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Keywords

α -box, disease management, *Fusarium solani* species complex, heterothallic, high mobility group, idiomorph, interspecific hybridization, *MAT1-2-3*, PCR assay, whole genome

Disciplines

Agriculture | Agronomy and Crop Sciences | Bioinformatics | Evolution | Genetics and Genomics | Plant Pathology

Comments

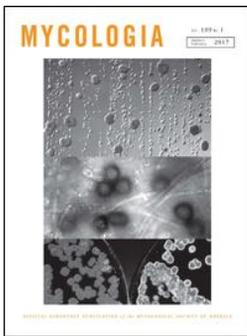
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Genetic architecture and evolution of the mating type locus in fusaria that cause soybean sudden death syndrome and bean root rot

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Abstract: *Fusarium tucumaniae* is the only known sexually reproducing species among the seven closely related fusaria that cause soybean sudden death syndrome (SDS) or bean root rot (BRR). In a previous study, laboratory mating of *F. tucumaniae* yielded recombinant ascospore progeny but required two mating-compatible strains, indicating that it is heterothallic. To assess the reproductive mode of the other SDS and BRR fusaria, and their potential for mating, whole-genome sequences of two SDS and one BRR pathogen were analyzed to characterize their mating type (*MAT*) loci. This bioinformatic approach identified a *MATI-1* idiomorph in *F. virguliforme* NRRL 22292 and *MATI-2* idiomorphs in *F. tucumaniae* NRRL 34546 and *F. azukicola* NRRL 54364. Alignments of the *MAT* loci were used to design PCR primers within the conserved regions of the flanking genes *APN1* and *SLA2*, which enabled primer walking

to obtain nearly complete sequences of the *MAT* region for six *MATI-1* and five *MATI-2* SDS/BRR fusaria. As expected, sequences of the highly divergent 4.7 kb *MATI-1* and 3.7 kb *MATI-2* idiomorphs were unalignable. However, sequences of the respective idiomorphs and those that flank *MATI-1* and *MATI-2* were highly conserved. In addition to three genes at *MATI-1* (*MATI-1-1*, *MATI-1-2*, *MATI-1-3*) and two at *MATI-2* (*MATI-2-1*, *MATI-2-3*), the *MAT* loci of the SDS/BRR fusaria also include a putative gene predicted to encode for a 252 amino acid protein of unknown function. Alignments of the *MATI-1-3* and *MATI-2-1* sequences were used to design a multiplex PCR assay for the *MAT* loci. This assay was used to screen DNA from 439 SDS/BRR isolates, which revealed that each isolate possessed *MATI-1* or *MATI-2*, consistent with heterothallism. Both idiomorphs were represented among isolates of *F. azukicola*, *F. brasiliense*, *F. phaseoli* and *F. tucumaniae*, whereas isolates of *F. virguliforme* and *F. cuneirostrum* were only *MATI-1* and *F. crassistipitatum* were only *MATI-2*. Finally, nucleotide sequence data from the *RPB1* and *RPB2* genes were used to date the origin of the SDS/BRR group, which was estimated to have occurred about 0.75 Mya (95% HPD interval: 0.27, 1.68) in the mid-Pleistocene, long before the domestication of the common bean or soybean.

Key words: α -box, disease management, *Fusarium solani* species complex, heterothallic, high mobility group, idiomorph, interspecific hybridization, *MATI-2-3*, PCR assay, whole genome

INTRODUCTION

Sudden death syndrome (SDS), which causes a root rot and premature defoliation of soybeans (*Glycine max*), severely effects yield in all major production regions of North and South America (Koening and Wrather 2010, Leandro et al. 2012). Four species of *Fusarium* are known to produce symptoms characteristic of SDS (Aoki et al. 2003, 2005, 2012a): *F. virguliforme*, which is known to occur in USA, Canada and Argentina; *F. tucumaniae* and *F. crassistipitatum* in Brazil and Argentina; and *F. brasiliense* in Brazil, Argentina and California (Li et al. 2003, O'Donnell et al. 2010, Aoki et al. 2012a). Similarly three additional species of *Fusarium*, *F. azukicola*, *F. cuneirostrum* and *F. phaseoli* are associated with bean root rot (BRR), a dry rot of

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Phaseolus spp. and *Vigna* spp. that occurs in most production areas throughout the world (Burke and Hall 2005; Aoki et al. 2012a, b). Although the seven SDS/BRR fusaria are morphologically and phylogenetically distinct species, they are closely related and together form a distinct lineage within Clade 2 of the *Fusarium solani* species complex (FSSC) (O'Donnell et al. 2008, Aoki et al. 2012b) that is theorized to have evolved in South or Mesoamerica approximately 0.15 Mya (O'Donnell et al. 2013).

Knowledge of the reproductive mode of the SDS/BRR pathogens has practical implications for control because sexual reproduction serves to increase the genotypic diversity within a species, thereby letting it adapt more quickly to changing environments. Asexual reproduction on the other hand allows for rapid propagation with minimal energy and generally is considered advantageous when a species is well adapted to its environment (Clay and Kover 1996, Heitman et al. 2013). Pathogens capable of both methods of reproduction pose the greatest risk to disease management because they are more likely to overcome genetic resistance and chemical controls (McDonald and Linde 2002). Among the seven known SDS/BRR fusaria, only *F. tucumaniae* has been shown to reproduce sexually and possesses high genetic diversity (Covert et al. 2007, Scandiani et al. 2012). A study by Mbofung et al. (2012) reported that collections of *F. virguliforme* from North America were more genetically diverse than previously shown (Achenbach et al. 1996, Rupe et al. 2001, Malvik and Bussey 2008, O'Donnell et al. 2010). However, it has not been determined whether this diversity is related to sexual reproduction. Moreover, no study to date has studied mating-type organization and distribution in the SDS/BRR fusaria.

