and n.d. indicates not determined, because detection was below the limit of detection.

Fig. 4. Representative isolates of **A**, *Fusarium brasiliense* and **B**, *Fusarium solani* species complex (FSSC) clade 3 isolates plated on potato dextrose agar 7 days post inoculation. **A**, *F. brasiliense* isolates represented are i, MI-Mtc-C1; ii, MI-Mtc-A13; iii, F-16-137; and iv, F-15-158. **B**, *F. solani* (FSSC 5) isolates represented are i, MI-Mtc-C17FS; ii, MI-Mtc-B16FS; and iii, MI-Mtc-B10, whereas iv, FSSC 11 isolate MI-Mtc-A2FS is also represented.

qPCR assay sensitivity. The *F. brasiliense* qPCR assay targeting the IGS region provided high sensitivity, reproducibly amplifying target DNA as low as 10 fg of target DNA of multiple isolates (Fig. 3). Although all four isolates tested here amplified at this low concentration, standard error of detection was highest at this concentration. Therefore, the limit of detection was conservatively determined to be 100 fg or a Ct $\geq$ 31 (Table 3) (Bustin et al. 2009). Although target DNA can be amplified at lower concentrations, quantities determined below this Ct may not be reliable.

Assay validation. To determine the applicability of this assay to field trials, roots from artificially inoculated dry beans were harvested, subjected to DNA extraction, and tested with the *F. brasiliense* qPCR assay developed here. *F. brasiliense* was detected consistently above the limit of detection in dry bean plots inoculated with *F. brasiliense* (average Ct = 29.6) but not in noninoculated plots or plots inoculated with *F. oxysporum* (average Ct = 36.3) (Fig. 5).

Soil samples obtained from 10 counties throughout Michigan were screened for the presence of *F. brasiliense*. All soil samples spiked with *F. brasiliense* conidia were successfully detected with Ct values ranging from 17.29 to 21.97, indicating relatively pure DNA samples lacking PCR inhibitors (Table 4). Additionally, *F. brasiliense* was detected in nonspiked soil samples from six counties, although only two were confidently above the limit of detection of 100 fg (Table 4). Using a known quantity of spores in the spiked samples, estimated 2,804 and 3,051 spores were present in Saginaw County and Sanilac County, respectively, per 500 mg of soil.

The primers and probes for the *F. brasiliense* assay and a *F. virguliforme* assay (Wang et al. 2015) were used simultaneously in a multiplexed reaction (Table 1). Amplification for each assay was only observed when target DNA was present, indicating no cross-reactivity between the assays (Table 5). Combinations of serial dilutions of *F. brasiliense* and *F. virguliforme* DNA were successfully amplified in pooled reactions, and an accurate quantification of each target was obtained (Table 5).

Discussion

Many of the clade 2 species in the FSSC are economically important pathogens capable of causing root rot and/or soybean SDS and significant yield losses. They are very similar morphologically, with a slow growth rate, and they are taxonomically distinguished by conidia measurements and number of septa (Aoki et al. 2014). However, because a pure culture is required to obtain conidia and because conidia measurements of these species overlap (Aoki et al. 2003), it can

be very difficult to obtain accurate identifications in a timely manner, if at all, using morphology alone. DNA extractions and qPCR assays can be performed in a single day, expediting accurate diagnoses. High-quality qPCR assays have high sensitivity and are capable of detecting very low quantities of target DNA. Designing an assay around a multicopy target, like the IGS region, can increase sensitivity. Previous assays designed around the IGS region have provided sensitivity to as low as 100 fg of target DNA (Rojas et al. 2017; Wang et al. 2015). The assay developed here has validated SNPs specific to *F. brasiliense* compared with other species within the FSSC clade 2 (Fig. 1 and Table 2). The assay performs at a high efficiency of 99.6%, and it consistently detected 10 fg of target DNA when obtained from pure culture and has a confident limit of detection of 100 fg (Fig. 3 and Table 3).