Mating in filamentous ascomycetes is regulated by the mating-type (*MAT*) locus. Homothallic species are self fertile and contain both idiomorphs, designated *MAT1-1* and *MAT1-2*. In contrast, heterothallic species are self sterile and sexual reproduction can occur only between individuals with opposite mating type (Metzenberg and Glass 2005, Ni et al. 2011). Genetic analysis of ascospore progeny obtained from laboratory crosses of *F. tucumaniae* provided direct evidence that it is heterothallic (Covert et al. 2007). In addition, the results suggested that the *F. virguliforme* mating system also may be heterothallic based on its abortive mating with only the + isolates of *F. tucumaniae*. Isolates of *F. tucumaniae* and *F. virguliforme* were identified as possessing a + or – mating type and each isolate was tested as both the female and male parent in both intra- and interspecific crosses. Among the 27 isolates of *F. tucumaniae* tested, 24 readily produced fertile perithecia in

intraspecific pairings, with 15 isolates identified as the + and nine identified as the – mating type. No perithecia were produced between isolates of *F. tucumaniae* with identical mating types. Among the 17 isolates of *F. virguliforme* tested, none produced perithecia in intraspecific pairings. Although no fertile perithecia were produced in *F. virguliforme* × *F. tucumaniae* pairings, the abortive mating reaction between *F. tucumaniae* + and *F. virguliforme* indicated that the latter pathogen likely comprised only the – mating type (Covert et al. 2007).

Given the discovery of + and – mating types of *F. tucumaniae* in Argentina and Brazil, but only the – mating type of *F. virguliforme* to date in North and South America, Covert et al. (2007) posited that the + mating type of *F. virguliforme* may also be present in South America. Because current management strategies for SDS and BRR rely predominately on host resistance, understanding the reproductive mode and distribution of mating types among the SDS/BRR fusaria is essential to disease management as is understanding how these species have evolved in response to the domestication of the common bean and soybean that they commonly are found to infect. Thus, the objectives of this research were to: (i) characterize the *MAT* loci in the seven *Fusarium* species associated with SDS/BRR; (ii) date the evolutionary origin of the SDS/BRR fusaria; (iii) develop a multiplex PCR assay for determining *MAT* idiomorph in the SDS/BRR fusaria; (iv) test the hypothesis that each of the seven SDS/BRR fusaria are heterothallic; and (5) determine whether SDS/BRR fusaria that are represented by both *MAT* idiomorphs can complete a sexual cycle.

MATERIALS AND METHODS

Isolates.—A total of 439 isolates comprising the seven SDS/BRR fusaria was used in this study (SUPPLEMENTARY TABLE I). They were obtained from the Agricultural Research Service Culture Collection (NRRL) at the National Center for Agricultural Utilization Research, Peoria, Illinois, and from culture collections at Purdue University, West Lafayette, Indiana, and the Centro de Referencia de Micología (CEREMIC), Fac. de Cs. Bioquímicas y Farmacéuticas, UNR, Rosario, Argentina. Isolates were cultured in yeast-malt broth 3–5 d on a rotary shaker at 200 rpm, and genomic DNA was extracted from lyophilized mycelium with a CTAB (cetyl trimethyl-ammonium bromide, Sigma-Aldrich, St Louis, Missouri) mini-prep (O'Donnell et al. 1998). Species identification of 264 isolates had been determined with a validated multilocus genotyping assay (MLGT; O'Donnell et al. 2010, Scandiani et al. 2010). The remaining 175 isolates, including the 61 SDS isolates collected during 2013 in Argentina, were identified to species with the MLGT assay employing a Luminex 100 flow cytometer (Luminex Corp., Austin, Texas).

Identification of mating loci.—The genome of *Fusarium virguliforme* NRRL 22292 (= Mont-1) (Srivastava et al. 2014) was obtained with a whole-genome shotgun sequencing approach on a 454 GS-FLX Titanium sequencer. Genome sequences for *F. tucumaniae* NRRL 34546 and *F. azukicola* NRRL 54366 were obtained with an Illumina HiSeq2000 sequencer. Genomes of *F. virguliforme* and *F. tucumaniae* were assembled with the parallel contig assembly program (PCAP) (Huang et al. 2003) and annotated with the gene prediction program AUGUSTUS (Stanke et al. 2008). Nucleotide and amino acid sequences of the *MAT1-1* locus from *F. solani* f. sp. *pisi* NRRL 45880 (Coleman et al. 2009) were used as a starting point for bioinformatic searches for *MAT* loci. Searches also were conducted for the DNA lyase (*SLA2*) and cytoskeletal protein (*APNI*), which typically flank *MAT* loci in ascomycetous fungi (Martin et al. 2011, Tsui et al. 2013). The DNA-protein search (DPS) program (Huang 1996) located the *MAT1-1* idiomorph in *F. virguliforme* NRRL 22292 and the *MAT1-2* idiomorph in *F. tucumaniae* NRRL 34546 and *F. azukicola* NRRL 54364. *MAT* sequences from these isolates were assembled with PCAP and aligned with GAP3 (Huang and Chao 2003). The structure of each *MAT* gene was determined with the DNA-protein alignment program NAP (Huang and Zhang 1996) and the AUGUSTUS gene prediction software, together with manual analysis.

PCR amplification and DNA sequencing.—Initially the aligned *MAT* loci of *F. virguliforme* NRRL 22292, *F. tucumaniae* NRRL 34546 and *F. azukicola* NRRL 54364 were used to design forward and reverse PCR primers within the conserved regions of *APNI* and *SLA2* (FIG. 1). Subsequently forward and reverse PCR primers were developed within *MAT1-1-1* and *MAT1-2-1* so that the *MAT* idiomorphs could be PCR amplified as two overlapping fragments (FIG. 1). Long-range PCR reactions were conducted in a total volume of 40 μ L, containing 20 μ L genomic DNA at approximately 30 ng/ μ L, 250 nM each primer, 200 nM dNTPs, 25 mM MgSO₄, 1 \times buffer and 1 U Platinum[®] Taq DNA high fidelity polymerase (Invitrogen Life Technologies, Carlsbad, California). PCR amplifications were conducted in an Applied Biosystems 9700 thermal-cycler (Applied Biosystems, Foster City, California) with this program: one cycle at 95 C for 90 s followed by 40 cycles at 94 C for 30 s, 52 C for 30 s and 68 C for 7 min, with a final elongation step at 68 C for 5 min. Amplicons were visualized over a UV transilluminator after electrophoresis through a 1.5% agarose gel and staining with ethidium bromide. PCR products were purified with Montage PCR₉₆ filter plates (Millipore, Billerica, Massachusetts). Amplicons were sequenced with ABI BigDye chemistry, purified with ABI XTerminator and run on an ABI-Hitachi 3730 automated DNA sequencer (Tokyo, Japan). A minimal set of 18 and 16 internal sequencing primers were used respectively to walk across *MAT1-1* in six species and *MAT1-2* in five species (SUPPLEMENTARY FIG. 1).

Diversification time estimates.—BEAST 1.7.5 (Drummond and Rambaut 2007) was used to generate Bayesian-derived divergence time estimates from partial *RPB1* and *RPB2* partitions (O'Donnell et al. 2013). The calibration points were taken from O'Donnell et al. (2013) and included the

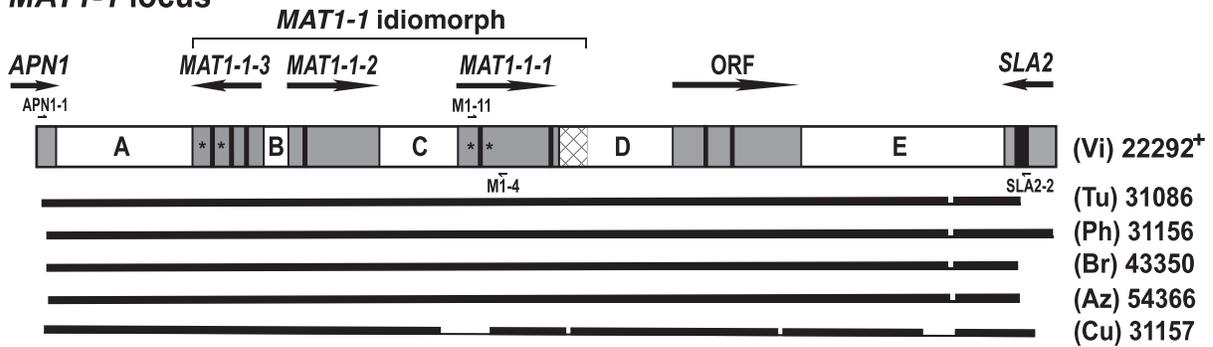
estimated ages for the last common ancestor of: (i) *F. decemcellulare* and *F. albosuccineum*; (ii) *F. falciforme*, *F. solani* f. sp. *pisi* (FSSC 3+4-e), *F. ambrosium* and *F. neocosmosporiellum*; (iii) all “solani clade” taxa (i.e. all except *F. decemcellulare*, *F. albosuccineum*, *F. setosum*); and (iv) all aforementioned taxa. The analysis was run under an uncorrelated lognormal relaxed molecular clock with a birth-death model (Gernhard 2008) as the tree prior. The nucleotide substitution model used was GTR+ Γ +I, which was chosen with the program Modeltest, as implemented in PHYLEMON 2.0 (Sánchez et al. 2011). The posterior distribution of rates and node ages was estimated with a Markov chain Monte Carlo (MCMC) algorithm, which was run 10 000 000 generations and sampled every 1000 generations. TRACER 1.5 (Rambaut and Drummond 2007) was used to visualize the BEAST output. MCMC was determined to have reached stationarity given that all of the effective sample sizes were > 1000. TreeAnnotator 1.7.5 was used to generate a maximum clade credibility (MCC) tree (Drummond and Rambaut 2007) in which 95% highest probability density (HPD) intervals were used to express statistical uncertainty in the divergence time estimates.

Nucleotide sequence accession numbers.—The DNA sequence data analyzed in the present study is available from GenBank as accessions Nos. KF706634–KF706657 and from TreeBASE as S14758: Tr68073, 68074, 66842, 66844, 66846–66851. This data also was deposited in the *Fusarium MLST* (<http://www.cbs.knaw.nl/fusarium>) and FUSARIUM-ID (<http://isolate.fusariumdb.org>) web-accessible databases.

Multiplex PCR for MAT1-1 and MAT1-2.—The aligned *MAT1-1-3* and *MAT1-2-1* sequences from six and five SDS/BRR fusaria respectively were used to identify conserved regions within these genes to design a multiplex PCR assay for the *MAT* idiomorphs (FIG. 2). Sequencing of the 496 bp *MAT1-1-3* or the 260 bp *MAT1-2-1* amplicon from each of the seven species verified that the PCR primers correctly amplified these genes. Once validated, the multiplex PCR assay was used to type 439 SDS/BRR isolates (TABLE I, SUPPLEMENTARY TABLE I). Each PCR reaction consisted of 20 μ L genomic DNA at approximately 30 ng/ μ L, 250 nM of each primer, 200 nM dNTPs, 25 mM MgSO₄, 1 \times buffer and 1 U Platinum[®] Taq DNA high fidelity polymerase (Life Technologies, Grand Island, New York) in 40 μ L total volume. Cycling conditions included an initial denaturing cycle at 95 C for 90 s followed by 40 cycles at 94 C for 30 s, 57 C for 30 s and 68 C for 50 s, with a final elongation step at 68 C for 5 min. PCR products were visualized after electrophoresis through a 1.5% agarose gel and ethidium bromide staining.

Sexual crosses.—At present *F. tucumaniae* is the only member of the SDS/BRR fusaria known to reproduce sexually (Covert et al. 2007, Scandiani et al. 2010). As such, intraspecific crosses were set between *MAT1-1* and *MAT1-2* isolates of *F. azukicola*, *F. brasiliense* and *F. phaseoli*, based on results of the *MAT* multiplex assay. All crosses and conditions followed the method of Klittich and Leslie (1988), which had been used to obtain fertile perithecia of *F. tucumaniae* (Covert et al. 2007).