Nomenclature within clade 3 of the FSSC is complicated, but progress has been made by incorporating multilocus genotyping and formal typification efforts (Coleman 2016; O'Donnell et al. 2010; Schroers et al. 2016). For instance, FSSC clade 3 species in this study represent FSSC 5, the true *F. solani*, and FSSC 11, an unnamed species. Interestingly, *F. solani* (FSSC 5) and FSSC 11 qPCR results showed variation among isolates, suggesting some IGS sequence diversity within these species. Sanger sequencing of the 149-bp IGS region where this qPCR assay was designed showed that FSSC 11 isolate MI-Mtc-C3FS (Wht) and *F. solani* isolates F-15-118 and MI-Mtc-C17FS share SNPs with other non-*F. brasiliense* species, confirming their negative reactions. Sanger sequencing of this region for FSSC 11 isolate MI-Mtc-A2FS and *F. solani* isolate MI-Mtc-B10 showed SNPs shared with *F. brasiliense*, thus explaining the positive reactions. Phylogenetic analyses of *F. solani* clade 3 isolates commonly use other molecular markers, such as *TEF1*, internal transcribed spacer (ITS) ribosomal DNA, and *RPB2* (Chitrampalam and Nelson 2016; Wang et al. 2019b), perhaps because of the difficulty of sequencing and assembling the repetitive IGS region. However, this assay provides specificity to *F. brasiliense* within *F. solani* clade 2, and qPCR-positive FSSC clade 3 strains can be distinguished from *F. brasiliense* through morphology (Fig. 4). However, the identity of all isolates should be confirmed through PCR and Sanger sequencing of multiple loci (Wang et al. 2019b).

When pure cultures and collections are not being obtained or maintained, this assay can still provide an initial screen for *F. brasiliense* in soil and root samples. The assay is not likely to produce false-positive results by cross-amplification of FSSC clade 3 species, because some

Fig. 5. Detection of *Fusarium brasiliense* within root tissues of dry bean **A**, cultivar Zenith or **B**, cultivar Red Hawk. Values listed over bars indicate the average femtograms of *F. brasiliense* DNA detected within roots of 12 plants per treatment. Noninoculated and *Fusarium oxysporum*-inoculated samples were not detected above the limit of detection of 100 fg.

Table 5. Amplification of serial dilutions of *Fusarium brasiliense* and/or *Fusarium virguliforme* DNA in a multiplexed system^a

<i>F. virguliforme</i> DNA added ^b	<i>F. brasiliense</i> DNA added ^b	<i>F. virguliforme</i> Ct ^c	<i>F. brasiliense</i> Ct ^d
1,000,000	0	17.35	—
100,000	0	20.95	—
10,000	0	24.46	—
1,000	0	28.10	—
100	0	31.53	—
0	1,000,000	—	17.57
0	100,000	—	21.02
0	10,000	—	24.17
0	1,000	—	26.60
0	100	—	31.80
1,000,000	1,000,000	17.49	18.86
10,000	1,000,000	24.74	17.26
10,000	100,000	24.52	20.73
100	100,000	33.34	20.87
1,000	10,000	28.02	23.61
100	1,000	31.82	27.00
0	0	—	—

^a Dashes represent no detection.

^b Quantity in femtograms.

^c Ct, cycle threshold (cycle at which fluorescent signal crossed the detection threshold for *F. virguliforme*).

^d Ct, cycle threshold (cycle at which fluorescent signal crossed the detection threshold for *F. brasiliense*).