MAT1-1 locus



MAT1-2 locus

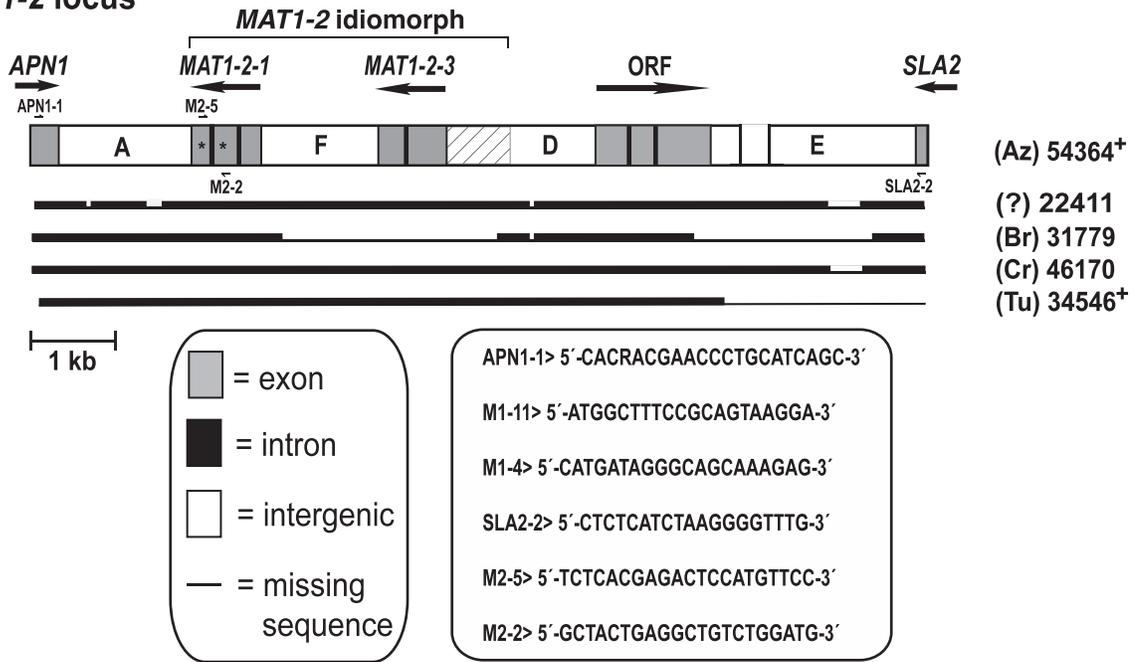


FIG. 1. Organization of the *MAT1-1* and *MAT1-2* loci in seven *Fusarium* species that cause soybean sudden death syndrome (SDS) or bean root rot (BRR). *MAT* locus organization is similar to other heterothallic ascomycetes in gene content, synteny and intron number and position. Bold arrows indicate the direction of transcription of the six genes at *MAT1-1* and five at *MAT1-2*. Locations of the four PCR primers used to amplify the *MAT* region of each idiormorph as two overlapping fragments are indicated by labeled half arrows, and their sequences are identified below the *MAT1-2* locus. Crisscrossed and diagonal lines identify regions within intergenic region D that are part of the divergent *MAT1-1* and *MAT1-2* idiormorphs respectively. A thin line is used to indicate regions where data is missing in the 11 *MAT* sequences. Species are identified to the right of each map by the following two-letter code, followed by the 5-digit NRRL strain number: Vi, *F. virguliforme*; Tu, *F. tucumaniae*; Ph, *F. phaseoli*; Br, *F. brasiliense*; Az, *F. azukicola*; Cu, *F. cuneirostrum*; Cr, *F. crassispitatum*; ?, *Fusarium* sp. Superscript + identifies three isolates where *MAT* was obtained from the whole-genome sequence. Asterisks indicate the location of a HMG motif in *MAT1-1-3* and *MAT1-2-1* and α -box motif in exons 1 and 2 of *MAT1-1-1*.

RESULTS

Structural organization of MAT loci.—Bioinformatic searches conducted with DPS loci successfully identified the *MAT1-1* locus in the whole-genome sequence of *F. virguliforme* NRRL 22292 and the *MAT1-2* locus in the genomes of *F. azukicola* NRRL 54364 and *F. tucumaniae* NRRL 34546 (FIG. 1). AUGUSTUS-based analyses of these genomes failed to find any *MAT*

genes outside the *MAT* locus. As is typical of other filamentous ascomycetes, the *MAT* loci were flanked by *APN1* (encoding a DNA lyase) and *SLA2* (encoding a cytoskeletal protein). Conserved regions within *APN1* and *SLA2* (FIG. 1, SUPPLEMENTARY FIG. 1) were used to design a PCR primer pair that amplified an 11.8 kb region from *MAT1-1* and a 10.4 kb region from *MAT1-2*. A second pair of PCR primers, within *MAT1-1-1* and *MAT1-2-1*, enabled amplification of

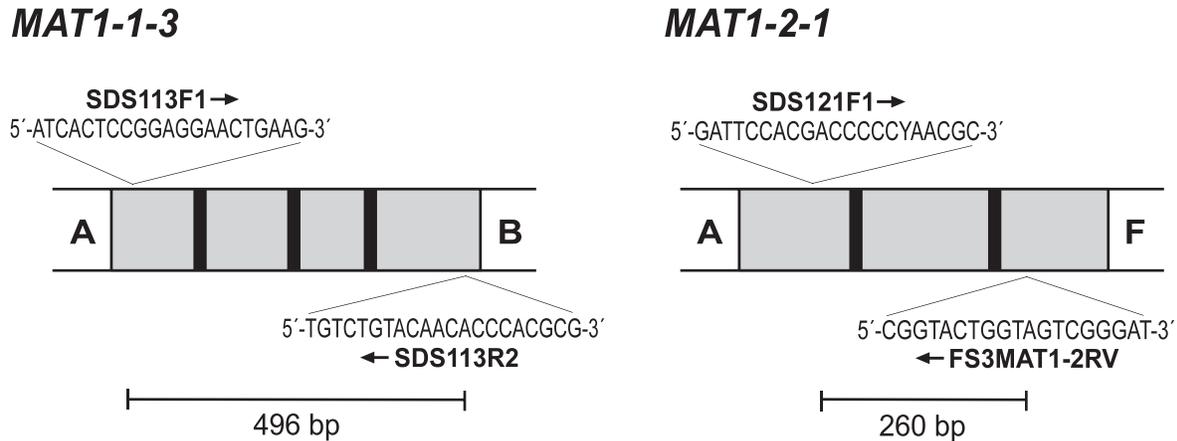


FIG. 2. Location of forward and reverse primers used in the multiplex PCR assay for *MAT* idiomorph. The *MAT1-1-3* and *MAT1-2-1* amplicons in the SDS/BRR fusaria were 496 and 260 bp respectively as determined by DNA sequencing. Results of the PCR assay are summarized (TABLE I) and strain histories of the 439 isolates typed are presented (SUPPLEMENTARY TABLE I).

both *MAT* regions as overlapping fragments (FIG. 1). Nearly complete sequences of the *MAT1-1* and *MAT1-2* loci were obtained from five and four additional SDS/BRR species respectively by walking across the region with internal sequencing primers (SUPPLEMENTARY FIG. 1).

Similar to other heterothallic *Fusarium* spp., the *MAT1-1* idiomorph contained the *MAT1-1-1*, *MAT1-1-2* and *MAT1-1-3* genes while the *MAT1-2* idiomorph contained the gene *MAT1-2-1* as well as the recently identified *MAT1-2-3* (Martin et al. 2011). Organization of the *MAT1-1* and *MAT1-2* loci for each of the seven SDS/BRR fusaria strongly suggests that these species are heterothallic and self sterile. BLASTx queries of GenBank, using the *MAT* gene sequences, identified a highly conserved α -box motif within the protein predicted to be encoded by *MAT1-1-1*, while high mobility group (HMG) domains were identified within the amino acid sequences of *MAT1-*

1-1, *MAT1-1-3* and *MAT1-2-1*. No conserved domains were detected within *MAT1-1-2* or *MAT1-2-3*.

The 806 bp *MAT1-2-3* gene was detected within the genome of a *MAT1-2* isolate of *F. tucumaniae* NRRL 34546 with the gene prediction program AUGUSTUS 2.5.5 (Stanke et al. 2008) and subsequently in the aligned *MAT1-2* sequences of four other SDS/BRR pathogens (FIG. 1). The *MAT1-2-3* gene was predicted to have one 47 bp intron and to encode for a protein 252 amino acids long. Similarly, the AUGUSTUS analysis also detected an ORF for a novel gene flanked by intergenic regions D and E in *F. tucumaniae* NRRL 31086, which is highly conserved in both *MAT1-1* and *MAT1-2* idiomorphs for the 11 SDS/BRR fusaria sequenced in this study (FIG. 1). An uncorrected (“p”) distance matrix calculated in PAUP (Swofford 2003) was used to estimate genetic divergence within intergenic A and D among the SDS/BRR fusaria (FIG. 1). These analyses revealed

TABLE I. Summary of *MAT* multiplex assay

<i>Fusarium</i> species	Disease ^a	No. of isolates			Percentage of isolates	
		Total	<i>MAT1-1</i>	<i>MAT1-2</i>	<i>MAT 1-1</i>	<i>MAT1-2</i>
<i>F. azukicola</i>	BRR	8	3	5	38	62
<i>F. brasiliense</i>	SDS	12	5	7	42	58
<i>F. crassistipitatum</i>	SDS	13	0	13	0	100
<i>F. cuneirostrum</i>	BRR	6	6	0	100	0
<i>F. phaseoli</i>	BRR	2	1	1	50	50
<i>Fusarium</i> sp. ^b	BRR	1	0	1	0	100
<i>F. tucumaniae</i>	SDS	268	92	176	34	66
<i>F. virguliforme</i>	SDS	129	129	0	100	0
Total		439	236	203	—	—

^aBRR = bean root rot; SDS = sudden death syndrome.

^bIsolate identified previously as *F. phaseoli* using a multilocus genotype assay (O'Donnell et al. 2010). Current study suggests isolate NRRL 22411 may be a hybrid with *F. phaseoli* and *F. brasiliense*-like parents.

that divergence within intergenic A ranged from as little as 0.06% in *F. brasiliense* NRRL 31779 × *Fusarium* sp. NRRL 22411 up to 1.1% in *F. tucumaniae* × *F. crassistipitatum*; divergence within intergenic D ranged from zero in *F. brasiliense* NRRL 31779 × *Fusarium* sp. 22411 up to 1.4% in *F. azukicola* NRRL 54366 × *F. brasiliense* NRRL 43350.

The hypothetical protein product of this putative gene showed highest identity to GenBank accession EEU47082.1, which is an unidentified protein expressed by *F. solani* f. sp. *pisi*. Comparisons of the ORF in the *MAT1-1* isolates *F. tucumaniae* NRRL 30186 and *F. virguliforme* NRRL 22292 revealed only three nonsynonymous substitutions (453/456 = 99.3% identity), which included one hydrophobic nonpolar-to-hydrophobic nonpolar (P to L at residue 145) and two hydrophilic-to-hydrophilic (D to G and G to D at residues 58 and 213 respectively) mutations. In addition, the ORF in *F. phaseoli* NRRL 31156 (*MAT1-1*) and *Fusarium* sp. NRRL 22411 (*MAT1-2*) had 99.8% identity (455/456), with the only difference being a single nonsynonymous substitution at residue 309 (R to H, both amino acids are charged and hydrophilic). Further comparisons revealed that the 0.5 kb UTR region 5' to the ORF in the *MAT1-1* isolates *F. virguliforme* 22292 and *F. tucumaniae* 31086 differed only by a single 11 bp indel (489/500 = 97.8%). By comparison, the 0.5 kb UTR region 3' to the stop codon in these two isolates contained five base mismatches and three indel gaps 2–6 bp long (483/500 = 96.6% identity).

Sequences of the highly divergent *MAT1-1* and *MAT1-2* idiomorphs were defined in part by their lack of synteny (FIG. 1). Separate pairwise comparisons of the 4.7 kb *MAT1-1* or the 3.7 kb *MAT1-2* idiomorphs revealed that they were highly conserved as were the flanking sequences. For example, no variation in nucleotide length, intron number or position was detected within the *MAT1-1-1*, *MAT1-1-2*, *MAT1-1-3* and *MAT1-2-3* genes for the isolates tested. However, a 30 bp deletion within exon 3 of *MAT1-2-1* was detected in *F. azukicola* and *F. crassistipitatum* (data not shown). As a result, the *MAT1-2-1* gene in these two species was predicted to encode for 10 fewer amino acids than *MAT1-2-1* in the other three *MAT1-2* fusaria. The in silico translations of the proteins predicted for *MAT1-1-1*, *MAT1-1-2* and *MAT1-1-3* showed highest identity to GenBank accessions EEU47077 (57%), EEU47076.1 (58%) and EEU47579 (58%) from the whole-genome sequence of the *MAT1-1* isolate of *Fusarium solani* f. sp. *pisi* NRRL 45880 (Coleman et al. 2009). By comparison, the predicted proteins of *MAT1-2-1* and *MAT1-2-3* showed highest identity respectively to GenBank accessions EFY88585 from *Metarhizium*

acidum (46%) and to JF776863 of *F. subglutinans* (28%).