do not cross-react even at high concentrations, and others that do cross-react do so with much lower efficiency, producing significantly higher Ct values (Fig. 2 and Table 2). For instance, with 5 ng of purified fungal DNA, cross-reactive FSSC clade 3 species showed higher Ct values by at least six cycles (Table 2). Therefore, obtaining a sufficient concentration of FSSC clade 3 DNA from root or soil samples for cross-reactivity is unlikely. In contrast, all *F. brasiliense* isolates from both the United States and Brazil were consistently detected with high accuracy and efficiency. Therefore, obtaining a sufficient concentration of *F. brasiliense* from root or soil samples for successful amplification and detection is possible as shown in Figure 5 and Table 4. Initial screens with this qPCR assay can help guide when and where to begin isolation attempts for pure cultures, and they can also help generate an understanding of the global distribution of *F. brasiliense*. For example, the *F. brasiliense* assay gave Ct values <31 from soil samples from Saginaw and Sanilac Counties, where *F. brasiliense* has already been successfully isolated from plant roots (K. A. Oudman et al., unpublished data). The assay also gave Ct values >31 but <36 with DNA from soil samples from Montcalm and Ingham Counties, where *F. brasiliense* has also been successfully isolated (K. A. Oudman et al., unpublished data). Finally, the assay gave Ct values >31 but <36 from Lenawee and St. Joseph soils, where *F. brasiliense* has not yet been isolated. Therefore, the assay provides evidence that warrants additional investigation and isolation attempts for *F. brasiliense* from these counties.

Soybean and dry bean are valuable crops in Michigan worth a combined \$1.08 billion in 2017 (USDA-NASS 2017). Fusarium root rot and SDS consistently rank in the top 10 most destructive soybean diseases in northern states, including Michigan (Allen et al. 2017). Genetic resistance to Fusarium root rot is partial, and only a few fungicides are effective against it (Sang et al. 2018; Wang et al. 2017). Therefore, accurate detection and integrating successful management practices, like using seed treatments (Kandel et al. 2018), crop rotations (Leandro et al. 2018), tillage practices, and planting partially resistant cultivars, are key to preventing yield loss. Additional understanding of a possible host preference is also needed to inform management strategies, because *F. brasiliense* is more commonly found in areas of dry bean production, whereas *F. virguliforme* is more commonly found in areas of soybean production in Michigan (K. A. Oudman et al., unpublished data). Preliminary experiments examining host preference have shown that both *F. brasiliense* and *F. virguliforme* cause disease on both soybean and dry bean and that disease severity and root colonization are not significantly different from one another in a growth chamber setting (M. G. Roth and M. I. Chilvers, unpublished data). Symptomatic roots infected by *F. brasiliense* and *F. virguliforme* are indistinguishable from one another, and coinfections may also be possible. Therefore, the ability to multiplex the *F. brasiliense* and *F. virguliforme* assays can also save time and effort in accurate diagnoses.

Partial genetic resistance will continue to be an important part of integrated management for Fusarium root rot pathogens. Although more research has been done with resistance to *Fusarium* spp. in soybean, resistance is complicated and partial (Chang et al. 2018). Dry beans (*P. vulgaris*) originated from Mesoamerica (Bitocchi et al. 2012), but they were traded through Central America and experienced a genetic bottleneck while reaching South America. Therefore, gene pools in current bean breeding lines originate from Mesoamerica or the northern Andean region of South America (Bellucci et al. 2014; Miklas et al. 2006; Singh 2001; Singh and Schwartz 2010). Dry bean varieties from Mesoamerican gene pools, like Zenith, are smaller in size and typically have higher resistance to root rot diseases (Román-Avilés and Kelly 2005; Schneider et al. 2001). Another Mesoamerican variety, Zorro, was shown to have more resistance to *F. virguliforme* than the Andean variety Red Hawk, a large-seeded red kidney bean cultivar (Michigan Dry Edible Bean Production Research Advisory Board 2017). Interestingly, more *F. brasiliense* was detected in inoculated Zenith roots than Red Hawk roots in this study. However, root colonization and disease severity are not always strongly correlated, particularly among the clade 2 FSSC species (Wang et al. 2019a). A formal

study should be conducted to determine the inherent resistance levels of multiple varieties from the two different gene pools to *F. brasiliense*.

Acknowledgments

The authors thank Z. A. Noel, M. Breunig, V. Ortiz, and A. G. McCoy for helpful discussions and manuscript revisions.

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