Divergence-time estimates of the SDS-BRR fusaria.—

The dated *RPB1/RPB2* phylogeny (FIG. 3) suggests that the *Fusarium solani* species complex (FSSC) diverged from its sister group during the early-middle Paleocene, 62 Mya (95% HPD interval: 41.2, 85.8). Subsequently clade 1 of the FSSC diverged from the ancestor of the sister clades 2 and 3 in the middle Oligocene approximately 28 Mya, followed by the split of clades 2 and 3 in the early Miocene 21.38 Mya (95% HPD interval: 17.58, 24.53). Last, *F. azukicola* diverged from the remaining SDS/BRR fusaria roughly 0.75 Mya (95% HPD: 0.27, 1.68), in the mid-Pleistocene (FIG. 3). No other dates could be obtained for the SDS/BRR fusaria due to the high conservation of the *RPB1/RPB2* genes.

Multiplex PCR assay for MAT1-1 and MAT1-2.—

Sequences of the 11 *MAT* regions generated in the present study were used to design a multiplex PCR assay to efficiently identify mating type for the SDS/BRR fusaria. Primers SDS113F1 and SDS113R2, targeting *MAT1-1-3*, and SDS121F1 and FS3MAT1-2RV, for *MAT1-2-1*, reproducibly yielded 496 bp and 260 bp amplicons (FIG. 2) respectively for 439 SDS/BRR fusaria isolates. Results of the *MAT* multiplex PCR assay indicated that each of the seven SDS/BRR fusaria possesses a heterothallic mating-type organization, with only one of the two idiomorphs present in each isolate tested. In addition to *F. tucumaniae* (n = 268), which has been shown to possess a heterothallic sexual reproductive mode (Covert et al. 2007, Scandiani et al. 2010), both idiomorphs were detected for the first time in *F. azukicola* from Hokkaido, Japan (n = 8), *F. brasiliense* from Brazil, Argentina and California in USA (n = 12) and *F. phaseoli* from USA (n = 2) (TABLE I). By contrast, only *MAT1-1* isolates of *F. virguliforme* from USA and Argentina (n = 129) and *F. cuneirostrum* from USA, Canada and Japan (n = 6), and *MAT1-2* isolates of *F. crassistipitatum* from Argentina and Brazil (n = 13) were detected within these SDS/BRR pathogens. *Fusarium tucumaniae* was the only SDS/BRR species in which isolates of both mating types were collected within the same region and year, Argentina 2001–2013 (SUPPLEMENTARY TABLE I). In addition, our MLGT analysis of 61 isolates collected during the 2013 growing season in Argentina revealed that *F. tucumaniae* was the dominant SDS pathogen, with both *MAT1-1* and *MAT1-2* isolates recovered in the provinces of Buenos Aires, Entre Ríos and Santa Fe (SUPPLEMENTARY TABLE I). Although reported from other provinces (O'Donnell et al. 2010), isolates of *F.*

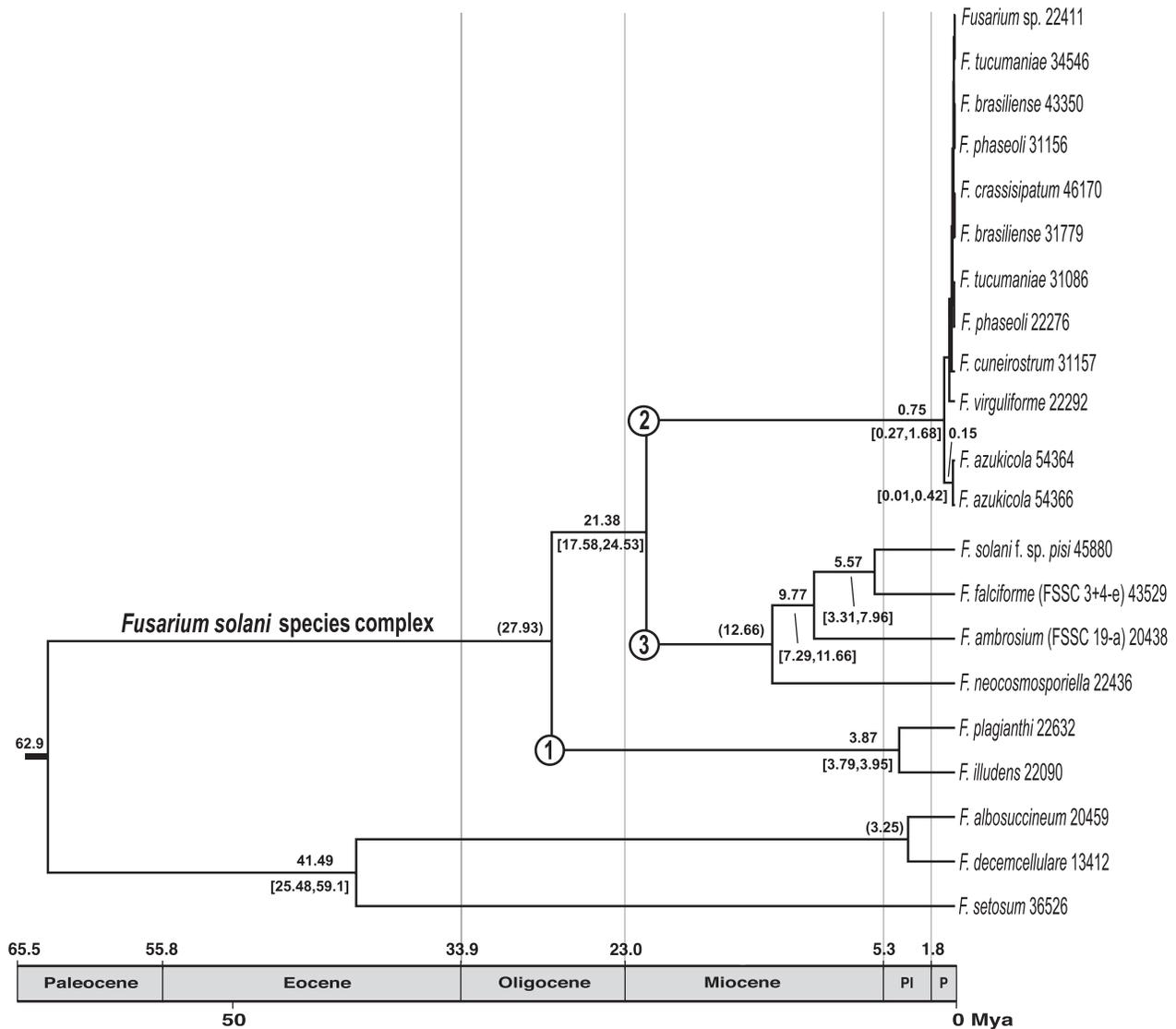


FIG. 3. Maximum clade credibility tree. For each node, the inferred age (median value) is shown above the branch immediately to the left. Numbers in brackets below the branch indicate the 95% highest posterior probability density (HPD) interval for the associated node. The divergence time estimates from O'Donnell et al. (2013) that were used as calibration points are in parentheses above the branch associated with the node in question. The three clades of the *Fusarium solani* species complex (1–3) are identified by a number within a circle. PI = Pliocene, P = Pleistocene.

virguliforme and *F. crassisipitatum* were discovered for the first time in Córdoba, Argentina, in 2013.

To correlate *MAT* idiomorph assignment in the present study with previous reports (Covert et al. 2007, Scandiani et al. 2010), the multiplex PCR assay was used to assign mating type to the nine pairs of isolates used to obtain fertile perithecia of *F. tucumaniae*, 28 random ascospore progeny from the laboratory cross of *F. tucumaniae* NRRL 34546 \times *F. tucumaniae* NRRL 31781 (SUPPLEMENTARY TABLE I, Covert et al. 2007) and 16 random ascospore progeny obtained from perithecia of *F. tucumaniae* collected on roots of soybean in Argentina (Scandiani et al.

2010). The results of this screen revealed that isolates previously assigned the – or + mating type comprise the *MATI-1* and *MATI-2* idiomorphs respectively. Chi-square goodness of fit tests (conducted online at <http://www.quantpsy.org>) indicated that the *MAT* idiomorph of the 28 (9 *MATI-1* to 19 *MATI-2*, $\chi^2 = 3.5714$; $df = 1$; $P = 0.0588$) and 16 (7 *MATI-1* to 9 *MATI-2*, $\chi^2 = 0.25$; $df = 1$; $P = 0.6171$) random ascospore progeny was statistically consistent with the expected 1 : 1 ratio of a single-gene locus. In addition, if the four anonymous loci (i.e. loci 1, 44, 81, 96 in Aoki et al. 2012b) and *MAT* segregate independently in the 28 progeny from the laboratory cross of NRRL

34546 × NRRL 31781, then 93.75% of the progeny should be recombinants. Genotyping revealed that 26/28 of the progeny were recombinants, which closely fit the expectation of five unlinked loci ($\chi^2 = 0.038$; $df = 1$; $P = 0.8452$).

Sexual crosses.—Based on the discovery that both idiomorphs were represented in our collection of *F. azukicola*, *F. brasiliense* and *F. phaseoli*, which are not known to reproduce sexually, intraspecific sexual crosses were established after the detailed published protocol (Klittich and Leslie 1988) that was used to obtain fertile perithecia of *F. tucumaniae* (Covert et al. 2007). However, none of the three intraspecific crosses yielded perithecia, suggesting that the conditions were inadequate and/or the collection lacked female-fertile isolates.

DISCUSSION

The primary goal of this project was to provide the first detailed information on the structural organization of the mating type (*MAT*) locus in the seven SDS/BRR fusaria and to test the hypothesis that all seven species are capable of sexual reproduction. Consistent with the discovery that the SDS pathogen *Fusarium tucumaniae* possesses a heterothallic or self-sterile sexual cycle (Covert et al. 2007, Scandiani et al. 2010), heterothallic *MAT* loci were identified in the whole-genome sequence of three SDS/BRR fusaria. *MAT* loci in the SDS/BRR fusaria shared several conserved features with phylogenetically diverse fusaria and other filamentous ascomycetes (Yun et al. 2000, Fraser et al. 2007, Martin et al. 2011, Kim et al. 2012, Tsui et al. 2013). These features included: (i) idiomorphs flanked by genes encoding a DNA lyase (*APNI*) and a cytoskeletal protein (*SLA2*), and non-coding intergenic A and D spacers that are highly conserved among the SDS/BRR fusaria (data not shown); (ii) highly divergent *MATI-1* and *MATI-2* idiomorphs that were defined by their lack of synteny (Menkis et al. 2010, Idnurm et al. 2011); (iii) presence of an α -box motif transcription factor within the predicted *MATI-1-1* protein and a high mobility group (HMG) domain transcription factor within the *MATI-2-1* and *MATI-1-3* genes; and (iv) high conservation of *MAT* gene complement, synteny and intron position.

Despite these similarities, the SDS/BRR *MAT* loci are distinct in three important respects. The first is the presence of the *MATI-2-3* gene within the *MATI-2* idiomorph. This gene was discovered in the *MAT* sequence of *Fusarium* sp. NRRL 22411 using the gene-prediction tool AUGUSTUS 2.5.5 (Stanke et al. 2008). *MATI-2-3* initially was discovered within the

MATI-2 locus of heterothallic members of the *F. fujikuroi* species complex (FFSC) as well as homothallic species in the *F. graminearum* species complex (FGSC) (Martin et al. 2011). However, the *MATI-2-3* gene in *Fusarium* sp. NRRL 22411 was predicted to encode a protein with only 53% identity to its homolog in *F. subglutinans*, a member of the FFSC. Two lines of evidence suggest that the *MATI-2-3* gene may have a broad phylogenetic distribution within the genus *Fusarium*. The first is the presence of *MATI-2-3* within the SDS/BRR fusaria, which are nested within the early diverging FSSC, while the second is the identification of this gene in distantly related members of the FFSC and *F. oxysporum* species complex (O'Donnell et al. 2013). Of note, most *Fusarium* genomes sequenced thus far indicate heterothallism by possessing only the *MATI-1* locus (Ma et al. 2013), so the phylogenetic distribution of *MATI-2-3* within *Fusarium* must await future phylogenomic analyses. Other than *Fusarium*, *MATI-2-3* has been found only within closely related members of the Hypocreales, including *Trichoderma* and *Metarhizium*, via BLASTx queries of GenBank using the *MATI-2-3* protein of *Fusarium* sp. NRRL 22411. Although the expression pattern of *MATI-2-3* in homothallic, *F. graminearum* is similar to *MATI-1-1* and *MATI-2-1*; fertile perithecia developed in a strain lacking *MATI-2-3*, indicating that this gene is not essential for sexual reproduction in this species (Kim et al. 2012).

The second unique feature of the SDS/BRR *MAT* loci is that they have expanded to include a gene of unknown function that is predicted to encode for a protein with 32% identity to an expressed protein from *F. solani* f. sp. *pisi* (GenBank accession No. EEU47082.1). Of note, this gene is not orthologous to either of the two divergently transcribed genes (GenBank accessions Nos. XP_003053293.1, XP_003052791.1) that are located between *MATI-1-1* and *SLA2* in the whole genome of *F. solani* f. sp. *pisi* NRRL 45880 (Ma et al. 2013). Results of the present study add to the growing number of examples of genes that have been integrated within *MAT* loci (Fraser et al. 2007), which is consistent with the hypothesis that mating-type loci are more susceptible to introgressive hybridization (Strandberg et al. 2010). In contrast to the reports of truncated or degenerated *MAT* genes that reside outside the *MAT* locus in the genomes of some ascomycetes (Tsui et al. 2013), none were found in the AUGUSTUS analysis of the SDS and BRR fusaria in the present study.

The third feature of the SDS/BRR *MAT* loci, specific to *MATI-2-1*, is the discovery of a 30 bp deletion within the coding region of *F. azukicola* NRRL 34546 and *F. crassistipitatum* NRRL 46170,

which is predicted to result in a protein with 10 fewer amino acid residues than the one produced by the other three *MATI-2* SDS/BRR fusaria. Therefore, functional studies are needed to assess whether this putative deletion has a negative effect on protein function. Heterologous expression of the *MATI-2-1* gene from *F. azukicola* NRRL 34546 or *F. crassistipitatum* NRRL 46170 in an isolate of *F. tucumaniae* that has been shown to function as a sexually competent parent (Covert et al. 2007) could provide a means for assessing whether the product from this truncated gene is functional.

While the FSSC is one of the earliest diverging lineages within *Fusarium*, estimated to have diverged from its sister group during the early-middle Paleocene, 62 Mya (95% HPD interval: 41.2, 85.8) (O'Donnell et al. 2013), the evolutionary radiation of the SDS/BRR fusaria had been dated to the late Pleistocene, approximately 0.15 Mya (95% HPD interval: 0.0127, 0.456) (O'Donnell et al. 2013). In that study only *F. virguliforme* and *F. phaseoli* were available for analysis. Here we included five additional taxa, including *F. azukicola*, which is more divergent at the *RPB1* and *RPB2* loci compared to the other SDS/BRR taxa. The inclusion of the *F. azukicola* isolates pushes the date of origin for the SDS/BRR clade back to roughly 0.75 Mya (95% HPD: 0.27, 1.68), in the mid-Pleistocene (FIG. 3). Although dated phylogenies such as the one for *Fusarium* generally have broad confidence intervals due to several potential sources of error (Berbee and Taylor 2010), the *Fusarium* time-calibrated phylogeny suggests that the SDS/BRR fusaria clade originated well before the domestication of common bean and soybean. Apart from *F. azukicola*, the species within this group display shallow divergence and the *RPB1* and *RPB2* gene sequence data cannot resolve their relationships. Clearly nucleotide sequence data from genes that evolve faster than *RPB1* and *RPB2* are needed to date the cladogenic events within this group of economically important plant pathogens subsequent to their origin in the mid-Pleistocene.

One of the main objectives of this work was to develop a multiplex PCR assay for rapidly typing SDS/BRR *MAT* idiomorphs. Similar assays have advanced our understanding of the reproductive mode of other agronomically important lineages of *Fusarium* (Wallace and Covert 2000, Kerényi et al. 2004, O'Donnell et al. 2004), which have provided invaluable information in support of agriculture biosecurity. Our *MAT* multiplex PCR screen of 439 SDS/BRR fusaria from North and South America and Japan have helped elucidate the genetic diversity, host range and geographic distribution of these pathogens by the discovery that, in addition to *F. tucumaniae*, three

additional SDS/BRR fusaria possess both *MAT* idiomorphs (*F. azukicola*, *F. brasiliense*, *F. phaseoli*), while only one idiomorph was detected in isolates of *F. crassistipitatum*, *F. cuneirostrum* and *F. virguliforme*. These results mirror phylogenetic analyses (Aoki et al. 2003, 2005, 2012a, b; O'Donnell et al. 2010) of these fungi that identified genetic diversity within the first four fusaria but little or no variation within the latter three pathogens, which is consistent with asexual reproduction (Achenback et al. 1996, Rupe et al. 2001, Mbofung et al. 2012). Our data support the hypothesis of Covert et al. (2007) that only *MATI-1* isolates of *F. virguliforme* have been introduced to North America. However, given the hypothesized recent evolutionary origin of the SDS/BRR lineage in South America, where relatively few pathogen surveys have been conducted, coupled with the *in silico* translations of the *MAT* genes that indicate they likely encode for functional proteins, we speculate that all seven SDS/BRR fusaria reproduce sexually on hosts endemic to South America or Mesoamerica.

In addition to tracking the global movement of *MATI-1* and *MATI-2* isolates of the SDS/BRR fusaria, the multiplex PCR assay also established that the – and + mating type testers reported in Covert et al. (2007) correspond to *MATI-1* and *MATI-2* respectively. Also, χ^2 goodness of fit tests on *MAT* idiomorph data obtained from typing random ascospore progeny of *F. tucumaniae* from a laboratory cross (Covert et al. 2007) and naturally occurring perithecia on soybean roots (Scandiani et al. 2010) fit the expectation of a sexually reproducing species segregating for alternate idiomorphs.

The final objective of the present project was to obtain fertile perithecia from heterothallic matings of SDS/BRR species for which a sexual cycle is not known. Results of the multiplex-PCR assay for *MAT* detected both idiomorphs in *F. azukicola*, *F. brasiliense* and *F. phaseoli* (TABLE I). Following the methods of Klittich and Leslie (1988), crosses were established between opposite mating types for each of these three SDS/BRR fusaria. Although this protocol was used to obtain fertile perithecia of *F. tucumaniae* with multiple pairs of *MATI-1* and *MATI-2* isolates as parents (Covert et al. 2007), no perithecia were produced in the three intraspecific pairings tested. Failure to obtain sexual reproduction could be due to a combination of factors, including absence of female-fertile isolates, especially because none produced protoperithecia (Leslie and Klein 1996) or because the conditions employed were not favorable for mating (Covert et al. 1999). Future efforts to assess whether the SDS/BRR fusaria reproduce sexually should benefit from more extensive surveys in South America that take advantage of the high-throughput MLGT assay for species identification

and the multiplex PCR assay for *MAT* idiomorph determination.

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